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Development of therapeutics for the treatment of diabetic brain complications

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DEVELOPMENT OF THERAPEUTICS FOR THE TREATMENT OF DIABETIC BRAIN COMPLICATIONS

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TO MY FAMILY

ABSTRACT

Type-2 diabetes (T2D) is characterized by hyperglycemia and hyperlipidemia, resulting in impaired insulin production and insulin resistance in peripheral tissues. Several studies have demonstrated an association between diabetes and central nervous system complications such as stroke and Alzheimer's disease. Due to the fact that T2D is one of the fastest growing chronic illnesses, there is an urgent need to improve our knowledge on the pathogenic mechanisms to why diabetes leads to brain complications as well as to identify novel drugable targets for therapeutic use.

Project 1: studies I-II

Pre-clinical studies have shown that adult neurogenesis is impaired in diabetic animal models. We hypothesized that diabetes leading to neurogenesis impairment plays a role in the development of neurological complications. If so, normalizing neurogenesis in diabetes/obesity could be therapeutically useful in counteracting neurological dysfunction. The aim of studies I-II was to establish an *in vitro* system where to study the effect of a diabetic *milieu* on adult neurogenesis. Furthermore, we determined the potential role of pituitary adenylate cyclase-activating polypeptide (PACAP) and galanin to protect adult neural stem cells (NSCs) from these diabetic-like conditions. Moreover, we determined whether apoptosis and the unfolded protein response (UPR) were induced by diabetic-like conditions and whether their regulation was involved in the PACAP/galanin-mediated protective effect. Finally, we studied the potential regulation of PACAP and galanin receptors in NSCs in response to diabetic-like conditions *in vitro* and *ex vivo*.

The viability of NSCs isolated from the mouse brain subventricular zone (SVZ) was assessed in presence of a diabetic *milieu*, as mimicked by high palmitate and glucose, which characterize diabetic glucolipotoxicity. The results show that high palmitate and glucose impair NSC viability in correlation to increased apoptosis (Bcl-2, cleaved caspase-3) and UPR signaling (CHOP, BIP, XBP1, JNK phosphorylation). We also show that PACAP and galanin counteract glucolipotoxicity *via* PAC1 receptor and GalR3 activation, respectively. Furthermore, we also report that PACAP and galanin receptors are regulated by diabetes in NSCs *in vitro* and in the SVZ *ex vivo*.

Project 2: study III

T2D is a strong risk factor for stroke and no therapy based on neuroprotection is currently available. Exendin-4 (Ex-4) is a glucagon-like peptide-1 receptor (GLP-1R) agonist in clinical use for the treatment of T2D, which has also been shown to mediate neuroprotection against stroke pre-clinically. However, the applicability of a therapy based on Ex-4 has not been investigated in a pre-clinical setting with clinical relevance.

The aim of this study was to determine the potential efficacy of Ex-4 against stroke in T2D rats by using a drug administration paradigm and a dose that mimics a diabetic patient on Ex-4 therapy. Moreover, we investigated inflammation and neurogenesis as potential cellular mechanisms at the basis of Ex-4 efficacy. T2D Goto-Kakizaki (GK) rats were treated peripherally for 4 weeks with daily clinical doses of Ex-4 (0.1, 1, 5 μg/kg body weight) before inducing stroke by transient middle cerebral artery occlusion. The Ex-4 treatment was continued for 2-4 weeks thereafter. The severity of ischemic damage was measured by evaluation of stroke volume and by stereological counting of neurons in the striatum and cortex. Evaluation of stroke-induced inflammation, stem cell proliferation and neurogenesis was also quantitatively assessed by immunohistochemistry. We show that peripheral administration of Ex-4 counteracts

ischemic brain damage in T2D GK rats. The results also show that Ex-4 decreased microglia infiltration and increased stroke-induced neural stem cell proliferation and neuroblast formation, while stroke-induced neurogenesis was not affected by Ex-4 treatment.

Together, our data in project 1 show that we have established an *in vitro* assay where to study the molecular mechanism on how diabetes impact adult neurogenesis. Furthermore, our results show that this assay has the potential to be developed into a screening platform for the identification of molecules that can regulate adult neurogenesis under diabetes. In project 2, we show neuroprotective efficacy against stroke by Ex-4 in a T2D rat model, by using a pre-clinical setting with clinical relevance. Ex-4 is an anti-diabetic drug in clinical use that has been reported to show limited side effects. Thus, at least in theory stroke patients should be able to easily receive this treatment, probably with minimal risks.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I. **Mansouri. S**, Ortsäter. H, Pintor Gallego. O, Darsalia. V, Sjöholm. A, Patrone. C. Pituitary adenylate cyclase-activating polypeptide counteracts the impaired adult neural stem cell viability induce by palmitate, *J Neurosci Res*, 2012, 90(4):759-68.

II. **Mansouri. S**, Barde. S, Ortsäter. H, Eweida. M, Darsalia. V, Sjöholm. Å, Hökfelt, T, Patrone. C. GalR3 activation promotes adult neural stem cell survival in response to a diabetic milieu, *J. Neurochem*. 2013, 10.1111/jnc.12396.

III. Darsalia. V, **Mansouri. S**, Ortsäter. H, Olvering. A, Nozadze. N, Kappe. C, Iverfeldt. K, Tracy. LM, Grankvist. N, Sjöholm. Å, Patrone. C. GLP-1R activation reduces ischemic brain damage following stroke in a type 2 diabetic animal model, *Clin Sci*, 2012, 122(10):473-83.

Other publications, not included in the thesis:

I. Darsalia. V, **Mansouri. S**, Wolbert. P, Barde. S, Sjöholm. Å, Patrone. C. The specific VPAC2 agonist Bay 55-9837 increases neuronal damage and hemorrhagic transformation after stroke in type 2 diabetic rats, *Neuropeptides*, 2012, 45(2):133-7.

II. Hussain. S, **Mansouri. S**, Sjöholm. Å, Patrone. C, Darsalia. V. Evidence for cerebral neurodegeneration in type 2 diabetic Goto-Kakizaki rats, *Journal of Alzheimer's disease*, 2013. (submitted)

III. Darsalia. V, Olverling. A, **Mansouri. S**, Larsson. M, Nathanson. D, Rosenbrock. H, Nyström. T, Klein. T, Sjöholm. Å, Patrone. C, Increased GLP-1 levels by linagliptin in diabetes correlate with enhanced NSC activation after stroke in the mouse, *Journal of Neuroscience Research*, 2013. (submitted)

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LIST OF ABBREVIATIONS

ASK-1	Apoptosis signal regulating kinase 1
AC	Adenylate cyclase
AD	Alzheimer's disease
AGE	Advanced glycation end products
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adenosinetrisphosphate
ABC kit	Avidin–biotin complex kit
BDNF	Brain-derived neurothrophic factor
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BW	Body weight
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CNS	Central nervous system
CVD	Cardiovascular disease
DAPI	4,6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DMEM	Dulbecco's modified eagle medium
DRN	Dorsal raphe nucleus
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
Ex-4	Exendin-4
bFGF	Basic fibroblast growth factor
FFA	Free fatty acid
GLP-1	Glucagon-like-peptide-1
GK-rat	Goto-Kakizaki rat
GPCR	G-protein-coupled-receptor
HbA_{1c}	Glycated haemoglobin
H_2O_2	Hydrogen peroxide
HD	Huntington disease
HFD	High fat diet
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
ID	International Dollars
IDF	International Diabetes Federation
IHC	Immunohistochemistry
JNK	c-Jun *N*-terminal kinase
K_{ATP} channels	ATP-sensitive potassium channels
LC	Locus coeruleus
MAPK	Mitogen activated protein kinase
Max-4	Maxidilan-4
MCA	Middle cerebral artery
MTT	3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NSC	Neural stem cell
NS	Neurosphere

OB	Olfactory bulb
PACAP	Pituitary adenylate cyclase-activating polypeptide
PD	Parkinson’s disease
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PKA	Protein kinase A
PVDF	Polyvinylidene fluoride
q-RT PCR	Quantitative reverse transcriptase PCR
RMS	Rostral migratory stream
ROS	Reactive oxygen species
r-TPA	Recombinant tissue plasminogen activator
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SEM	Standard error of the mean
SNpc	Substantia nigra pars companda
SGZ	Subgranular zone
STZ	Streptozotocin
SVZ	Subventricular zone
T1D	Type 1 diabetes
T2D	Type 2 diabetes
tMCAO	Transient unilateral middle cerebral artery occlusion
TNFα	Tumor necrosis factor α
VIP	Vasoactive intestinal peptide
VEFG	Vascular endothelial growth factors
VDCC	Voltage-dependent Ca^{2+} channel
WB	Western blot
WHO	World Health Organization
UPR	Unfolded protein response

INTRODUCTION

1. Diabetes

Diabetes mellitus is a complex metabolic disorder mainly characterized by chronic hyperglycemia and later associated with dysfunction or failure of various organs (1). According to the World Health Organization (WHO), diabetes is one of the most prevalent and fastest growing chronic illnesses, globally affecting more than 347 million people worldwide. Cardiovascular diseases (CVD) accounts for 50-80 % of deaths in people with diabetes, a number expected to rise by more than 50 % in the next 10 years. For these reasons, diabetes has become a major cause of premature illness and death in most countries and is predicted to become the seventh leading cause of death in the world by 2030. Moreover, diabetes accounted for ~ 12 % of total health-care expenditure in the world in 2010 (2), and it's estimated that the global health-care costs to treat and prevent diabetes and its complications to increase from International Dollars (ID) 218 billion in 2010 to ID 561 billion in 2030 (3).

There are two main forms of diabetes, classified by etiology: type 1 diabetes (T1D) and type 2 diabetes (T2D).

1.1 Type 1 diabetes

T1D most often develops in childhood or adolescence but can occur at any age (1). T1D's characterization is based on a cell-mediated autoimmune disease causing destruction of pancreatic β-cells leading to absolute or near total loss of insulin production, to such a degree that insulin therapy becomes necessary (4). In the much less common T1Db, there is also β-cell destruction; however, on a non-autoimmune base. Several studies have suggested that various pro-inflammatory cytokines, such as interleukin (IL)-1, IL-1β and tumor necrosis factor α (TNFα), interferon gamma (IFNγ) and reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide and nitric oxide, may play important role in pancreatic β-cell destruction (5, 6). Certain viral infections and human leukocyte antigen (HLA) subtypes have also been connected to T1D (7, 8).

1.2 Type 2 diabetes

T2D accounts for 90-95 % of diabetic patients (9). Previously a disease of middle-aged and older adults, T2D is rapidly increasing among young people. The main causes of the syndrome appear to be multiple and include obesity (especially abdominal obesity), physical inactivity, insulin resistance, aging, and a genetic predisposition (10, 11). T2D is characterized by insulin resistance leading to progressive decline in functional β-cell mass and impaired insulin production (12). Normally, β-cells compensate for insulin resistance by increasing insulin secretion and β-cell mass. In addition, along the progression of T2D, pancreatic β-cells initially go through a proliferative state in order to compensate for insulin resistance in peripheral tissues (11). Later, higher levels of glucose and free fatty acid (FFA) (glucotoxicity and lipotoxicity) create an unnatural environment; these events are believed to trigger β-cell apoptosis which results in a decreased β-cell mass, relative insulin deficiency and the onset of overt diabetes symptoms (13, 14). However, this is a slow and insidious process that may take 15-20 years until diabetes eventually is diagnosed, under which time organ damage occurs. It is estimated that, by the time of diagnosis, 50 % of β-cell mass is lost. Additionally, ROS have been shown to trigger

the progression of islet dysfunction (5). T2D is diagnosed by fasting plasma glucose levels >7 mmol/l on two different occasions, or by plasma glucose concentration >11.1 mmol/l 2 hours following an oral glucose tolerance test, or a random plasma glucose level exceeding 11.1 mmol/l combined with typical symptoms, according to the criteria by the WHO.

The pathophysiology of T2D involves defects in several organs, *i.e.* liver, pancreas, adipose and skeletal muscle tissue that "conspire" together to produce abnormal glucose and lipid metabolism. T2D often co-exists with other disorders, including obesity, hypertension and dyslipidemia (a.k.a. the metabolic syndrome), which may contribute to the severity of its complications. It has been well documented that people with diabetes suffer from peripheral neuropathy (15). However, substantial epidemiological evidence suggests that both T1D and T2D are also strongly associated with cognitive impairment (16-19).

2. T2D and CNS complications

In addition to the better characterized peripheral neuropathies, several studies have demonstrated an association between diabetes and central nervous system (CNS) complications, such as stroke, dementia, Alzheimer's disease (AD) and Parkinson's disease (PD) (20-22). However, the molecular and cellular mechanisms at the basis of why this occurs have not yet been fully understood. T2D is a complex disease that extends beyond its dysfunctional glucose regulation. People with T2D generally have a "metabolic syndrome" (23), which comprises a cluster of abnormalities that occur as a result of disturbances in multiple metabolic pathways, such as hyperinsulinemia, insulin resistance, hyperglycemia, hypoglycemia, dyslipidemia, hypertension, vascular dysfunction, pro-inflammatory conditions (oxidative stress) and obesity. These conditions may play a role in the development of CNS complications in diabetes (24, 25).

2.1 Dementia

Significant strides in the treatment of T2D have improved the life expectancy of T2D patients. However, the improved longevity has increased the risk of developing geriatric health complications prematurely, including cognitive impairment and dementia (26, 27). Compared to the general population, the risk of developing dementia is 1.5-2.5 times higher in people with T2D (28). Although controlling some of the diabetes parameters, such as hypertension, has shown promising results (29), currently there is no effective treatment available. Moreover, diabetes-related factors such as the age of onset of diabetes, diabetes duration, type of mechanism of action of anti-diabetic medications, presence of other diabetes-related complications, comorbidities and diabetes-specific complications could interact with the clinical expression and neuropathology of dementia, as well as with the rate of cognitive decline (30). However, several mechanisms have been proposed to explain the association between diabetes and dementia, such as brain vascular lesion (31), recurrent hypoglycemia (32), advanced glycation end products (AGE) (33), inflammation (34), oxidative stress (35), competition of insulin and β-amyloid in the insulin-degrading enzyme (36) and insulin resistance (37).

2.1.1 Alzheimer's disease (AD)

AD is the most common neurodegenerative disease characterized by chronic dementia with around 50-80 % of all dementia cases (38-40). The most common risk factor is increased age (41). Currently affecting around 36 million people worldwide with a global cost of $600 billion in 2010, AD is the biggest unmet medical need in neurology. Current drugs improve symptoms, but do not have profound disease-modifying effects; this is mainly due to un-elucidated etiology of the disease (42). AD is characterized by progressive memory deficiency and neuronal loss (43). The main basic pathological processes affecting the disease are deposition of senile plaques (insoluble fibrous protein aggregations) and neurofibrillary tangles (NFTs), characterized primarily by hyperphosphorylated *tau* protein in the frontal cortex (21). Familial AD is caused by genetic factors, such as gene mutation of presenilin 1 (PSEN1), presenilin 2 (PSEN2), amyloid precursor protein (APP), and the apolipoprotein E4 (44). Yet, the late-onset AD might be caused by environmental and/or lifestyle factors (45). Epidemiological studies of patient data sets have clearly identified T2D as a risk factor for developing AD (25, 32, 46, 47).

Hyperglycemia, increased oxidative stress levels, alterations of microvasculature and not least recurrent hypoglycemia following T2D treatments are the main players at the basis of T2D that could induce AD (40), but also comorbid conditions such as hypertension, small brain infarcts, dyslipidemia and obesity (39). An important role connecting diabetes and decreased cognitive function is insulin resistance (48), where a compensatory chronic elevation of circulating insulin may exert a negative influence on memory and other cognitive functions (49). While it is generally agreed that glucose uptake by the brain does not require insulin, insulin may serve other functions in the CNS. In fact, T2D has been associated with impaired learning in both animal and human studies (50). Moreover, it has been shown that even non-diabetics who are insulin resistant show an increase risk to develop dementia/AD (21, 30).
Desensitization of insulin receptors has been observed in the brains of AD patients and appears to directly contribute to the development of AD (51, 52). A major component of NFTs is hyperphosphorylated *tau*; cleaved *tau* is detected in brains of patients with AD (40). Interestingly, in mouse models of diabetes, increased *tau* phosphorylation has been shown (53). Moreover, insulin may affect *tau* phosphorylation (54) where a study has shown an increase of *tau* phosphorylation in the CNS of conditional knockout mouse for the insulin receptor (55). Therefore, some current therapeutical strategies in order to prevent or treat AD, aim to normalize insulin signaling in the brain (49).

2.2 Parkinson's disease (PD)

PD is the second most common neurodegenerative disease, affecting 1-2 % of humans aged 60 years and older and it's characterized by a loss of dopaminergic neurons in the *substantia nigra pars companda* (*SNpc*) region of the brain, with display of intracytoplasmic Lewy bodies that consist of aggregated filamentous α-synuclein. The clinical symptoms of PD patients are muscle rigidity, bradykinesia, resting tremor and postural instability (56). These symptoms are caused by dopamine deficiency in the striatum due to the degeneration in the *SNpc*. Depression and dementia may also occur (57, 58). Diabetes and PD patients share disruption in common mechanistic pathways. For example, 60 % of the PD patients have impaired insulin signaling and are glucose intolerant (59), where recent studies have shown insulin resistance in 62 % of PD patients with dementia, of which 30 % were glucose intolerant (60). In addition, a study

showed diabetes associated with parkinsonian symptoms (61). Moreover, recent studies show PD and diabetes share genetic susceptibility that put individuals at risk for both diseases (62, 63). Interestingly, heavy metal exposure is associated with islet dysfunction and disease progression in both PD and diabetes (59, 64). Additionally, mitochondrial dysfunction, endoplasmic reticulum (ER) stress and inflammation play a role in the etiology and progression of both diseases (65-67). Therefore, many factors that put individuals at risk of PD also put them at risk for diabetes. However, these findings have been disputed (68) and therefore more studies are required to demonstrate if there is a link between PD and T2D.

2.3 Stroke

Stroke cause long-term disability and mortality in the Western population. This has led to a large financial burden on the health systems worldwide (69). There are two types of strokes: ischemic, which accounts for 85% of stroke cases, and hemorrhagic. Hemorrhages are caused by the rupture of blood vessels, and hypertension is the primary risk factor (70, 71). Ischemic stroke can be further subdivided into thrombotic and embolic stroke (70). Thrombotic stroke occurs when a blood clot (thrombus) blocks the blood flow of a specific brain area. A thrombus may form in an artery affected by atherosclerosis. Embolic stroke occurs when a piece of clot (embolus) breaks loose, for instance from the heart if there is atrial fibrillation and it's carried through the bloodstream to the brain, where large vessels branch off into small vessels. When it reaches a point where it cannot travel further, blood supply to the part of the brain is cut off (69). The reduction of blood flow can lead to insufficient supply of oxygen and glucose to the tissue, causing ATP depletion, ionic homeostasis alterations, inflammation, release of multiple cytokines and abnormal release of neurotransmitters, as a consequence leading to energy failure and mitochondrial dysfunction, and eventually cell death (72).

There are several risk factors associated with stroke, *e.g.* hypertension, arterial fibrillation, smoking, hyperlipidemia and diabetes (69). T2D is an independent risk factor for stroke, particularly ischemic stroke, where studies have reported a 2-6 fold increase risk for stroke in diabetic patients *versus* those without (20). Also, stroke typically occurs prematurely in diabetic patients. In addition, T2D also doubles the risk of stroke recurrence, which generally shows a worse functional outcome than the first stroke (20, 73), increasing the mortality rate (74). Moreover, diabetes-associated risk factors for stroke do not only include hyperglycemia or vascular risk factors (*e.g.* hypertension, dyslipidemia), but also genetic, demographic and lifestyle factors can contribute (74). Even after these risk factors have been adjusted with conventional therapies, a doubling of ischemic stroke cases is still seen in T2D patients compared with non-diabetic individuals (73). The risk of stroke in T2D patients arises already in pre-diabetic stages, where insulin resistance is a strong risk factor for stroke (73). In addition, amounts of HbA_{1c} levels have been proposed as a measure of risk for stroke occurrence in pre-diabetic patients, where a level greater than 42 mmol/mol (6 %) can increase the risk of stroke 2-3 times compared to normoglycemic individuals (75).

2.3.1 GLP-1R, a neuroprotective target for the potential treatment of stroke in T2D

The major limitation in the development of neuroprotective therapeutic strategies against stroke is contingent upon how quickly neurons die ("time is brain"). Because of this, several pre-clinically successful candidate drugs with neuroprotective properties (used before or few minutes after stroke in rodents) have failed in clinical trials once

administered to patients few hours after stroke (76, 77). Thrombolytic treatment with recombinant tissue plasminogen activator (r-TPA) is the only method that is partially effective against stroke if given within 3-4 hours from the onset of stroke (78). However, the majority of the patients do not reach the hospital in time or have counter-indications to receive this treatment (79). In addition, r-TPA has been associated with increased risk of hemorrhagic transformation and r-TPA is performed even less frequently in diabetic patients with stroke, since elevated blood glucose and diabetes are associated with increased risk of intracerebral hemorrhages after r-TPA use (EU Stroke Guidelines 2009) (80, 81). While it is still unclear if glucose lowering can be beneficial against stroke, it is well understood that hypoglycemic events (that can result from glucose lowering treatments) should be avoided in T2D patients suffering stroke (74) and therefore insulin might not the best drug in that respect. An ideal therapy for the treatment of stroke in T2D would be a drug that is effective in rapidly normalizing glycemia (devoid of propensity for hypoglycemic side effects) in combination with a neuroprotective property.

Glucagon-like peptide-1 (GLP-1) is an insulinotropic hormone and member of the proglucagon-derived peptide family, which is secreted from enteroendocrine L-cells upon nutrient ingestion (82). GLP-1 exerts its physiological effects following the binding to its receptor (GLP-1R), a 463-amino acid, G-protein-coupled receptor (GPCR). The signal transduction pathways of GLP-1 and its analogues in the pancreatic β-cell are mediated through adenylate cyclase (AC) and the cAMP/PKA pathways to potentiate glucose-induced closure of ATP-sensitive K^+ channels, thereby generating cellular depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs) and influx of Ca^{2+} that sets in motion insulin exocytosis (83). GLP-1 has multifunctional effects according to its sites of synthesis and release (84). In addition to stimulate glucose-dependent insulin secretion, GLP-1 is associated with a number of cellular survival and plasticity effects, including β–cell islet neogenesis, protection against apoptosis (85), cardioprotective (86) and anti-inflammatory effects (87). Exenatide [synthetic exendin-4 (Ex-4)] is a stable GLP-1 analogue isolated from the saliva of the "Gila monster" lizard (88). It is resistant to degradation and is approved in both Europe and the U.S. for the clinical treatment of T2D (82).

Although the GLP-1 receptor is pre-dominantly located in the pancreatic islets, in the CNS GLP-1 is synthesized largely in the brainstem and transported along axonal networks to diverse CNS regions. Several studies have shown its expression in the rodent (89) and human (90) brain, *e.g.* in the hypothalamus, neocortex, brainstem and the hippocampus, where early findings showed its role in food intake regulation (91). Recently, a study showed that small amounts of GLP-1 are also produced by the microglia cells in the brain (92). Moreover, GLP-1 and Ex-4 can cross the blood-brain barrier (93). Studies have shown that exogenous Ex-4/GLP-1 also acts as a neuroprotectant in models of AD, PD (87, 94) and stroke, where intracerebroventricular administration of Ex-4 15 min before stroke reduced ischemic damage in mice (95). Additionally, intraperitoneal injection of Ex-4 2 hours before ischemia and 1 hour after reperfusion protected hippocampal CA1 neurons (96). Ex-4 has also shown neurogenic properties (97), enhancing synaptic plasticity and improve different forms of learning (98).

Although the effects of GLP-1R activation in the brain are promising, the field is still in its infancy and more research is needed to understand the mechanisms behind its action

and whether GLP-1R activation in diabetes could promote beneficial effects in the CNS.

2.4 Endoplasmic reticulum stress: a mechanism at the basis of CNS complications in T2D?

Emerging evidence suggests that ER stress may play a pivotal role in the development and pathology of many neurodegenerative diseases (66, 67, 99). In recent years, ER stress has been implicated in metabolic disorders, such as obesity, T2D and atherosclerosis (100, 101) In response to such conditions, cells initiate pro-survival signaling pathways collectively known as the unfolded protein response (UPR) (101, 102), which consists of three signaling pathways each controlled by a specific protein, *viz*. CHOP, GADD34, Grp78/BIP and spliced XBP1. Activation of the UPR pathways is often used as an indicator of ER stress (103). The UPR normally serves to alleviate ER stress by inhibiting general protein translation, increasing folding capacity and promoting degradation of misfolded proteins. Normally, the efforts mounted by the UPR restore the intra-organelle *milieu* and are therefore cytoprotective (104). If these actions fail to rescue the cells from ER stress, then apoptosis can occur (105). It is not clear exactly when and how a cell "decides" between pro-survival and pro-apoptotic signaling, nor is the mechanism behind ER stress-induced apoptosis fully elucidated but it includes reduced Bcl-2 expression. Excessive activity in the UPR does, however, converge in the mitochondria and results in opening of the permeability transition pore, loss of mitochondrial membrane potential, leakage of cytochrome c, formation of the apoptosome and activation of caspases (105). Moreover, elevated levels of circulating fatty acids and hyperglycemia increase the production of ROS (13, 106), and it may lead to cellular apoptosis. The mitogen-activated protein kinase (MAPK) signaling cascade transduces this apoptotic message. Two mammalian MAPKs, c-Jun *N*-terminal kinase (JNK) and p38 MAPK are known to be activated by various environmental stressors and inflammation (107). Apoptosis signal-regulating kinase (ASK) 1 can activate the JNK and p38 pathways, and thus tightly regulate the kinase activity of ASK1(108).

3. Adult neurogenesis

"*Once development was ended, the fonts of growth and regeneration of the axons and the dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, and immutable: everything may die, nothing may be regenerated.*"- Santiago Ramón y Cajal, 1928

We know today that the generation of new neurons continuously occurs in the adult brain in selected regions, namely the subgranular zone of hippocampus and the subventricular zone of the lateral ventricle wall (SGZ and SVZ, respectively) (109-111). Recently, adult neurogenesis was also described in the hypothalamic region (112). However, until only a few decades ago, the prevailing idea was that all neurons were developed before birth in the human brain and no new neurons were added to the CNS in adult life. It was specifically the work of Altman and colleagues in the 1960s (113-116) that showed that new neurons were added in the olfactory bulb (OB) and the hippocampus of adult rodents, and in 1998 Eriksson and colleagues showed for the first time neurogenesis in the adult human brain (117).

Neurogenesis in the hippocampus occurs in the SGZ adjacent to the dentate gyrus (DG). The newly born neurons in the SGZ differentiate and integrate into the local neural network as granule cells of the DG. In the DG, specialized GFAP-positive

astrocyte-like cells express stem cell markers like nestin, Sox2 and Hes5 (118, 119). See figure 1 for more detailed description. This area has been extensively studied, mainly due to the importance of hippocampus in relation to brain functions such as memory (120) and emotional behaviors (121). To date, the specific role of hippocampal neurogenesis is still under debate. Nonetheless, this process has been associated with learning (122), stress and certain brain disorders, such as depression. Interestingly anti-depressant therapy has been shown to increase adult DG neurogenesis (123).

Recent studies have indicated adult neurogenesis also in the hypothalamus, a brain area responsible for central homeostatic regulation of numerous physiological and behavioral functions such as feeding, metabolism, body temperature, sleep, circadian rhythms and sexual behavior (124). Among the first studies to describe adult neurogenesis in the hypothalamus were a series of experiments in which co-intraventricular infusion of brain-derived neurothrophic factor (BDNF) and the proliferative marker 5-bromo-2'-deoxyuridine (BrdU) led to an increased level of BrdU-positive cells co-labeled with the neuronal marker βIII-tubulin in the parenchymal part of the hypothalamus (125). This was further confirmed by other authors who showed differential distribution of hypothalamic neurogenesis between the hypothalamic regions (126). Moreover, a high fat diet (HFD) altered regions of the hypothalamic neurogenesis (126-128). In addition, recent pre-clinical reports have indicated a role of hypothalamic adult neurogenesis in maintaining energy balance in response to environmental and physiological insults (129).

In the ventricular zone, NSCs differentiate into cortical neurons during embryonic development (130). At development completion, the ventricular zone becomes the SVZ of the lateral ventricle where an ependymal layer separates these two regions (72). Ependymal cells have been proposed to be the NSCs responsible for SVZ neurogenesis (131). However, other studies have refuted this hypothesis, showing that ependymal cells are quiescent and do not have NSCs *in vitro* properties (132, 133). Today we know that the forebrain ependymal cells lining in the lateral walls are normally quiescent but can give rise to astrocytes and neuroblasts following stroke (134). Whether this property can be used for therapeutic purposes remains to be determined. In general, it is accepted within the research community that there are three types of cells existing in the rodent and human SVZ, type A, B, C cells. The NSCs in SVZ form special subpopulation of GFAP-expressing cells (type B) that originate from highly proliferative/amplifying cells known as C cells expressing the marker nestin. Type C cells differentiate into type A cells or so-called neuroblasts and migrate radially *via* the rostral migratory stream (RMS) towards the OB. It has also been shown that neurons born in the SVZ can migrate into the cortical regions in the primate (135). Type A cells express beta-3-tubulin, polysialylated neural cellular adhesion molecule, doublecortin (DCX) and Dlx-2 transcription factor. Once they have reached the OB, they differentiate into different subtypes of mature interneurons, mainly GABAergic granule neurons (136). The function of OB neurogenesis is not fully understood; however, several studies point towards an important role in olfaction (137, 138). This process has been described in humans (139) and, while the number of migrating neuroblasts is high during infancy, it decreases dramatically upon ageing (134, 140).

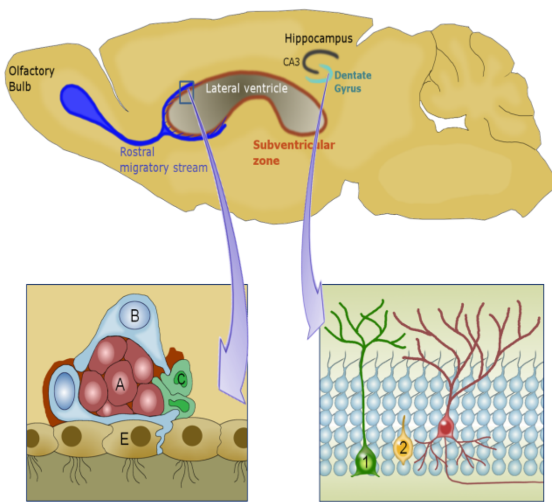

Figure 1. Illustration of neurogenesis in the adult brain; SVZ and the DG of the hippocampus. There are three types of progenitor cells lying adjacent to the ependymal cell layer (E) in the SVZ. Type B cells are the quiescent GFAP+ cells. They function as precursors of rapidly dividing transit-amplifying type C cells. The type A cells migrate through the rostral migratory stream towards the olfactory bulb. In the SGZ, there is a population of type 1 cells that are GFAP+ (Sox2+ nestin, and Hes5) quiescent NSCs. They co-exist with actively proliferating; GFAP+ Sox2+ NSCs the type 2 cells that give rise to both astrocytes and neuroblasts. Neuroblasts then migrate into the granule cell layer and mature into neurons (red cell). *Permission from Sandrine Thuret and Hindawi Publishing Corporation*.

3.1 SVZ neurogenesis and CNS disorders

Adult neurogenesis can be modulated by different factors, *e.g.* growth factors, peptides and neurotransmitters, but also by traumatic brain injuries and neurological diseases and disorders, suggesting that this process can play an important role not only under physiological but also under pathological conditions (141). For example, studies on Huntington disease (HD) animal models and *post mortem* human tissues have shown impairment in the hippocampal stem cell proliferation (130), suggesting an involvement of neurogenesis in depression and other mood disturbances that occur in up to 50 % of the HD population (142). Moreover, HD is a disease affecting striatum and studies both in animals and humans have shown that there is an up-regulation of progenitor cells in the HD SVZ, where cells proliferate and migrate towards the injured striatum (143, 144).

Of relevance for this thesis, SVZ proliferation and striatal neurogenesis have been extensively documented after stroke both in the rodent (145, 146) and human (147) brain. In particular, stem cell proliferation in the SVZ is increased in response to stroke, and a small fraction of neuroblasts deviates their migration towards the damaged striatal area, away from their normal migratory stream to the OB (146, 148). In the striatum they express markers of developing and mature (striatal medium-sized spiny) neurons, which have been suggested to play a role in functional recovery (149). Whether this is a causal link, rather than a correlation, remains to be determined (150).

Another important observation linking SVZ adult neurogenesis and neurological disorders derives from observations in both AD animal models and *post mortem* tissues from the human AD brain. Although different animal models have shown contradictory results, a recent study in the human brain showed that SVZ neurogenesis is impaired in AD (151). The data were also confirmed recently by employing an animal model where the Aβ and *tau* pathologies are reproduced (152). Whether decreased neurogenesis in AD plays a role in the development of the disease remains to be determined.

Reduced OB neurogenesis has been observed in PD model animals and resulted in impaired odor discrimination, which is also common in PD in human (153, 154). If impaired odor discrimination is related to the disease, is still unknown. However, the data about SVZ neurogenesis regulation in PD are contradictory since in a PD study in human (*post mortem* analysis) showed a profound increase in the proliferative capacity of SVZ (155), whilst another study showed opposite results (156, 157).

In conclusion, a large number of studies have shown that adult SVZ neurogenesis is regulated in response to brain pathologic states. However, the functional significance of SVZ neurogenesis regulation in neurological disorders has yet to be understood.

3.2 SVZ neurogenesis and diabetes

Recent pre-clinical studies have showed impaired adult neurogenesis in diabetes [reviewed by Bachor *et al*. (158)]. One study demonstrated a marked decrease of proliferating BrdU-labeled cells both in the SVZ and DG of streptozotocin (STZ)-treated rats, which develop a type-1 like-diabetes (159). Another study showed a significant increase of proliferating neural progenitors in SVZ and DG of a T2D animal model, *viz*. the Goto-Kakizaki (GK) rat (160). However, the survival of the newly formed cells rapidly and significantly decreased. One reason could be their lack of responsiveness to growth factors, since NSC cultures derived from lean control animals formed neurospheres (NS) when exposed to growth factors, whereas the GK rat NSCs did not (160). The precise nature of the mechanisms that mediate dysfunction of the neurogenic niches in diabetes is unknown. However, the diabetic impact on neurogenesis may change according to the magnitude of the metabolic derangement (158) and it is also possible that T1D and T2D impose different effects. In addition, diabetes can cause chronic inflammation and oxidative stress, which may independently promote neuronal death and inhibit neurogenesis (161, 162). In support of the hypothesis that impairment of SVZ neurogenesis in diabetes has physiological relevance are recent data showing that dysfunctional hippocampal neurogenesis in diabetic and obese animal models (163-165) resulted in cognitive impairment (166).

3.2.1 Pharmacological normalization of SVZ neurogenesis for the treatment of diabetic CNS complications

As explained in the chapters above, diabetic patients suffer from several neurological disorders at a very high rate and often prematurely. Adult neurogenesis is impaired in experimental models of T1D and T2D, as well as in experimental models of CNS disorders/diseases, and in *post mortem* human brain tissues. It remains to be determined whether the pharmacological normalization of adult neurogenesis in diabetes can decrease the rate of CNS complications. In support of this hypothesis are results showing that pharmacological stimulation of adult neurogenesis correlates with beneficial effects in animal models of PD, AD and amyotrophic lateral sclerosis (157). Furthermore, a great number of studies on the efficacy of candidate drugs in animal models of stroke have correlated improved histological and behavioral recovery with increased SVZ/hippocampal neurogenesis (146, 167). Some of the identified factors that can regulate neurogenesis are Noggin (168), vascular endothelial growth factors (VEGF) (169) and BDNF (170), where intraventricularly infused BDNF increased the number of newly born neurons in the OB of the adult rat brain, and moreover promoted neuronal migration into other parts of the brain, such as striatum, septum, thalamus and hypothalamus. Extrinsic stimuli, such as voluntary exercise, have also shown positive results to counteract the decline in neurogenesis in aged animals (171).

3.2.2 PACAP

The pituitary adenylate cyclase-activating polypeptide (PACAP) (172) is a 38-amino acid neuropeptide that is member of the VIP/secretin/glucagon peptide family, also including the vasoactive intestinal peptide (VIP) (173). PACAP and VIP share 67 %

amino acid sequence. PACAP and VIP are pleiotropic and multifunctional peptides, possessing neurotrophic and neuroprotective properties (174-176). They are involved in regulating important biological functions in the brain, digestive tract, cardiovascular system, airway, reproductive systems, and the immune system (176). In addition, studies *in vitro* and *in vivo* have shown their role in insulin secretion in both rodents and human islets (176-181). PACAP/VIP have three receptor subtypes named PAC1, VPAC1 and VPAC2. VIP binds with high affinity to VPAC1 and VPAC2 receptors but with lower affinity to the PAC1 receptor. PACAP and VIP receptors are both member of the class B (class II) GPCR family (182). PAC1, VPAC1 and VPAC2 receptors are coupled to the $G_{\alpha s}$ protein, regulating intracellular concentration of cyclic adenosine monophosphate (cAMP) through stimulation of AC. PACAP receptor also modulates intracellular calcium and inositol triphosphate levels through the phospholipase C pathway (173). PACAP receptors are widely distributed in the body and high levels of PAC1 receptors have also been reported in neurological disorders such as brain trauma, PD and AD (183-186). PACAP is found in the hypothalamic area, pituitary and the adrenal gland (176, 187), while VPAC receptors are expressed mainly in the lung, liver, and the testis (178, 188-190). Moreover, PAC1 and VPAC1/VPAC2 receptors play critical roles in early development of the CNS (190-192). Neuroprotection through PAC1 signaling is well documented (193-196) and PAC1 expression levels have been shown to be increased in pathological states, such as ischemic brain injury (196-199). However, the neuroprotective actions of VPAC1 and VPAC2 have also been reported in neurological disorders such as brain trauma, PD and AD (183-186). In addition, targeting each of these receptor subtypes has recently been proposed for the treatment of multiple sclerosis (200).
Germane for this thesis, recent data have shown an important role of PACAP in the regulation of adult and embryonic neurogenesis, where studies have shown that PACAP induces NSC proliferation/survival and self-renewal both *in vitro* and *in vivo* (201-203).

3.2.3 Galanin

Galanin is a 28 (mouse) or 30 (human) amino acid peptide with a wide range of biological effects. Galanin is involved in metabolism and reproduction (204, 205), survival, regeneration (206), cognition (207, 208) nociception (209, 210), but also pathological conditions such as AD (211), addiction (212) and epilepsy (213). Galanin triggers cellular responses *via* at least three seven transmembrane GPCRs, namely GalR1-R2-R3; coupled *via* different G_i/G_0-proteins to inhibit AC, causing hyperpolarization *via* opening of K^+ channels (GalR1/R3) and increase intracellular Ca^{2+} *via* stimulation of phospholipase C (GalR2) (214, 215). Both GalR1 and GalR2 are abundantly expressed in the rat brain (216-219). Individual galanin receptors have been associated with specific physiological functions. For instance, the activation of GalR1 promotes overeating and weight gain (220), while GalR2 activation confers neuroprotection (221, 222). The GalR3 expression pattern has a more restricted distribution in rodents with transcript levels most abundant in the dorsal raphe nucleus (DRN) locus coeruleus (LC), amygdala and the hypothalamus (219, 223). In addition, the physiological and pathological role of GalR3 in the brain is less well characterized (224, 225). Recent studies have shown the expression of galanin and galanin receptor mRNA in neurogenic areas of the adult mouse brain, but also in the SVZ and in hippocampal-derived NSCs cultures (226, 227). In addition, a study in mice has shown that GalR2 and/or GalR3 activation can regulate NSC differentiation (228).

AIMS

The overall goal of this thesis was to identify new potential therapeutic targets for the treatment of diabetic brain complications. We addressed this by running two projects, one *in vitro* and one *in vivo*.

In project 1 we studied the effect of diabetes-like conditions on adult neurogenesis. We hypothesized that adult neurogenesis impairment can be an important player linking diabetes and its neurological complications. Thus, we aimed to identify compounds able to increase the viability of adult NSCs in response to a diabetes-like *milieu in vitro*. The specific aims of the projects were:

1) To establish an *in vitro* assay in which to study the effects of stimulated hyperglycemia and hyperlipidemia on adult NSCs isolated from the mouse SVZ.
2) To identify some of the mechanisms by which a diabetes-like state (*i.e.* glucolipotoxicity) negatively impacts adult NSC viability *in vitro.*
3) To determine the putative protective effects of PACAP and galanin on adult NSC viability against diabetic glucolipotoxicity *in vitro*.

In project 2 we studied whether a neuroprotective strategy based on the activation of GLP-1R was efficacious against stroke in T2D rats. The specific objective of the projects was:

1) To prove efficacy of the specific GLP-1R agonist Ex-4 against stroke in the Goto-Kakizaki (GK) T2D rat model.

MATERIALS AND METHODS

1. *In vitro* and *ex vivo* experiments (Project 1: studies I-II)

1.1 SVZ isolation and primary NSC cultures

To obtain NSCs from the adult brain, the SVZ of the lateral brain ventricle of adult male mice 5 to 7 weeks of age (five C57 BL6/SCA mice in each experiment) was micro-dissected by using a micro-dissector scissor and enzymatically dissociated in 0.5 mg/ml trypsin, 0.8 mg/ml hyaluronidase and 80 U/ml deoxyribonuclease I (Sigma-Aldrich; St Louis, MO) in Dulbecco's modified Eagle's medium/ F12 (DMEM/F12; Gibco, Life Technologies; Sweden) containing B27 supplement (Gibco, Life Technologies; Sweden), 4.5 mg/ml glucose, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate and 12.5 mM HEPES buffer solution (Invitrogen, Stockholm, Sweden). The enzymatic digestion was carried out at 37 °C for 20 min. After a gentle trituration with a pipette and mixing, cells were passed through a 70 µm strainer (BD Biosciences; Sweden) and pelleted at 1,000 rpm for 12 min. The centrifugation step was repeated once more after removing the supernatant by adding fresh cold DMEM/F12. The supernatant was then removed, and cells were re-suspended in DMEM/F12 supplemented with B27 and 18 ng/ml human epidermal growth factor (EGF; R&D systems; Oxon, U.K.). Cells were plated in a 10 cm Petri dish and incubated at 37 °C for 7 days in order for NS to develop. After 7 days, the NS were collected and centrifuged at 1,000 rpm for 10 min. To obtain NSCs, NS were re-suspended in 0.5% trypsin/EDTA (Invitrogen; Sweden), incubated at 37 °C for 2 min and triturated gently to aid dissociation. After a further 2 min incubation at 37 °C, the cell preparation was diluted 1/20 in DMEM/F12 at 37 °C. NSCs were then pelleted at 1,000 rpm for 10 min and re-suspended in fresh DMEM/F12 containing 18 ng/ml EGF and 16 ng/ml human basic fibroblast growth factor (bFGF; R&D systems; Oxon, U.K.) before plating. NSCs were then expanded for 5 days to form new NS before the next passage and splitted again. All experiments were performed between passage 2 and 5 **(studies I-II).**

1.2 NSC viability assays

1.2.1 Fatty acid-enriched media

To mimic a hyperglycemic and hyperlipidemic *milieu in vitro*, DMEM/F12 (19 mM glucose) containing B27 and sodium palmitate 0.1-0.5 mM (Sigma-Aldrich; St Louis, MO) was used. In the experiments, cells were maintained in 0.01 ng/ml of EGF. To obtain the desired palmitate concentration, the NSC medium was supplemented with 0.25% BSA (Roche Diagnostics; Mannheim, Germany) before adding the palmitate (from a 100 mM palmitate stock solution dissolved in 12.5% EtOH) **(studies I-II).** All groups received the same concentration of vehicle.

1.2.2 ATP assay

Previous reports have shown that intracellular ATP levels correlate to cell number (229). Thus, an assay based on the quantification of ATP was employed to assess NSC viability. NSCs were plated as single cells from NS (see above) into 96-well plates (Corning B.V. Life Sciences; Amsterdam, Netherlands) at the final concentration of 50,000 cells/well in DMEM/F12 supplemented with B27 **(study I-II)**.

PACAP 38 (1-100 nM) (Phoenix Pharmaceuticals; Burlingame, CA), the specific PAC1 agonist Maxidilan-4 (Max-4; 30 nM; graciously provided by Dr. R.G. Titus),

Bay 55-9837 (500 nM) (VPAC2-specific agonist) or [Ala^{2,8,9,11,19,22,24,25,27,28}]-VIP (1 μM; VPAC1-specific agonist) (Tocris Biosciences; Bristol, U.K.) were added to NSCs 15 min before exposing the cells to 0.1-0.5 mM palmitate depending on the experiments. The cultures were maintained for 24 hours until NSC viability was assessed. In order to study whether NSC viability was increased by PAC1 activation, the specific PAC1 antagonist PACAP 6-38 (1 μM; Tocris Biosciences; Bristol, U.K.) was added to the cells 5 min before adding PACAP 38 (100 nM) **(study I)**.

Native galanin (0.1-10 nM; Gal 1-29, Tocris Biosciences; Bristol, U.K.), the GalR2-3 agonist Gal2-11 (AR-M 1896 0.1-10 nM; Tocris Biosciences, Bristol, U.K.), the selective GalR3 antagonist SNAP-3788 (1 μM; KO Key Organics; London, UK), the selective GalR2 antagonist M871 (1 μM; kindly given by Prof. Ülo Langel, Stockholm University), the ER stress inducer Salubrinal (50 μM; Calbiochem; La Jolla, CA) or PACAP 38 (100 nM) were added to NSCs 15 min prior to exposing the cells to the desired palmitate concentrations **(study II)**.

After 24 hours of incubation at 37 °C (5% CO_2, 98% humidity), intracellular ATP levels were measured using the Cellular ATP Kit HTS according to the manufacturer's instructions (BioThema; Stockholm, Sweden). In these experiments, the effect of each treatment at a certain concentration was determined in quadruplicates or octuplicates in 3-7 different sets of experiments. The experimental design is illustrated in Figure 2 **(studies I-II)**.

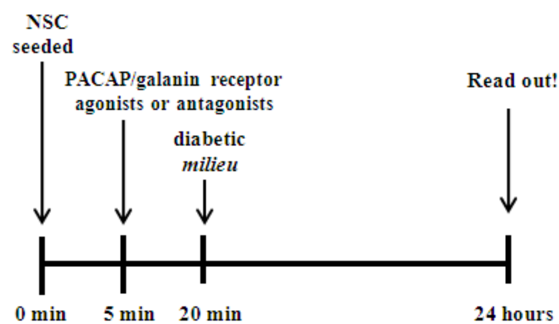

Figure 2. Experimental design of *in vitro* experiments

1.2.3 MTT assay

To confirm the cell viability results of the ATP assay, 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) levels (230) were also measured in some experiments. MTT levels were measured using the CellTiter96® non-radioactive cell viability assay according to the manufacturer's instructions (Promega; Madison, WI). In these experiments, the effect of each treatment at a certain concentration was determined in quadruplicates in 3 different experiments **(study I)**.

1.3 NSC ^{3}H-thymidine incorporation

To measure NSC proliferation in response to PACAP, ^{3}H-thymidine incorporation into DNA was assessed. NS were dissociated to a single-cell suspension and plated as single cells in 6-well plates (1,000,000 cells/well). PACAP (100 nM) alone or palmitate (0.2 mM) plus PACAP were added in duplicates and cells incubated at 37 °C for 24 hours **(study I)**.

To measure NSC proliferation in response to AR-M 1896 (GalR2/R3 agonist), ^{3}H-thymidine incorporation was also assessed. AR-M 1896 (10 nM) or PACAP (100 nM) (serving as positive control) were added in duplicates and cells incubated at 37 ^{o}C for 24 hours **(study II)**.

^{3}H-thymidine (1 µCi/ml) (Amersham Biosciences; Piscataway, NJ) was present from after plating till when the cells were harvested 24 hours later. Cells were harvested and radioactivity was measured using a microplate scintillation and luminescence counter (Wallac MicroBeta® Trilux; PerkinElmer; Waltham, MA) **(study I-II)**.

1.4 Western blotting experiments for apoptosis and ER stress markers

To assess whether PACAP/galanin modulate apoptosis and UPR signaling, Western blot (WB) experiments were performed. NSCs were plated as single cells and expanded in a 10 cm Petri dish with EGF/bFGF (see 1.1) for 3-4 days. When NS were formed, a 15 minute pre-treatment of PACAP (100 nM) or the equally affinity agonist for GalR2/3, ARM18-96 (10 nM) was carried out before exposing the cells to palmitate (0.3 mM). NSCs were then incubated for 24 hours. After NS formation, NS were harvested, washed twice with PBS, centrifuged at 1,000 rpm and homogenized on ice. Samples were clarified by centrifugation. The supernatants were transferred into new tubes and the total protein concentration was determined by Lowry protein assay (Bio-Rad Laboratories: Stockholm, Sweden). Samples were then mixed with reducing SDS-PAGE sample buffer and boiled for 5 min before performing SDS-PAGE. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories; Stockholm, Sweden). Immunoblot analyses were performed with antibodies against the cleaved form of caspase-3 (1:1,000), phosphorylation/total of JNK (1:3,000) (Cell Signaling Technology; Danvers, MA), Bcl-2 (1:200) (Abcam; Cambridge, MA) and CHOP (1:2,000) (Santa Cruz, Biotechnology; Stockholm, Sweden). Immuno-reactive bands were developed using ECL (GE Healthcare; Stockholm, Sweden), imaged with a GelDoc system and quantified with Quantity One software (Bio-Rad Laboratories; Stockholm, Sweden). After imaging, to verify equal protein loading, the PDVF membranes were stained with Coomassie blue (Fermentas; St. Leon-Rot, Germany).

In all these experiments, the effect of each treatment at a certain concentration was determined in single or double samples in 2-5 different set of experiments **(study I-II)**.

1.5 mRNA quantitative expression studies (RT-PCR)

1.5.1 PAC1, VPAC1, VPAC2, Galanin, GalR1, GalR2, GalR3, CHOP/GADD153, BIP/GRP78

To quantify the mRNAs of galanin and PACAP receptors and ER stress markers from NSCs *in vitro,* we used NSCs grown in EGF/bFGF (see 1.1) at passages 2-4 in presence or absence of palmitate (0.3 mM) and/or the different treatments for 24 hours. Effects were determined in single samples in 3-6 different sets of experiments.

In the *ex vivo* quantitative experiments, tissue from the SVZ of the lateral ventricle (less than 1 millimeter of tissue facing the lateral ventricle and including the SVZ) or the deeper part of striatum (not including the SVZ) was isolated for analysis (see above). Three to twelve brains, isolated from 5 week-old (young-adult pre-diabetic) and 36 week-old (middle aged diabetic) *ob/ob* mice plus their cognate lean littermates, were pooled in each experiment.

Total RNA was extracted using Aurum total RNA-mini kit (Bio-Rad Laboratories; Stockholm, Sweden) and the RNA was treated with DNase I (Bio-Rad Laboratories; Stockholm, Sweden) to eliminate possible DNA contamination, according to the manufacturer's protocol. Total mRNA was reversely transcribed into cDNA by using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories; Stockholm, Sweden). The expression levels of mRNAs were measured by SYBR green based quantitative RT-PCR (iQ™ SYBR® Green Supermix; Fermentas; St. Leon-Rot, Germany) using mouse-specific primer pairs for PAC1 transcript variant 1, VPAC1 and VPAC2 (Invitrogen; Stockholm, Sweden) **(study I)** and mouse-specific primer pairs for Galanin, GalR1, GalR2, GalR3, CHOP/GADD153, BIP/GRP78 (Invitrogen; Stockholm, Sweden) **(study II)**.

β-actin was used as an internal standard in all experiments. Reactions were resolved on a 1 % agarose gel containing ethidium bromide and the bands were visualized under UV light to verify the correct sizes of the amplification products.

1.5.2 Measurement of XBP1 mRNA splicing

To determine whether diabetic glucolipotoxicity induces UPR signaling, *e.g.* the IRE1 pathway, total RNA was isolated and cDNA synthesized from NSCs as described above after exposure to palmitate (0.3 mM) for 24 hours. Mouse XBP1 cDNA was amplified using iProof Master Mix (Fermentas; St. Leon-Rot, Germany) and XBP1 primers, flanking the 26 bp splicing site (231), which contains a PstI restriction site (CTGCAG). The PCR product was incubated with 1 µl fast digest PstI per reaction (Fermentas) for 30 min at 37 ^{o}C and the digested product was then separated on a 2 % agarose gel and visualized using ethidium bromide. The gel shows one band at 746 bp for spliced XBP1 and two bands at 458 and 314 bp for the non-spliced XBP1 **(study II)**.

1.6 The ob/ob T2D mouse model

The Lep $^{ob/ob}$ mouse is a monogenic model of obesity defective in leptin signaling; a commonly model used in T2D research (232). It derives from a spontaneous mutation of the leptin gene (233). Their weight gain begins to increase already at 2 weeks at which they develop hyperinsulinemia. At 4 weeks, hyperglycemia is reached, with blood glucose continuing to rise, peaking at 3-5 months, after which it falls when the mouse becomes older (234). Other metabolic derangements include hyperlipidemia, disturbance of temperature regulation and lower physical activity. Moreover, the pancreatic volume is increased, where some moderate insulin secretion is maintained (235); therefore, this model of diabetes is not completely representative of human T2D.

1.7 Statistical analysis

For both *ex vivo* and *in vitro* experiments, data are presented as mean ± SEM. Student's *t*-test was used when comparing the difference between two groups, while multiple comparisons were made by one-way ANOVA followed by *post hoc* Fisher LSD test or Kruskal-Wallis (Sigma Plot v. 11 software). $P < 0.05$ was considered statistically significant.

1.8 Ethical considerations

All experiments were conducted according to the 'Guide for the Care and Use of Laboratory Animals' published by U.S. National Institutes of Health (NIH publication # 85-23, revised 1985) and approved by the regional ethics committee for animal experimentation

2. ***In vivo*** experiments (Project 2: study III)

2.1 The Goto-Kakizaki (GK) T2D rat model

To determine the potential anti-stroke efficacy of the GLP-1R agonist Ex-4 in T2D, the GK-rat was employed. The GK-rat is a genetic animal model of T2D, originated via a spontaneous mutation by repetitive breeding of Wistar rats with the poorest glucose tolerance (236). The GK-rat is characterized by moderate hyperglycemia (12-16 mM), predominantly prandial in nature expected from its insulinopenic phenotype, glucose intolerance and defect in glucose-induced insulin secretion, whereas insulin resistance is a less prominent feature (237).

2.2 Experimental design

A total of 42 7–9-month-old male diabetic GK-rats were used. Before the start of the Ex-4/PBS treatments, baseline fasting blood glucose concentrations were measured and the animals were assigned to the different treatment groups so that mean blood glucose values were equalized. Treatment groups thus created were tested for normality using the D'Agostino and Pearson omnibus normality test. All rats received intraperitoneal injections of Ex-4 [0.1, 1 or 5 μg/kg of bw (body weight) (*n*=10)] or PBS (*n*=12) for 4 weeks twice daily before being subjected to stroke (see below). Ex-4 and PBS injections were continued for another 2 or 4 weeks following the stroke before sacrifice. To assess neurogenesis, all rats received daily intraperitoneal injections of the thymidine analogue bromodeoxyuridine (BrdU; 50 mg/kg of bw) for 2 weeks following stroke. Rats were sacrificed for immunohistochemical analyses at 2 or 4 weeks after stroke. The experimental design is illustrated in Figure 3.

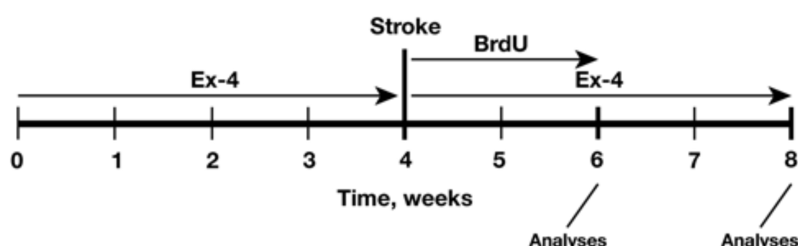

Figure 3. Experimental design and drug administration paradigm

2.3 Transient MCAO [MCA (middle cerebral artery) occlusion]

To induce stroke in T2D GK-rats, we employed one of the most common stroke experimental models, which is based on the transient unilateral middle cerebral artery occlusion (MCAO) (238), in which a round headed tip filament is inserted in from the

common carotid artery through the internal carotid artery into the circle of Willis. By so doing, the blood flow is blocked in one brain hemisphere. Reperfusion is achieved by removing the filament. This model provides reproducible MCA territory infarctions, involving the cortex and striatum. Moreover, blood reperfusion simulating **thrombolysis** is allowed by withdrawing the filament at a desired time. As a result, the length of ischemia and the severity of the neuronal damage can be adjusted according to the needs of the experiments (239, 240).
To perform MCAO, rats were anesthetized by spontaneous inhalation of 1.5% isofluorane through a snout-mask. Body temperature was maintained at 37–38 ˚C using a heating pad. To occlude the MCA in our experiments, the common and external carotid arteries were ligated, and the internal carotid artery was temporarily closed. A monofilament was advanced through the internal carotid artery to the origin of the MCA, the wound was closed and the animal was allowed to wake up and was placed in its cage. After 90 min of occlusion, the animals were anaesthetized again and the filament was withdrawn to allow for blood reperfusion. The surgeon performing the operation was blinded to the treatment groups.

2.4 Immunocytochemistry (IHC)

To quantify brain neurons, glia, neurogenesis and GLP-1R expression, several markers were assessed by IHC. Animals were deeply anesthetized and perfused transcardially with 4 % paraformaldehyde. The brains were extracted and submersed in 20 % sucrose in phosphate buffered saline overnight. 40-μm-thick coronal sections were cut by using a sliding microtome and stained as free-floating sections. The following primary antibodies were used: mouse anti-Ki67 (1:200; Novocastra; Sweden), a marker of cell proliferation; goat anti-DCX (doublecortin; 1:400 dilution; SantaCruz Biotechnology), a marker for migrating neuroblasts; mouse anti-NeuN (1:100; Merck Millipore; Billerica, MA), a neuronal marker; rabbit anti-Iba1 (1:1,000; Wako Chemicals GmbH; Germany), a marker for microglia; mouse anti-ED1 (1:200; ABd Serotec; Düsseldorf, Germany), a marker of activated microglia; rat anti-BrdU (1:200;Santa Cruz Biotechnology), to assess neurogenesis in combination with NeuN; rabbit anti-GLP-1R (1:50 dilution) (Abcam; Cambridge, MA). In the sections stained for GLP-1R, DAPI (4,6-diamidino-2-phenylindole) was used to visualize all the cells.

Sections were incubated with primary antibodies overnight at 4 ˚C in phosphate buffer saline (PBS) containing 3 % appropriate serum and 0.25 % Triton X-100. Primary antibodies were detected by the use of appropriate fluorescent Cy3 (indocarbocyanine), Cy2 (carbocyanine) (both from Jackson ImmunoResearch) or biotin-conjugated (Vector) secondary antibodies (1:200 dilution). Sections were incubated with secondary antibodies for 2 hours at room temperature (approx. 21 ˚C) in phosphate buffer containing 3 % of the appropriate serum and 0.25 % Triton X-100. For chromogenic visualization, biotinylated secondary antibodies, avidin–boitin complex (ABC kit) (Vector Laboratories; Burlingame, CA) and diaminobenzidine were used.

2.5 Tissue damage evaluation and cell quantification

Tissue damage evaluation and cell counting were performed blindly. For tissue damage evaluation, the NeuN-labeled tissue sections were displayed live on the computer screen and the area of contralateral hemisphere and the area of the intact ipsilateral tissue were measured in every section containing stroke damage using the NewCast

(Visiopharm) software. To compensate for the stroke-induced morphological tissue changes, the infarct volume was calculated by subtracting the volume of remaining tissue in the ipsilateral hemisphere from the volume of the contralateral hemisphere. Immunoreactive cells were counted using either a computerized non-biased setup for stereology, driven by the NewCast software, or by using an Olympus BX40 epifluorescence/light microscope. The number of neurons (NeuN) and microglia (Iba1 and ED1) cells was quantified using the optical fractionator method (241, 242). Briefly, brain sections were displayed live on the computer monitor and the striatum and cortex delineated at low magnification. Quantifications were performed using a 100 × oil-immersion lens. Ten evenly spaced sections in parallel-cut series through the entire striatum were included. Random sampling was carried out using the counting frame; this was systematically moved at predefined intervals so that ~200 immunoreactive cells were counted. The total number of cells was estimated according to the optical fractionator formula (241, 242). For evaluation of SVZ cell proliferation, SVZ cells immunoreactive for Ki67 were counted. DCX is a marker for newly produced neuroblasts from neurogenic brain areas and DCX-positive cells have been shown to migrate into the ischemic striatum (145). Hence, neuroblast production was quantified by counting cells positive for DCX in stroke-damaged striatum. NeuN is a ubiquitous nuclear marker for mature neurons. Thus, neurogenesis was assessed by counting NeuN/BrdU-positive cells in stroke-damaged striatum. These quantifications were performed in three evenly spaced (400 μm) brain sections on the side ipsilateral to stroke, starting at 0.7 mm anterior to Bregma.

2.6 Statistical analysis

Statistical analyses were performed using one-way or two-way ANOVA, followed by *post hoc* Bonferroni's or Dunnet's test (Prism 5). Differences between groups were considered statistically significant when $P<0.05$. Data are presented as mean ± SEM.

2.7 Ethical considerations

All experiments were conducted according to the 'Guide for the Care and Use of Laboratory Animals'published by U.S. National Institutes of Health (NIH publication # 85-23, revised 1985) and approved by the regional ethics committee for animal experimentation.

RESULTS

1. Project 1

1.1 Study I

Pituitary adenylate cyclase-activating polypeptide counteracts the impaired adult neural stem cell viability induced by palmitate

Our results show that a diabetic-like, *e.g.* high glucose and fat-enriched *milieu,* impaired NSC viability in a dose-dependent manner after 24 hours *in vitro*. We also show that this glucolipotoxicity was associated with an induction of apoptosis, as evidenced by increased and decreased cellular levels of the pro-apoptotic cleaved caspase-3 and of the anti-apoptotic Bcl-2 markers, respectively. We also demonstrate that PACAP was able to counteract the glucolipotoxic effect through the specific activation of PAC1 receptor, but not of VPAC1 or VPAC2 receptors. This effect was entirely mediated through cell protection, since NSC proliferation was not affected. This was further confirmed by another cell viability assay, the MTT assay. We also show that the neuroprotective effect of PACAP correlated with decreased apoptosis as measured by Bcl-2 protein levels. To study whether glucolipotoxicity impacted the expression of PACAP receptors *in vitro* and *ex vivo*, regulation studies of PACAP receptors were performed. The results of these studies reveal that PAC1 and VPAC2 receptors are expressed by NSCs *in vitro* and are up-regulated under the duress of a glucolipotoxic *milieu*. In addition, we show that PAC1, VPAC1 and VPAC2 receptors are expressed in the SVZ/striatum *ex vivo* and are up-regulated in *ob/ob* mice (both young and middle-aged) *versus* their cognate lean litter-mates.

In conclusion, our results indicate that a diabetic *milieu* -- mimicked by high glucose and palmitate -- decreases NSC viability. Furthermore, we show that this effect can be partially counteracted by PACAP through the activation of PAC1 receptors. Finally, we show that PACAP receptors in NSCs are regulated by a fat-enriched *milieu* both *in vitro* and *ex vivo*.

1.2. Study II

GalR3 activation promotes adult neural stem cell survival in response to a diabetic milieu

In study I, we showed that high glucose and palmitate decrease NSC viability in conjunction with increased apoptosis. In this study, we determined whether the UPR signaling/ER stress was increased by high glucose and palmitate in NSCs *in vitro*. Our results show that a diabetic *milieu* up-regulates NSC mRNA and protein levels of the ER stress markers CHOP and BIP and activates the IRE1 pathway by phosphorylation of JNK and by enhancing the alternative splicing of XBP1. In the second part of this study, we aimed to determine whether the neuropeptide galanin (and by which receptor) could counteract a diabetic glucolipotoxicity in NSCs and -- if so -- by regulating apoptosis and the UPR signaling pathways. We demonstrate that galanin, *via* GalR3 activation, counteracts NSC glucolipotoxicity. This neuroprotective effect correlated with decreased cleaved caspase 3, indicating decreased apoptosis. Furthermore galanin led to decreased CHOP levels, indicating decreased ER stress. Finally, we studied the regulation of galanin and its receptor subtypes under diabetes-like conditions in NSCs *in vitro* and in the SVZ *ex vivo*. Our results revealed that GalR3 mRNA expression levels remained unchanged in response to high glucose and palmitate in NSCs *in vitro* as well as in young pre-diabetic *ob/ob* mice *ex vivo*. However, GalR3 expression was strongly down-regulated in middle-aged *ob/ob* mice

in comparison with their lean littermates. In addition, galanin and its receptors (GalR1-R2) were regulated by diabetes-like conditions *in vitro* and *ex vivo* both in young pre-diabetic and middle-aged *ob/ob* mice. In summary, we show that glucolipotoxicity -- mimicked by a diabetic *milieu* -- correlates with increased ER stress as indicated by increased levels of CHOP, BIP, spliced form of XBP1 and phosphorylation of JNK. Our results also show that galanin, *via* GalR3 activation, can counteract this glucolipotoxic effect. Finally, our results show that galanin and its receptors are regulated by a glucolipotoxic *milieu in vitro* and by T2D/obesity *ex vivo*.

2. Project 2

2.1 Study III

Glucagon-like peptide-1 receptor activation reduces ischemic brain damage following stroke in type 2 diabetic rats

Our results show that peripheral administration of the stable GLP-1R agonist Ex-4 (for 4 weeks before stroke and for 2 or 4 weeks after) counteracts ischemic brain damage in aged T2D GK rats, conferring a profound (~ 70 %) anti-stroke effect. The effect was dose-dependent and already statistically significant at the clinically used dose for T2D of 0.1 μg/kg bw. The lowest neuroprotective employed dose of Ex-4 was ineffective on glycemia, suggesting that the neuroprotective effect occurred independently of glycemia. Moreover, Ex-4 induced an arrested microglia infiltration and an increase of stroke-induced NSC proliferation and neuroblast formation, whereas stroke-induced neurogenesis was not affected.

In conclusion, our findings indicate that the GLP-1R agonist Ex-4, at clinically relevant doses, confers neuroprotection in T2D rats subjected to stroke. The effect correlates with decreased inflammation and increased NSC activation and appears essentially independent of glycemic changes.

GENERAL DISCUSSION

Modern lifestyle, with excessive energy intake and lowered physical activity, has caused an alarming increase in obesity-related pathological conditions, including T2D. Moreover, the incidence of neurodegenerative disorders is increasing in the Western world accompanying the growing number of obese and elderly people (158). Diabetic patients have a substantially higher risk to prematurely develop neurological complications such as stroke, dementia, AD, and PD [see Intro and (20, 22, 243-246)]. Furthermore, there is a strong co-morbidity between T2D and AD (247), sometimes referred to as type 3 diabetes, the reasons for which are largely unknown. Increasing our knowledge on the pathogenic mechanisms as to why diabetes is such a strong risk factor for premature CNS disorders will be required to be able to find novel drugable targets for therapeutic use.

1. Project 1

In T2D, there is a plethora of changes that alone or together can impair brain metabolism and affect neuronal viability, such as glucose toxicity, hypoglycemia, hyperlipidemia, hypertension, increased inflammation, oxidative stress and insulin resistance (40). These events may lead to damage of cerebral microvasculature and/or neural tissue, which in turn could influence the development of brain disorders, including neurodegeneration (see Intro). In addition to these more characterized factors, recent pre-clinical studies have shown that adult NSC populations in the SGZ and SVZ in the adult CNS respond to different brain diseases/disorders (143, 151, 155, 156, 248). Furthermore, other studies have demonstrated adult neurogenesis impairment both in SVZ and hippocampus of obese and diabetic animal models (159, 160, 163, 165, 166, 249-251). This suggests that dysfunctional endogenous cell replacement in diabetes through impaired adult neurogenesis could also play a role linking diabetes to its neurological complications. Consequently, pharmacological normalization of impaired neurogenesis in diabetes could be therapeutically useful in preventing or limiting diabetic CNS complications.

Although some animal models of diabetes have shown neurogenesis impairment, an *in vitro* system where to study and characterize at cellular and molecular level how diabetes impairs NSCs has not yet been established. To understand and exploit the potential therapeutic use of NSCs, methodological limitations need to be overcome. NSCs are difficult to study *in vivo*; this is mainly due to the lack of specific cell markers of these cells (252), but also to the low number of NSCs in the SVZ and SGZ (253). An excellent platform to overcome these limitations is to grow adult NSCs *in vitro* (254). In this thesis, we have developed an *in vitro* system that has been previously reported under normal (non-diabetic) conditions by Bertilsson *et al*. (97). In studies I-II, we determined the effect of diabetes-like conditions on NSC viability in this assay. We demonstrated that a hyperglycemic (high glucose) and hyperlipidemic (high palmitate) *milieu* impairs NSC viability in a dose-dependent manner, and that this glucolipotoxicity correlates with increased apoptosis. Since ER stress may play a fundamental role in the development and pathology of certain forms of both diabetes and neurodegenerative diseases (66, 101), we determined whether a sustained activation of UPR by a diabetic *milieu*, leading to ER stress, correlated to NSC death. We demonstrated that glucolipotoxicity induced the activation of UPR signaling through its known pathways [*e.g.* PERK, IRE1 and ATF6 (101)]. Specifically, we showed that mRNA and protein levels of CHOP and BIP, phosphorylation of JNK and enhanced alternative splicing of XBP1 were increased

by glucolipotoxic conditions. SVZ neurogenesis has been suggested to play an important role in stroke recovery [see Intro and (145-147)]. Moreover, diabetic patients show increased stroke incidence and decreased functional recovery. Thus, our *in vitro* assay could reveal NSCs as a useful tool where to identify molecules able to normalize neurogenesis in diabetes; this in view to develop new potential therapies against stroke in diabetes. Furthermore, due to the similar features of SVZ and hippocampal neurogenesis (118), our results could also be of relevance to determine the potential role of hippocampal neurogenesis in diabetes. Impairment of hippocampal neurogenesis in diabetes has been suggested to play a role in decreased cognition (164-166). Thus, we believe that our assay could represent a useful tool to also progress the knowledge in these research areas.

The next goal of this thesis was to identify potential molecules able to counteract loss of NSC viability under diabetes-like conditions. We identified PACAP (paper 1) and galanin (paper 2) as such molecules. Previous work has shown that both neuropeptides promote neurogenesis in naïve rodents (201, 203, 255) and have neuroprotective properties (182, 196, 199, 221, 256-258). The pleiotropic features of these peptides have been the main reasons of our selection. We demonstrated that PACAP and galanin significantly protect NSCs from glucolipotoxicity-induced cell death, by decreasing apoptosis. We were also interested to determine by which receptors the protective effect of PACAP and galanin is mediated. By taking advantage of several selective agonists and antagonists of both PACAP and galanin receptors, we showed that the protective effects of PACAP and galanin are mediated by PAC1 and GalR3, respectively. Further, we showed that activation of GalR3 modulated the UPR signaling; suggesting that galanin -- *via* GalR3 -- can reduce NSC apoptosis by decreasing ER stress. Whether the attenuation of ER stress by GalR3 activation could provide opportunities to advantage the therapeutic efforts aiming at preventing and treating ER-stress associated diseases is an attractive hypothesis for further studies.
In view of the potential therapeutic importance of the PACAP and galanin "systems", we were interested to determine PACAP and galanin receptor expression in SVZ as well as their potential regulation in response to diabetes-like states, both *in vitro* and *ex vivo*. We believe this type of information could be of potential pharmacological importance. While PAC1 mRNA expression in NSCs from the SVZ has been previously reported (201, 202), we demonstrated for the first time VPAC2 mRNA expression in NSCs. Further, we showed that the mRNA of each of the three PACAP receptors was present and up-regulated by both a glucolipotoxic *milieu in vitro* and in *ob/ob* T2D mice (both young and middle-aged) *versus* their controls. These types of data are difficult to interpret. However, in view of our results showing that PAC1 counteracts NSC glucolipotoxicity, we speculate that PAC1 up-regulation might be a cellular attempt to prevent glucolipoapoptosis. With regard to our studies on galanin and its receptor subtype expression and regulation, our results show that diabetes led to an overall modulation of the "galanin system" both *in vitro* and *ex vivo*. To note is that GalR3 mRNA expression was strongly down-regulated in aged diabetic *ob/ob* mice in comparison with their non-diabetic controls. Recent data has shown that galanin regulates survival and differentiation of NSCs (228). We speculate that GalR3 down-regulation in diabetes-like states could play a role in the decreased NSC survival and/or proliferative and differentiative capacity. As for PACAP receptor studies, these observations remain an interesting finding to be further investigated *in vivo*.

1.1 Conclusion Project 1

It is estimated that around 13 % of the adult U.S. population suffers from diabetes. The global number of people with diabetes is projected to double by 2030 (259, 260); a worrying scenario when considering the magnitude and severity of premature neurological complications in these individuals. Thus, we need to understand exactly how T2D negatively affects the brain and studies focused at the interface of neuroscience and diabetes research are crucial in this respect. Based on the hypothesis that adult neurogenesis is impaired in diabetes, and that this impairment could play a role in the development of the neurological complications present in diabetic patients, we have characterized an *in vitro* system in which it is possible to study this process under diabetes-like states. Although the results that we obtained are *in vitro,* and will have to be confirmed *in vivo*, our findings provide novel insights as to how a diabetic micro-environment has a negative impact on NSC viability. Furthermore, we show that PACAP and galanin protect NSCs from the toxicity of a diabetic *milieu*. We believe that these protective effects mediated by PACAP and galanin could be of relevance in the context of future drug development aimed at preventing and/or treating CNS complications in diabetes.

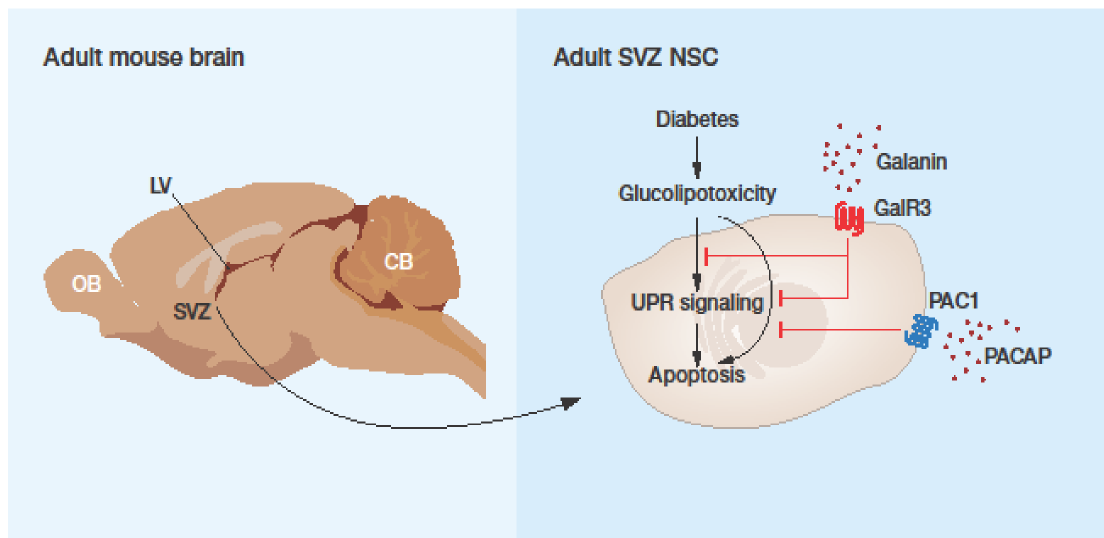

Figure 4. Adult neurogenesis impairment in diabetes could play a role in the development of neurological complications. We showed that PAC1 and GalR3 activation counteracts glucolipotoxicity in adult neural stem by decreasing apoptosis. We also show that at least part of the protective effect mediated by GalR3 activation occurs through the modulation of the unfolded protein response (UPR) signaling in the endoplasmic reticulum. The data supports a potential therapeutic development, based on increased neurogenesis by PAC1 and GalR3 activation, for the treatment of diabetic brain disorders. *Illustration by Mattias Karlén*.

2. Project 2

As extensively discussed in the Intro, stroke is over-represented in T2D and no therapy based on neuroprotection is currently available clinically (20). An ideal therapy for the treatment of stroke in T2D would be a drug that is effective in normalizing glycemia (without causing hypoglycemic effects) in combination with neuroprotective properties. Recent studies have suggested that GLP-1R agonists indeed show this type of profile (see Intro). However, although preclinical data have shown neuroprotection against stroke mediated by the specific GLP-1R agonist Ex-4 (95, 96, 261, 262), the applicability of such a therapy has not been investigated in a pre-clinical setting with clinical relevance. Furthermore no study aimed at proving the efficacy of Ex-4 against stroke has been carried out in diabetic rodents. Therefore, in study III, we aimed to closely mimic the way Ex-4 is used in the clinical practice against T2D by exploiting its neuroprotective properties against stroke. To do so, we administered clinical doses of Ex-4 for 4 weeks to T2D GK rats before inducing stroke by MCAO. We then continued with the Ex-4 treatment (or vehicle) for 2-4 weeks thereafter. Our goal was to mimic the situation of a T2D patient on GLP-1R therapy who then suffers a stroke. Here we demonstrated that a dose of Ex-4 clinically relevant for the treatment of T2D showed a significant increase in neuronal survival in both striatum and cortex. Moreover, the effects were found to be dose-dependent with the most effective response at a higher dose of Ex-4 (5 μg/kg). Interestingly, our data indicated that the effect was independent of glycemia. We also show that the highest dose of Ex-4 delayed pro-inflammatory microglia infiltration in the cortex, suggesting that at least part of the neuroprotective effect mediated by Ex-4 could occur by the regulation of microglia. Finally, the results show that, as expected, stroke-induced increase in the stem/progenitor cell proliferation. However, Ex-4 further increased the stroke-induced NSC proliferative response despite the fact that this increase of proliferation did not result in increased neurogenesis. Whether this increased proliferation NSC contributes to the reduction of the infarct volume and neuronal survival after stroke remains to be investigated.

2.1 Conclusion project 2

Our results show neuroprotective efficacy against stroke by Ex-4 in a T2D rat model. The results let us speculate that our findings could have clinical relevance for T2D patients who are at high risk to suffer a stroke. Diabetic patients could be kept on a therapy based on GLP-1R activation primarily against their diabetes (*e.g.* anti-hyperglycemic), while at the same time improving recovery after stroke by limiting brain injury. Indeed, recent clinical results showed that diabetic patients on GLP-1R therapy show a better cardiovascular profile and decreased stroke compared to those on sulfonylurea therapy (263). The molecular mechanisms at the basis of GLP-1R-mediated neuroprotection have been poorly investigated. Although we provided data showing that Ex-4 reduced microglia infiltration and increased NSC proliferation, more experiments are needed to precisely identify the mechanism(s) of action underlying the observed neuroprotection.

OVERALL CONCLUSIONS

The results of this thesis provide new knowledge about a potential mechanism by which T2D could lead to CNS complications: by regulating adult neurogenesis. In the established assay described in project 1, mechanistic aspects about how diabetes can negatively impact adult neurogenesis could be studied. If our hypothesis will be validated, this assay has also the potential to be developed into a screening platform for the identification of drugs against brain diabetic complications.

The results of this thesis also achieve new knowledge for the potential use of GLP-1R activation against stroke in diabetes. Previous data have showed GLP-1R-mediated neuroprotection against stroke by using experimental paradigms difficult to be translated to the clinical reality. Our results indicate the potential use of a GLP-1R activation therapy highly suitable for T2D patients suffering from a stroke.

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REFERENCES

1. Meetoo D, McGovern P, Safadi R. An epidemiological overview of diabetes across the world. Br J Nurs. 2007;16(16):1002-7.
2. IDF [Internet]. Available from: http://www.idf.org/diabetesatlas.
3. Dagens diabetes [Internet]. Available from: http://dagensdiabetes.se/home/forskning/1317-idf-2035-new-higher-figures-600-million-persons-with-diabetes-25-of-them-in-china.html.
4. Tisch R, McDevitt H. Insulin-dependent diabetes mellitus. Cell. 1996;85(3):291-7.
5. Kaneto H, Kawamori D, Matsuoka TA, Kajimoto Y, Yamasaki Y. Oxidative stress and pancreatic beta-cell dysfunction. American journal of therapeutics. 2005;12(6):529-33.
6. Kim KA, Lee MS. Recent progress in research on beta-cell apoptosis by cytokines. Frontiers in bioscience (Landmark edition). 2009;14:657-64.
7. Eringsmark Regnell S, Lernmark A. The environment and the origins of islet autoimmunity and Type 1 diabetes. Diabetic medicine : a journal of the British Diabetic Association. 2013;30(2):155-60.
8. Todd JA. Etiology of type 1 diabetes. Immunity. 2010;32(4):457-67.
9. American Diabetes A. Diagnosis and classification of diabetes mellitus. Diabetes care. 2012;35 Suppl 1:S64-71.
10. Callaghan BC, Hur J, Feldman EL. Diabetic neuropathy: one disease or two? Current opinion in neurology. 2012;25(5):536-41.
11. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes. 2004;53 Suppl 3:S16-21.
12. Diagnosis and classification of diabetes mellitus. Diabetes care. 2012;35 Suppl 1:S64-71.
13. Koshkin V, Wang X, Scherer PE, Chan CB, Wheeler MB. Mitochondrial functional state in clonal pancreatic beta-cells exposed to free fatty acids. The Journal of biological chemistry. 2003;278(22):19709-15.
14. Donath MY, Ehses JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, et al. Mechanisms of beta-cell death in type 2 diabetes. Diabetes. 2005;54 Suppl 2:S108-13.
15. Cavanagh PR, Lipsky BA, Bradbury AW, Botek G. Treatment for diabetic foot ulcers. Lancet. 2005;366(9498):1725-35.
16. Brands AM, Biessels GJ, de Haan EH, Kappelle LJ, Kessels RP. The effects of type 1 diabetes on cognitive performance: a meta-analysis. Diabetes care. 2005;28(3):726-35.
17. Cukierman T, Gerstein HC, Williamson JD. Cognitive decline and dementia in diabetes--systematic overview of prospective observational studies. Diabetologia. 2005;48(12):2460-9.
18. Stewart R, Liolitsa D. Type 2 diabetes mellitus, cognitive impairment and dementia. Diabetic medicine : a journal of the British Diabetic Association. 1999;16(2):93-112.
19. Strachan MW, Deary IJ, Ewing FM, Frier BM. Is type II diabetes associated with an increased risk of cognitive dysfunction? A critical review of published studies. Diabetes care. 1997;20(3):438-45.
20. Sander D, Kearney MT. Reducing the risk of stroke in type 2 diabetes: pathophysiological and therapeutic perspectives. J Neurol. 2009;256(10):1603-19.
21. Yang Y, Song W. Molecular links between Alzheimer's disease and diabetes mellitus. Neuroscience. 2013.

22. Vanitallie TB. Parkinson disease: primacy of age as a risk factor for mitochondrial dysfunction. Metabolism. 2008;57 Suppl 2:S50-5.
23. Alberti KG, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. Lancet. 2005;366(9491):1059-62.
24. Viscogliosi G, Marigliano V. Alzheimer's disease: how far have we progressed? Lessons learned from diabetes mellitus, metabolic syndrome, and inflammation. Journal of the American Geriatrics Society. 2013;61(5):845-6.
25. Biessels GJ, van der Heide LP, Kamal A, Bleys RL, Gispen WH. Ageing and diabetes: implications for brain function. European journal of pharmacology. 2002;441(1-2):1-14.
26. Strachan MW, Reynolds RM, Marioni RE, Price JF. Cognitive function, dementia and type 2 diabetes mellitus in the elderly. Nature reviews Endocrinology. 2011;7(2):108-14.
27. Fratiglioni L, Qiu C. Prevention of common neurodegenerative disorders in the elderly. Experimental gerontology. 2009;44(1-2):46-50.
28. Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. Lancet neurology. 2006;5(1):64-74.
29. Peters R, Beckett N, Forette F, Tuomilehto J, Clarke R, Ritchie C, et al. Incident dementia and blood pressure lowering in the Hypertension in the Very Elderly Trial cognitive function assessment (HYVET-COG): a double-blind, placebo controlled trial. Lancet neurology. 2008;7(8):683-9.
30. Ravona-Springer R, Schnaider-Beeri M. The association of diabetes and dementia and possible implications for nondiabetic populations. Expert review of neurotherapeutics. 2011;11(11):1609-17.
31. Miyakawa T. Vascular pathology in Alzheimer's disease. Psychogeriatrics : the official journal of the Japanese Psychogeriatric Society. 2010;10(1):39-44.
32. Messier C, Gagnon M. Glucose regulation and cognitive functions: relation to Alzheimer's disease and diabetes. Behavioural brain research. 1996;75(1-2):1-11.
33. Munch G, Schinzel R, Loske C, Wong A, Durany N, Li JJ, et al. Alzheimer's disease--synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. Journal of neural transmission (Vienna, Austria : 1996). 1998;105(4-5):439-61.
34. Tuppo EE, Arias HR. The role of inflammation in Alzheimer's disease. The international journal of biochemistry & cell biology. 2005;37(2):289-305.
35. Kapogiannis D, Mattson MP. Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. Lancet neurology. 2011;10(2):187-98.
36. Fernandez-Gamba A, Leal MC, Morelli L, Castano EM. Insulin-degrading enzyme: structure-function relationship and its possible roles in health and disease. Current pharmaceutical design. 2009;15(31):3644-55.
37. Craft S, Watson GS. Insulin and neurodegenerative disease: shared and specific mechanisms. Lancet neurology. 2004;3(3):169-78.
38. Bauduceau B, Doucet J, Bordier L, Garcia C, Dupuy O, Mayaudon H. Hypoglycaemia and dementia in diabetic patients. Diabetes & metabolism. 2010;36 Suppl 3:S106-11.
39. Luchsinger JA. Type 2 diabetes and cognitive impairment: linking mechanisms. Journal of Alzheimer's disease : JAD. 2012;30 Suppl 2:S185-98.
40. Sims-Robinson C, Kim B, Rosko A, Feldman EL. How does diabetes accelerate Alzheimer disease pathology? Nature reviews Neurology. 2010;6(10):551-9.
41. Cholerton B, Baker LD, Craft S. Insulin, cognition, and dementia. European journal of pharmacology. 2013.

42. Citron M. Strategies for disease modification in Alzheimer's disease. Nature reviews Neuroscience. 2004;5(9):677-85.
43. Gilman S. Alzheimer's disease. Perspectives in biology and medicine. 1997;40(2):230-45.
44. Gotz J, Schild A, Hoerndli F, Pennanen L. Amyloid-induced neurofibrillary tangle formation in Alzheimer's disease: insight from transgenic mouse and tissue-culture models. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience. 2004;22(7):453-65.
45. Rocchi A, Pellegrini S, Siciliano G, Murri L. Causative and susceptibility genes for Alzheimer's disease: a review. Brain research bulletin. 2003;61(1):1-24.
46. Haan MN. Therapy Insight: type 2 diabetes mellitus and the risk of late-onset Alzheimer's disease. Nature clinical practice Neurology. 2006;2(3):159-66.
47. Keller L, Xu W, Wang HX, Winblad B, Fratiglioni L, Graff C. The obesity related gene, FTO, interacts with APOE, and is associated with Alzheimer's disease risk: a prospective cohort study. Journal of Alzheimer's disease : JAD. 2011;23(3):461-9.
48. Qiu C, Xu W, Fratiglioni L. Vascular and psychosocial factors in Alzheimer's disease: epidemiological evidence toward intervention. Journal of Alzheimer's disease : JAD. 2010;20(3):689-97.
49. Cholerton B, Baker LD, Craft S. Insulin resistance and pathological brain ageing. Diabetic medicine : a journal of the British Diabetic Association. 2011;28(12):1463-75.
50. Cotman CW, Poon WW, Rissman RA, Blurton-Jones M. The role of caspase cleavage of tau in Alzheimer disease neuropathology. Journal of neuropathology and experimental neurology. 2005;64(2):104-12.
51. Han W, Li C. Linking type 2 diabetes and Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(15):6557-8.
52. Scheen AJ. Central nervous system: a conductor orchestrating metabolic regulations harmed by both hyperglycaemia and hypoglycaemia. Diabetes & metabolism. 2010;36 Suppl 3:S31-8.
53. Kim B, Backus C, Oh S, Hayes JM, Feldman EL. Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. Endocrinology. 2009;150(12):5294-301.
54. Hong M, Lee VM. Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neurons. The Journal of biological chemistry. 1997;272(31):19547-53.
55. Schubert M, Gautam D, Surjo D, Ueki K, Baudler S, Schubert D, et al. Role for neuronal insulin resistance in neurodegenerative diseases. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(9):3100-5.
56. Willis AW. Parkinson disease in the elderly adult. Missouri medicine. 2013;110(5):406-10.
57. Korczyn AD. Mild cognitive impairment in Parkinson's disease. Journal of neural transmission (Vienna, Austria : 1996). 2013;120(4):517-21.
58. Gazewood JD, Richards DR, Clebak K. Parkinson disease: an update. American family physician. 2013;87(4):267-73.
59. Santiago JA, Potashkin JA. Shared dysregulated pathways lead to Parkinson's disease and diabetes. Trends in molecular medicine. 2013;19(3):176-86.
60. Bosco D, Plastino M, Cristiano D, Colica C, Ermio C, De Bartolo M, et al. Dementia is associated with insulin resistance in patients with Parkinson's disease. Journal of the neurological sciences. 2012;315(1-2):39-43.
61. Arvanitakis Z, Wilson RS, Bienias JL, Bennett DA. Diabetes and parkinsonian signs in older persons. Alzheimer disease and associated disorders. 2007;21(2):144-9.

62. Qi L, Cornelis MC, Kraft P, Stanya KJ, Linda Kao WH, Pankow JS, et al. Genetic variants at 2q24 are associated with susceptibility to type 2 diabetes. Human molecular genetics. 2010;19(13):2706-15.
63. Kooner JS, Saleheen D, Sim X, Sehmi J, Zhang W, Frossard P, et al. Genome-wide association study in individuals of South Asian ancestry identifies six new type 2 diabetes susceptibility loci. Nature genetics. 2011;43(10):984-9.
64. Chen YW, Yang CY, Huang CF, Hung DZ, Leung YM, Liu SH. Heavy metals, islet function and diabetes development. Islets. 2009;1(3):169-76.
65. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. Nature reviews Drug discovery. 2013;12(9):703-19.
66. Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. Cell death and differentiation. 2006;13(3):385-92.
67. Doyle KM, Kennedy D, Gorman AM, Gupta S, Healy SJ, Samali A. Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. Journal of cellular and molecular medicine. 2011;15(10):2025-39.
68. Cereda E, Barichella M, Pedrolli C, Klersy C, Cassani E, Caccialanza R, et al. Diabetes and risk of Parkinson's disease: a systematic review and meta-analysis. Diabetes care. 2011;34(12):2614-23.
69. Allen CL, Bayraktutan U. Risk factors for ischaemic stroke. International journal of stroke : official journal of the International Stroke Society. 2008;3(2):105-16.
70. Lees KR, Bath PM, Naylor AR. ABC of arterial and venous disease. Secondary prevention of transient ischaemic attack and stroke. BMJ (Clinical research ed). 2000;320(7240):991-4.
71. Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2013.
72. Sanchez-Mendoza E, Bellver V, Merino JJ, Gonzalez M, Martinez-Murillo R, Oset-Gasque MJ. Review: Could Neurotransmitters Influence Neurogenesis and Neurorepair After Stroke? Neuropathology and applied neurobiology. 2013.
73. Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, Di Angelantonio E, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet. 2010;375(9733):2215-22.
74. Luitse MJ, Biessels GJ, Rutten GE, Kappelle LJ. Diabetes, hyperglycaemia, and acute ischaemic stroke. Lancet neurology. 2012;11(3):261-71.
75. Sone H, Tanaka S, Iimuro S, Tanaka S, Oida K, Yamasaki Y, et al. Long-term lifestyle intervention lowers the incidence of stroke in Japanese patients with type 2 diabetes: a nationwide multicentre randomised controlled trial (the Japan Diabetes Complications Study). Diabetologia. 2010;53(3):419-28.
76. Sena E, van der Worp HB, Howells D, Macleod M. How can we improve the pre-clinical development of drugs for stroke? Trends in neurosciences. 2007;30(9):433-9.
77. Wahlgren NG, Ahmed N. Neuroprotection in cerebral ischaemia: facts and fancies--the need for new approaches. Cerebrovascular diseases (Basel, Switzerland). 2004;17 Suppl 1:153-66.
78. Hacke W, Donnan G, Fieschi C, Kaste M, von Kummer R, Broderick JP, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. Lancet. 2004;363(9411):768-74.
79. Stroke forum. Available from: http://www.strokeforum.com/acute-stroke-treatment/importance-of-time.html.
80. Tanne D, Kasner SE, Demchuk AM, Koren-Morag N, Hanson S, Grond M, et al. Markers of increased risk of intracerebral hemorrhage after intravenous recombinant

tissue plasminogen activator therapy for acute ischemic stroke in clinical practice: the Multicenter rt-PA Stroke Survey. Circulation. 2002;105(14):1679-85.
81. ESO stroke [Internet]. Available from: www.eso-stroke.org.
82. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet. 2006;368(9548):1696-705.
83. Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, et al. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. The Journal of biological chemistry. 1993;268(26):19650-5.
84. Gupta V. Pleiotropic effects of incretins. Indian journal of endocrinology and metabolism. 2012;16 Suppl 1:S47-56.
85. Perfetti R, Hui H. The role of GLP-1 in the life and death of pancreatic beta cells. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2004;36(11-12):804-10.
86. Ban K, Noyan-Ashraf MH, Hoefer J, Bolz SS, Drucker DJ, Husain M. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation. 2008;117(18):2340-50.
87. Holst JJ, Burcelin R, Nathanson E. Neuroprotective properties of GLP-1: theoretical and practical applications. Current medical research and opinion. 2011;27(3):547-58.
88. Eng J, Kleinman WA, Singh L, Singh G, Raufman JP. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. The Journal of biological chemistry. 1992;267(11):7402-5.
89. Goke R, Larsen PJ, Mikkelsen JD, Sheikh SP. Distribution of GLP-1 binding sites in the rat brain: evidence that exendin-4 is a ligand of brain GLP-1 binding sites. Eur J Neurosci. 1995;7(11):2294-300.
90. Satoh F, Beak SA, Small CJ, Falzon M, Ghatei MA, Bloom SR, et al. Characterization of human and rat glucagon-like peptide-1 receptors in the neurointermediate lobe: lack of coupling to either stimulation or inhibition of adenylyl cyclase. Endocrinology. 2000;141(4):1301-9.
91. Hayes MR, De Jonghe BC, Kanoski SE. Role of the glucagon-like-peptide-1 receptor in the control of energy balance. Physiology & behavior. 2010;100(5):503-10.
92. Kappe C, Tracy LM, Patrone C, Iverfeldt K, Sjoholm A. GLP-1 secretion by microglial cells and decreased CNS expression in obesity. Journal of neuroinflammation. 2012;9:276.
93. Holscher C. Potential role of glucagon-like peptide-1 (GLP-1) in neuroprotection. CNS drugs. 2012;26(10):871-82.
94. Perry T, Greig NH. The glucagon-like peptides: a new genre in therapeutic targets for intervention in Alzheimer's disease. Journal of Alzheimer's disease : JAD. 2002;4(6):487-96.
95. Li Y, Perry T, Kindy MS, Harvey BK, Tweedie D, Holloway HW, et al. GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(4):1285-90.
96. Lee CH, Yan B, Yoo KY, Choi JH, Kwon SH, Her S, et al. Ischemia-induced changes in glucagon-like peptide-1 receptor and neuroprotective effect of its agonist, exendin-4, in experimental transient cerebral ischemia. Journal of neuroscience research. 2011;89(7):1103-13.

97. Bertilsson G, Patrone C, Zachrisson O, Andersson A, Dannaeus K, Heidrich J, et al. Peptide hormone exendin-4 stimulates subventricular zone neurogenesis in the adult rodent brain and induces recovery in an animal model of Parkinson's disease. Journal of neuroscience research. 2008;86(2):326-38.
98. Isacson R, Nielsen E, Dannaeus K, Bertilsson G, Patrone C, Zachrisson O, et al. The glucagon-like peptide 1 receptor agonist exendin-4 improves reference memory performance and decreases immobility in the forced swim test. European journal of pharmacology. 2011;650(1):249-55.
99. Dave KR, Tamariz J, Desai KM, Brand FJ, Liu A, Saul I, et al. Recurrent hypoglycemia exacerbates cerebral ischemic damage in streptozotocin-induced diabetic rats. Stroke. 2011;42(5):1404-11.
100. Engin F, Hotamisligil GS. Restoring endoplasmic reticulum function by chemical chaperones: an emerging therapeutic approach for metabolic diseases. Diabetes, obesity & metabolism. 2010;12 Suppl 2:108-15.
101. Ortsater H, Sjoholm A. A busy cell--endoplasmic reticulum stress in the pancreatic beta-cell. Mol Cell Endocrinol. 2007;277(1-2):1-5.
102. Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev. 2008;29(1):42-61.
103. Basseri S, Austin RC. Endoplasmic reticulum stress and lipid metabolism: mechanisms and therapeutic potential. Biochemistry research international. 2012;2012:841362.
104. Malo A, Kruger B, Goke B, Kubisch CH. 4-Phenylbutyric Acid Reduces Endoplasmic Reticulum Stress, Trypsin Activation, and Acinar Cell Apoptosis While Increasing Secretion in Rat Pancreatic Acini. Pancreas. 2012.
105. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxidants & redox signaling. 2007;9(12):2277-93.
106. Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, et al. Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. Biochem Biophys Res Commun. 2003;300(1):216-22.
107. Peti W, Page R. Molecular basis of MAP kinase regulation. Protein science : a publication of the Protein Society. 2013;22(12):1698-710.
108. Hayakawa R, Hayakawa T, Takeda K, Ichijo H. Therapeutic targets in the ASK1-dependent stress signaling pathways. Proceedings of the Japan Academy Series B, Physical and biological sciences. 2012;88(8):434-53.
109. Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. Neuron. 2004;41(5):683-6.
110. Falk A, Frisen J. New neurons in old brains. Ann Med. 2005;37(7):480-6.
111. Gage FH, Ray J, Fisher LJ. Isolation, characterization, and use of stem cells from the CNS. Annual review of neuroscience. 1995;18:159-92.
112. Kokoeva MV, Yin H, Flier JS. Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science (New York, NY). 2005;310(5748):679-83.
113. Altman J, Das GD. Post-natal origin of microneurones in the rat brain. Nature. 1965;207(5000):953-6.
114. Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. The Journal of comparative neurology. 1965;124(3):319-35.
115. Altman J, Das GD. Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. The Journal of comparative neurology. 1966;126(3):337-89.

116. Altman J. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. The Journal of comparative neurology. 1969;137(4):433-57.
117. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. Nature medicine. 1998;4(11):1313-7.
118. Kempermann G, Wiskott L, Gage FH. Functional significance of adult neurogenesis. Current opinion in neurobiology. 2004;14(2):186-91.
119. Dias GP, Cavegn N, Nix A, do Nascimento Bevilaqua MC, Stangl D, Zainuddin MS, et al. The role of dietary polyphenols on adult hippocampal neurogenesis: molecular mechanisms and behavioural effects on depression and anxiety. Oxidative medicine and cellular longevity. 2012;2012:541971.
120. Squire LR. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychological review. 1992;99(2):195-231.
121. Sahay A, Hen R. Adult hippocampal neurogenesis in depression. Nature neuroscience. 2007;10(9):1110-5.
122. Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, et al. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature. 2011;472(7344):466-70.
123. David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I, et al. Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. Neuron. 2009;62(4):479-93.
124. Lee DA, Blackshaw S. Functional implications of hypothalamic neurogenesis in the adult mammalian brain. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience. 2012;30(8):615-21.
125. Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2001;21(17):6706-17.
126. Lee DA, Bedont JL, Pak T, Wang H, Song J, Miranda-Angulo A, et al. Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. Nature neuroscience. 2012;15(5):700-2.
127. Kokoeva MV, Yin H, Flier JS. Evidence for constitutive neural cell proliferation in the adult murine hypothalamus. The Journal of comparative neurology. 2007;505(2):209-20.
128. Ahmed EI, Zehr JL, Schulz KM, Lorenz BH, DonCarlos LL, Sisk CL. Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nature neuroscience. 2008;11(9):995-7.
129. Pierce AA, Xu AW. De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. J Neurosci. 2010;30(2):723-30.
130. Curtis MA, Low VF, Faull RL. Neurogenesis and progenitor cells in the adult human brain: a comparison between hippocampal and subventricular progenitor proliferation. Developmental neurobiology. 2012;72(7):990-1005.
131. Johansson CB, Svensson M, Wallstedt L, Janson AM, Frisen J. Neural stem cells in the adult human brain. Experimental cell research. 1999;253(2):733-6.
132. Capela A, Temple S. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron. 2002;35(5):865-75.

133. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell. 1999;97(6):703-16.
134. Goritz C, Frisen J. Neural stem cells and neurogenesis in the adult. Cell stem cell. 2012;10(6):657-9.
135. Gould E, Reeves AJ, Graziano MS, Gross CG. Neurogenesis in the neocortex of adult primates. Science (New York, NY). 1999;286(5439):548-52.
136. Lois C, Alvarez-Buylla A. Long-distance neuronal migration in the adult mammalian brain. Science (New York, NY). 1994;264(5162):1145-8.
137. Konefal S, Elliot M, Crespi B. The adaptive significance of adult neurogenesis: an integrative approach. Frontiers in neuroanatomy. 2013;7:21.
138. Bergmann O, Liebl J, Bernard S, Alkass K, Yeung MS, Steier P, et al. The age of olfactory bulb neurons in humans. Neuron. 2012;74(4):634-9.
139. Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, et al. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science (New York, NY). 2007;315(5816):1243-9.
140. Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai HH, Wong M, et al. Corridors of migrating neurons in the human brain and their decline during infancy. Nature. 2011;478(7369):382-6.
141. Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron. 2011;70(4):687-702.
142. Slaughter JR, Martens MP, Slaughter KA. Depression and Huntington's disease: prevalence, clinical manifestations, etiology, and treatment. CNS spectrums. 2001;6(4):306-26.
143. Curtis MA, Penney EB, Pearson AG, van Roon-Mom WM, Butterworth NJ, Dragunow M, et al. Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(15):9023-7.
144. Curtis MA, Waldvogel HJ, Synek B, Faull RL. A histochemical and immunohistochemical analysis of the subependymal layer in the normal and Huntington's disease brain. J Chem Neuroanat. 2005;30(1):55-66.
145. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nature medicine. 2002;8(9):963-70.
146. Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Annals of neurology. 2002;52(6):802-13.
147. Marti-Fabregas J, Romaguera-Ros M, Gomez-Pinedo U, Martinez-Ramirez S, Jimenez-Xarrie E, Marin R, et al. Proliferation in the human ipsilateral subventricular zone after ischemic stroke. Neurology.74(5):357-65.
148. Jin K, Sun Y, Xie L, Peel A, Mao XO, Batteur S, et al. Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Molecular and cellular neurosciences. 2003;24(1):171-89.
149. Lindvall O, Kokaia Z. Stem cell research in stroke: how far from the clinic? Stroke; a journal of cerebral circulation. 2011;42(8):2369-75.
150. Lagace DC. Does the endogenous neurogenic response alter behavioral recovery following stroke? Behavioural brain research. 2012;227(2):426-32.
151. Ziabreva I, Perry E, Perry R, Minger SL, Ekonomou A, Przyborski S, et al. Altered neurogenesis in Alzheimer's disease. Journal of psychosomatic research. 2006;61(3):311-6.
152. Rodriguez JJ, Jones VC, Verkhratsky A. Impaired cell proliferation in the subventricular zone in an Alzheimer's disease model. Neuroreport. 2009;20(10):907-12.

153. Lledo PM, Alonso M, Grubb MS. Adult neurogenesis and functional plasticity in neuronal circuits. Nature reviews Neuroscience. 2006;7(3):179-93.
154. Curtis MA, Faull RL, Eriksson PS. The effect of neurodegenerative diseases on the subventricular zone. Nature reviews Neuroscience. 2007;8(9):712-23.
155. Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I, et al. Dopamine depletion impairs precursor cell proliferation in Parkinson disease. Nature neuroscience. 2004;7(7):726-35.
156. van den Berge SA, van Strien ME, Korecka JA, Dijkstra AA, Sluijs JA, Kooijman L, et al. The proliferative capacity of the subventricular zone is maintained in the parkinsonian brain. Brain : a journal of neurology. 2011;134(Pt 11):3249-63.
157. Abdipranoto A, Wu S, Stayte S, Vissel B. The role of neurogenesis in neurodegenerative diseases and its implications for therapeutic development. CNS & neurological disorders drug targets. 2008;7(2):187-210.
158. Bachor TP, Suburo AM. Neural stem cells in the diabetic brain. Stem cells international. 2012;2012:820790.
159. Guo J, Yu C, Li H, Liu F, Feng R, Wang H, et al. Impaired neural stem/progenitor cell proliferation in streptozotocin-induced and spontaneous diabetic mice. Neuroscience research. 2010;68(4):329-36.
160. Lang BT, Yan Y, Dempsey RJ, Vemuganti R. Impaired neurogenesis in adult type-2 diabetic rats. Brain research. 2009;1258:25-33.
161. Cacci E, Ajmone-Cat MA, Anelli T, Biagioni S, Minghetti L. In vitro neuronal and glial differentiation from embryonic or adult neural precursor cells are differently affected by chronic or acute activation of microglia. Glia. 2008;56(4):412-25.
162. Russo I, Barlati S, Bosetti F. Effects of neuroinflammation on the regenerative capacity of brain stem cells. Journal of neurochemistry. 2011;116(6):947-56.
163. Park HR, Park M, Choi J, Park KY, Chung HY, Lee J. A high-fat diet impairs neurogenesis: involvement of lipid peroxidation and brain-derived neurotrophic factor. Neurosci Lett. 2010;482(3):235-9.
164. Alvarez EO, Beauquis J, Revsin Y, Banzan AM, Roig P, De Nicola AF, et al. Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes. Behavioural brain research. 2009;198(1):224-30.
165. Zhang WJ, Tan YF, Yue JT, Vranic M, Wojtowicz JM. Impairment of hippocampal neurogenesis in streptozotocin-treated diabetic rats. Acta Neurol Scand. 2008;117(3):205-10.
166. Stranahan AM, Arumugam TV, Cutler RG, Lee K, Egan JM, Mattson MP. Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nature neuroscience. 2008;11(3):309-17.
167. Lichtenwalner RJ, Parent JM. Adult neurogenesis and the ischemic forebrain. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2006;26(1):1-20.
168. Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron. 2000;28(3):713-26.
169. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(18):11946-50.
170. Zigova T, Pencea V, Wiegand SJ, Luskin MB. Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Molecular and cellular neurosciences. 1998;11(4):234-45.
171. van Praag H, Shubert T, Zhao C, Gage FH. Exercise enhances learning and hippocampal neurogenesis in aged mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005;25(38):8680-5.

172. Arimura A. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol. 1998;48(5):301-31.
173. Dickson L, Finlayson K. VPAC and PAC receptors: From ligands to function. Pharmacology & therapeutics. 2009;121(3):294-316.
174. Lindholm D, Skoglosa Y, Takei N. Developmental regulation of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptor 1 in rat brain: function of PACAP as a neurotrophic factor. Ann N Y Acad Sci. 1998;865:189-96.
175. Gonzalez-Rey E, Varela N, Chorny A, Delgado M. Therapeutical approaches of vasoactive intestinal peptide as a pleiotropic immunomodulator. Curr Pharm Des. 2007;13(11):1113-39.
176. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacological reviews. 2009;61(3):283-357.
177. Moody TW, Ito T, Osefo N, Jensen RT. VIP and PACAP: recent insights into their functions/roles in physiology and disease from molecular and genetic studies. Curr Opin Endocrinol Diabetes Obes. 2011;18(1):61-7.
178. Jamen F, Puech R, Bockaert J, Brabet P, Bertrand G. Pituitary adenylate cyclase-activating polypeptide receptors mediating insulin secretion in rodent pancreatic islets are coupled to adenylate cyclase but not to PLC. Endocrinology. 2002;143(4):1253-9.
179. Bertrand G, Puech R, Maisonnasse Y, Bockaert J, Loubatieres-Mariani MM. Comparative effects of PACAP and VIP on pancreatic endocrine secretions and vascular resistance in rat. Br J Pharmacol. 1996;117(4):764-70.
180. Jamen F, Persson K, Bertrand G, Rodriguez-Henche N, Puech R, Bockaert J, et al. PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. J Clin Invest. 2000;105(9):1307-15.
181. Filipsson K, Tornoe K, Holst J, Ahren B. Pituitary adenylate cyclase-activating polypeptide stimulates insulin and glucagon secretion in humans. J Clin Endocrinol Metab. 1997;82(9):3093-8.
182. Bourgault S, Vaudry D, Dejda A, Doan ND, Vaudry H, Fournier A. Pituitary adenylate cyclase-activating polypeptide: focus on structure-activity relationships of a neuroprotective Peptide. Current medicinal chemistry. 2009;16(33):4462-80.
183. Rangon CM, Goursaud S, Medja F, Lelievre V, Mounien L, Husson I, et al. VPAC2 receptors mediate vasoactive intestinal peptide-induced neuroprotection against neonatal excitotoxic brain lesions in mice. J Pharmacol Exp Ther. 2005;314(2):745-52.
184. Nishimoto M, Miyakawa H, Wada K, Furuta A. Activation of the VIP/VPAC2 system induces reactive astrocytosis associated with increased expression of glutamate transporters. Brain research. 2011;1383:43-53.
185. White CM, Ji S, Cai H, Maudsley S, Martin B. Therapeutic potential of vasoactive intestinal peptide and its receptors in neurological disorders. CNS Neurol Disord Drug Targets. 2010;9(5):661-6.
186. Reglodi D, Kiss P, Lubics A, Tamas A. Review on the protective effects of PACAP in models of neurodegenerative diseases in vitro and in vivo. Curr Pharm Des. 2011;17(10):962-72.
187. Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacological reviews. 2000;52(2):269-324.
188. Arimura A, Somogyvari-Vigh A, Miyata A, Mizuno K, Coy DH, Kitada C. Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. Endocrinology. 1991;129(5):2787-9.

189. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun. 1989;164(1):567-74.
190. Shneider Y, Shtrauss Y, Yadid G, Pinhasov A. Differential expression of PACAP receptors in postnatal rat brain. Neuropeptides. 2010;44(6):509-14.
191. Waschek JA, Casillas RA, Nguyen TB, DiCicco-Bloom EM, Carpenter EM, Rodriguez WI. Neural tube expression of pituitary adenylate cyclase-activating peptide (PACAP) and receptor: potential role in patterning and neurogenesis. Proc Natl Acad Sci U S A. 1998;95(16):9602-7.
192. Watanabe J, Ohba M, Ohno F, Kikuyama S, Nakamura M, Nakaya K, et al. Pituitary adenylate cyclase-activating polypeptide-induced differentiation of embryonic neural stem cells into astrocytes is mediated via the beta isoform of protein kinase C. J Neurosci Res. 2006;84(8):1645-55.
193. Brenneman DE. Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Peptides. 2007;28(9):1720-6.
194. Ohtaki H, Nakamachi T, Dohi K, Aizawa Y, Takaki A, Hodoyama K, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. Proc Natl Acad Sci U S A. 2006;103(19):7488-93.
195. Reglodi D, Somogyvari-Vigh A, Vigh S, Kozicz T, Arimura A. Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. Stroke. 2000;31(6):1411-7.
196. Stumm R, Kolodziej A, Prinz V, Endres M, Wu DF, Hollt V. Pituitary adenylate cyclase-activating polypeptide is up-regulated in cortical pyramidal cells after focal ischemia and protects neurons from mild hypoxic/ischemic damage. J Neurochem. 2007;103(4):1666-81.
197. Shioda S, Ohtaki H, Nakamachi T, Dohi K, Watanabe J, Nakajo S, et al. Pleiotropic functions of PACAP in the CNS: neuroprotection and neurodevelopment. Ann N Y Acad Sci. 2006;1070:550-60.
198. Chen Y, Samal B, Hamelink CR, Xiang CC, Chen M, Vaudry D, et al. Neuroprotection by endogenous and exogenous PACAP following stroke. Regul Pept. 2006;137(1-2):4-19.
199. Ohtaki H, Nakamachi T, Dohi K, Shioda S. Role of PACAP in ischemic neural death. J Mol Neurosci. 2008;36(1-3):16-25.
200. Tan YV, Waschek JA. Targeting VIP and PACAP receptor signaling: New therapeutic strategies in multiple sclerosis. ASN Neuro. 2011.
201. Mercer A, Ronnholm H, Holmberg J, Lundh H, Heidrich J, Zachrisson O, et al. PACAP promotes neural stem cell proliferation in adult mouse brain. Journal of neuroscience research. 2004;76(2):205-15.
202. Ohta S, Gregg C, Weiss S. Pituitary adenylate cyclase-activating polypeptide regulates forebrain neural stem cells and neurogenesis in vitro and in vivo. Journal of neuroscience research. 2006;84(6):1177-86.
203. Dicicco-Bloom E, Lu N, Pintar JE, Zhang J. The PACAP ligand/receptor system regulates cerebral cortical neurogenesis. Annals of the New York Academy of Sciences. 1998;865:274-89.
204. Merchenthaler I. Galanin and the neuroendocrine axes. Exs. 2010;102:71-85.
205. Barson JR, Morganstern I, Leibowitz SF. Galanin and consummatory behavior: special relationship with dietary fat, alcohol and circulating lipids. Exs. 2010;102:87-111.

206. Hobson SA, Bacon A, Elliot-Hunt CR, Holmes FE, Kerr NC, Pope R, et al. Galanin acts as a trophic factor to the central and peripheral nervous systems. Exs. 2010;102:25-38.
207. Crawley JN. The role of galanin in feeding behavior. Neuropeptides. 1999;33(5):369-75.
208. Ogren SO, Kuteeva E, Elvander-Tottie E, Hokfelt T. Neuropeptides in learning and memory processes with focus on galanin. European journal of pharmacology. 2010;626(1):9-17.
209. Liu HX, Hokfelt T. The participation of galanin in pain processing at the spinal level. Trends in pharmacological sciences. 2002;23(10):468-74.
210. Xu XJ, Hokfelt T, Wiesenfeld-Hallin Z. Galanin and spinal pain mechanisms: past, present, and future. Exs. 2010;102:39-50.
211. Counts SE, Perez SE, Ginsberg SD, Mufson EJ. Neuroprotective role for galanin in Alzheimer's disease. Exs. 2010;102:143-62.
212. Picciotto MR. Galanin and addiction. Exs. 2010;102:195-208.
213. Lerner JT, Sankar R, Mazarati AM. Galanin and epilepsy. Exs. 2010;102:183-94.
214. Gundlach AL, Burazin TC, Larm JA. Distribution, regulation and role of hypothalamic galanin systems: renewed interest in a pleiotropic peptide family. Clinical and experimental pharmacology & physiology. 2001;28(1-2):100-5.
215. Kask K, Berthold M, Bartfai T. Galanin receptors: involvement in feeding, pain, depression and Alzheimer's disease. Life sciences. 1997;60(18):1523-33.
216. O'Donnell D, Ahmad S, Wahlestedt C, Walker P. Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: distinct distribution from GALR1. J Comp Neurol. 1999;409(3):469-81.
217. Burazin TC, Larm JA, Ryan MC, Gundlach AL. Galanin-R1 and -R2 receptor mRNA expression during the development of rat brain suggests differential subtype involvement in synaptic transmission and plasticity. Eur J Neurosci. 2000;12(8):2901-17.
218. Smith KE, Forray C, Walker MW, Jones KA, Tamm JA, Bard J, et al. Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. The Journal of biological chemistry. 1997;272(39):24612-6.
219. Waters SM, Krause JE. Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. Neuroscience. 2000;95(1):265-71.
220. Fang P, Yu M, Guo L, Bo P, Zhang Z, Shi M. Galanin and its receptors: a novel strategy for appetite control and obesity therapy. Peptides. 2012;36(2):331-9.
221. Elliott-Hunt CR, Marsh B, Bacon A, Pope R, Vanderplank P, Wynick D. Galanin acts as a neuroprotective factor to the hippocampus. Proc Natl Acad Sci U S A. 2004;101(14):5105-10.
222. Pirondi S, Giuliani A, Del Vecchio G, Giardino L, Hokfelt T, Calza L. The galanin receptor 2/3 agonist Gal2-11 protects the SN56 cells against beta-amyloid 25-35 toxicity. J Neurosci Res. 2010;88(5):1064-73.
223. Mennicken F, Hoffert C, Pelletier M, Ahmad S, O'Donnell D. Restricted distribution of galanin receptor 3 (GalR3) mRNA in the adult rat central nervous system. J Chem Neuroanat. 2002;24(4):257-68.
224. Webling KE, Runesson J, Bartfai T, Langel U. Galanin receptors and ligands. Front Endocrinol (Lausanne). 2012;3:146.
225. Barreto SG, Bazargan M, Zotti M, Hussey DJ, Sukocheva OA, Peiris H, et al. Galanin receptor 3--a potential target for acute pancreatitis therapy. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2011;23(3):e141-51.

226. Ma J, Shan L, Yu LL, Yuan CG. [Expression of galanin and galanin receptors in neurogenesis regions of adult mouse brain and effect of galanin on the neural stem cell's differentiation]. Fen Zi Xi Bao Sheng Wu Xue Bao. 2008;41(5):359-66.
227. Abbosh C, Lawkowski A, Zaben M, Gray W. GalR2/3 mediates proliferative and trophic effects of galanin on postnatal hippocampal precursors. J Neurochem. 2011;117(3):425-36.
228. Agasse F, Xapelli S, Coronas V, Christiansen SH, Rosa AI, Sarda-Arroyo L, et al. Galanin promotes neuronal differentiation in murine subventricular zone cell cultures. Stem cells and development. 2013;22(11):1693-708.
229. Crouch SP, Kozlowski R, Slater KJ, Fletcher J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods. 1993;160(1):81-8.
230. van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods in molecular biology (Clifton, NJ). 2011;731:237-45.
231. Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature. 2002;415(6867):92-6.
232. King AJ. The use of animal models in diabetes research. British journal of pharmacology. 2012;166(3):877-94.
233. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372(6505):425-32.
234. Lindstrom P. The physiology of obese-hyperglycemic mice [ob/ob mice]. TheScientificWorldJournal. 2007;7:666-85.
235. Lavine RL, Voyles N, Perrino PV, Recant L. Functional abnormalities of islets of Langerhans of obese hyperglycemic mouse. The American journal of physiology. 1977;233(2):E86-90.
236. Goto Y, Kakizaki M, Masaki N. Production of spontaneous diabetic rats by repetition of selective breeding. The Tohoku journal of experimental medicine. 1976;119(1):85-90.
237. Portha B, Lacraz G, Kergoat M, Homo-Delarche F, Giroix MH, Bailbe D, et al. The GK rat beta-cell: a prototype for the diseased human beta-cell in type 2 diabetes? Molecular and cellular endocrinology. 2009;297(1-2):73-85.
238. Zhao Q, Pahlmark K, Smith ML, Siesjo BK. Delayed treatment with the spin trap alpha-phenyl-N-tert-butyl nitrone (PBN) reduces infarct size following transient middle cerebral artery occlusion in rats. Acta physiologica Scandinavica. 1994;152(3):349-50.
239. Liu F, McCullough LD. Middle cerebral artery occlusion model in rodents: methods and potential pitfalls. Journal of biomedicine & biotechnology. 2011;2011:464701.
240. Durukan A, Tatlisumak T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacology, biochemistry, and behavior. 2007;87(1):179-97.
241. West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. The Anatomical record. 1991;231(4):482-97.
242. West MJ. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. Trends in neurosciences. 1999;22(2):51-61.
243. Harmsen P, Lappas G, Rosengren A, Wilhelmsen L. Long-term risk factors for stroke: twenty-eight years of follow-up of 7457 middle-aged men in Goteborg, Sweden. Stroke. 2006;37(7):1663-7.

244. Nelson TJ, Sun MK, Hongpaisan J, Alkon DL. Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. Eur J Pharmacol. 2008;585(1):76-87.
245. Biessels GJ, De Leeuw FE, Lindeboom J, Barkhof F, Scheltens P. Increased cortical atrophy in patients with Alzheimer's disease and type 2 diabetes mellitus. J Neurol Neurosurg Psychiatry. 2006;77(3):304-7.
246. Carro E, Torres-Aleman I. The role of insulin and insulin-like growth factor I in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. European journal of pharmacology. 2004;490(1-3):127-33.
247. Kalaria RN. Neurodegenerative disease: Diabetes, microvascular pathology and Alzheimer disease. Nature reviews Neurology. 2009;5(6):305-6.
248. Lindvall O, Kokakia, Z. Neurogenesis following Stroke Affecting the Adult Brain. In: Gage FH, Kempermann, G., Song, H., editor. Adult Neurogenesis. New York: Cold Spring Harbor Laboratory Press; 2008. p. 549-70.
249. Beauquis J, Roig P, Homo-Delarche F, De Nicola A, Saravia F. Reduced hippocampal neurogenesis and number of hilar neurones in streptozotocin-induced diabetic mice: reversion by antidepressant treatment. Eur J Neurosci. 2006;23(6):1539-46.
250. Suh SW, Fan Y, Hong SM, Liu Z, Matsumori Y, Weinstein PR, et al. Hypoglycemia induces transient neurogenesis and subsequent progenitor cell loss in the rat hippocampus. Diabetes. 2005;54(2):500-9.
251. Saravia F, Revsin Y, Lux-Lantos V, Beauquis J, Homo-Delarche F, De Nicola AF. Oestradiol restores cell proliferation in dentate gyrus and subventricular zone of streptozotocin-diabetic mice. J Neuroendocrinol. 2004;16(8):704-10.
252. Gil-Perotin S, Duran-Moreno M, Cebrian-Silla A, Ramirez M, Garcia-Belda P, Garcia-Verdugo JM. Adult neural stem cells from the subventricular zone: a review of the neurosphere assay. Anatomical record. 2013;296(9):1435-52.
253. Bottai D, Fiocco R, Gelain F, Defilippis L, Galli R, Gritti A, et al. Neural stem cells in the adult nervous system. Journal of hematotherapy & stem cell research. 2003;12(6):655-70.
254. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science (New York, NY). 1992;255(5052):1707-10.
255. Shen PJ, Yuan CG, Ma J, Cheng S, Yao M, Turnley AM, et al. Galanin in neuro(glio)genesis: expression of galanin and receptors by progenitor cells in vivo and in vitro and effects of galanin on neurosphere proliferation. Neuropeptides. 2005;39(3):201-5.
256. Dejda A, Jolivel V, Bourgault S, Seaborn T, Fournier A, Vaudry H, et al. Inhibitory effect of PACAP on caspase activity in neuronal apoptosis: a better understanding towards therapeutic applications in neurodegenerative diseases. J Mol Neurosci. 2008;36(1-3):26-37.
257. Chen WH, Tzeng SF. Pituitary adenylate cyclase-activating polypeptide prevents cell death in the spinal cord with traumatic injury. Neurosci Lett. 2005;384(1-2):117-21.
258. Sokolowska P, Dejda A, Nowak JZ. [Neuroprotective role of PACAP, VIP, and PHI in the central nervous system]. Postepy Hig Med Dosw (Online). 2004;58:416-27.
259. Ginter E, Simko V. Type 2 diabetes mellitus, pandemic in 21st century. Advances in experimental medicine and biology. 2012;771:42-50.
260. Farag YM, Gaballa MR. Diabesity: an overview of a rising epidemic. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2011;26(1):28-35.

261. Teramoto S, Miyamoto N, Yatomi K, Tanaka Y, Oishi H, Arai H, et al. Exendin-4, a glucagon-like peptide-1 receptor agonist, provides neuroprotection in mice transient focal cerebral ischemia. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2011;31(8):1696-705.
262. Briyal S, Gulati K, Gulati A. Repeated administration of exendin-4 reduces focal cerebral ischemia-induced infarction in rats. Brain Res. 2012;1427:23-34.
263. Best JH, Hoogwerf BJ, Herman WH, Pelletier EM, Smith DB, Wenten M, et al. Risk of cardiovascular disease events in patients with type 2 diabetes prescribed the glucagon-like peptide 1 (GLP-1) receptor agonist exenatide twice daily or other glucose-lowering therapies: a retrospective analysis of the LifeLink database. Diabetes care. 2011;34(1):90-5.