Aus der Klinik für Innere Medizin III

Direktor: Herr Prof. Dr. Stefan R.Bornstein

Titel

The role of endogenous neural stem cells (eNSCs) in metabolic syndrome and aging

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"To boldly go where no one has gone before"

Stephen Hawking

To my family, our precious small genius and to my good friends…

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Thanks to everyone that stood by me during my PhD life.

Σας ευχαριστώ!!!

Summary

The role of endogenous neural stem cells (eNSCs) in metabolic syndrome and aging

Introduction

The adult brain exhibits low regenerative ability. Stem cell-based transplantation approaches have been largely unsuccessful, due to the difficulty to recapitulate the complex cytoarchitecture of the central nervous system (CNS). eNSCs are a new therapeutic option as pharmacological activation and increase of their number *in vivo* is accompanied by powerful neuroprotection in various disease models.

Hes3 is expressed in both proliferating and quiescent NSCs, which makes it a useful biomarker for NSC identification. Direct injections of insulin in the adult brain increase the number of eNSCs and promote rescue of injured neurons via a novel molecular mechanism, the STAT3-Ser/Hes3 Signaling Axis. This molecular pathway with the STAT3-Ser phosphorylation at its core regulates Hes3 and together they form a merging point for several signals including insulin receptor activation.

Main aim and Hypothesis

Beyond the brain, STAT3-Ser/Hes3 signaling regulates various plastic cell populations in other organs of the endocrine/neuroendocrine system. In the pancreas, Hes3 is expressed in islets cells and regulates their growth, regeneration, and insulin release. Hes3 is also expressed in mouse hypothalamic tanycytes, which are diet responsive cells and play a very crucial role for the communication between the brain and the endocrine system. Also, Hes3 is expressed in the adrenal gland (both in the cortex and medulla); cultured adrenal progenitors express Hes3 and various treatments that induce Hes3 expression promote their growth. Therefore, STAT3-Ser/Hes3 Signaling may be involved in tissue problems that result from metabolic dysfunction.

Metabolic syndrome often results in diabetes (Type I, Type II) and insulin resistance, suggesting that eNSCs may be affected by the condition. There is evidence that obesity induces inflammatory reactions in the hypothalamus, leading to NSC loss. However, it is not clear if damage to NSCs is also directly linked to insulin signaling disruption.

Materials and Methods

***In vivo animal models*:** For the aging experiments, mice were kept under normal diet conditions and were sacrificed in certain timepoints. Type I diabetes was induced by 1 single injection of high dose streptozotocin (STZ). Type II diabetes was induced by High Fat Diet

(HFD) administration. Metformin was administered in the drinking water. ***In vitro NSC cultures:*** Fetal Neural Stem Cells (fNSCs) were isolated and cultured from mouse embryos at embryonic day 13.5 (E13.5) and grown under established conditions for NSC maintenance. To assess possible effects of common pharmacological treatments prescribed for metabolic related dysfunctions on NSCs we treated the cultures with metformin and exendin-4. Proliferation was assessed by EdU incorporation. Cells were studied in their self-renewal state and their differentiation potential into different cell types was also assessed following mitogen withdrawal from the cell culture medium. ***Immunocytochemistry-Biochemistry:*** Standard immunofluorescence, X-gal staining techniques and Western Blotting were used to investigate protein expression changes in the genes of interest. ***Molecular Biology:*** PCR and qPCR were used to assess mRNA expression levels in the genes of interest. ***Phenotypic Analysis:*** Phenotypic characterization experiments were performed in house and through collaboration with the German mouse Clinic (GMC) in Munich.

Results

Our results show that various parameters affect Hes3 levels in the brain. Aging decreased Hes3 mRNA expression. Type I diabetes increased Hes3 expression. Type II diabetes decreased Hes3 expression. Thus, we conclude that eNSCs are modulated by diabetes in an age-dependent manner.

We also investigated whether common medication for metabolic related dysfunction also affects Hes3 expression in the adult brain. Indeed, our results show that metformin decreases Hes3 expression in the mouse hypothalamus.

To address whether metformin has a direct effect on NSCs we treated primary mouse fNSCs with metformin. Metformin decreases cell number, proliferation and affects cell morphology, giving a more differentiated appearance (large, flat cell body with wider projections). Hes3 expression increases significantly at 72 hours of treatment.

The metformin result opens the question if the increase in the Hes3 expression is a direct effect of the signal transduction pathways activated by metformin or due to a stress reaction. To address this we treated NSCs with exendin-4, another diabetes drug that we previously showed to both elevate Hes3 expression and cell number using a mouse insulinoma cell line (MIN6). Exendin-4 increases fNSC cell number but it did not affect the morphology. Similar to metformin proliferation was not affected. Hes3 expression increased significantly at 72 hours of treatment as well. This result indicates the distinctive action of the drugs on the STAT3-Ser/Hes3 signaling pathway. Specifically it dissociates Hes3 levels from other cellular

parameters. Importantly it shows that two common diabetes medications have very different effects on NSCs.

Because Hes3 is strongly regulated by metabolic parameters and medication we addressed potential roles of Hes3 using an established Hes3 null mouse line. Hes3 null mice exhibit no obvious phenotypes under normal conditions. However, we previously showed that when stressed by chemical induced damage, they exhibit low regenerative potential in the pancreas and brain. To identify additional phenotypes, we performed a phenotypic analysis of the Hes3 null mouse line under normal diet and HFD conditions (which induced type II diabetes). We found mild phenotypes that relate to the nervous system, the immune system and metabolism. At the molecular level, Hes3 deletion affects the expression of other genes within the Hes superfamily in the adult mouse brain. However, we did not observe these molecular differences in the HFD condition, suggesting an interplay between metabolic parameters (possibly, circulating insulin) and the regulation of Hes/Hey genes in the brain. Our data suggest a broad range of roles for Hes3, particularly under abnormal conditions.

Conclusions

Our work establishes that multiple parameters of metabolic state as well as diabetes medication affect Hes3 expression in the brain. Metabolic syndrome is a risk factor for many neurological disorders such as Alzheimer's disease, Parkinson's disease and Multiple Sclerosis. It is important to understand at the molecular and cellular level how metabolic dysfunction affects the brain. Here, we introduced a new cellular biomarker and signaling component that is greatly regulated in metabolic dysfunction.

Zusammenfassung

Die Rolle endogener neuraler Stammzellen (eNSZs) im metabolischen Syndrom und in Alterungsprozessen

Einleitung

Die geringe Regenerationsfähigkeit des erwachsenen Gehirns verursacht zahlreicher neurologischer Erkrankungen. Aufgrund der komplizierten zellulären Architektur des Zentralnervensystems erwiesen sich dabei rasch auch moderne Therapieansätze wie Stammzelltransplantation weitgehend als erfolglos. Neue Hoffnung ruht derzeit auf dem gezielten Einsatz endogener neuraler Stammzellen (eNSZs), bei deren pharmakologische Aktivierung und Vermehrung *in vivo* bereits in diversen Krankheitsmodellen eine neuroprotektive Wirkung zeigte.

In jüngster Forschung zeigte sich Hes3 durch seine Expression in sowohl proliferierend wie auch quieszente NSZs als idealer Biomarker zur Identifizierung von NSZs. Bedeutend dabei war die Etablierung der STAT3-Ser/Hes3 Signalachse, welche Aktivierung mittels einer lokalen Injektion von Insulin zu einem direkten zahlenmäßigen Anstieg der eNSZs und gleichzeitiger Verheilung verletzter Neuronen führte.

Hypothese

Zusätzlich zu seiner Rolle im Gehirn reguliert der STAT3-Ser/Hes3 Signalweg eine Vielzahl plastischer Zellpopulation des endokrinen/neuroendokrinen Systems. So wird Hes3 beispielsweise von den Inselzellen des Pankreas exprimiert und beeinflusst deren Wachstum, Regeneration und Insulinsekretion.

Desweiteren produzieren hypothalamische Maus-Tanyzyten Hes3 nahrungs-sensorische Zellen, die als eine entscheidende Schrittstelle in der Kommunikation zwischen Hirn und endokrinem System gelten. Ferner wird Hes3 in der Nebenniere und in der Nebennierenrinde als auch in der Medulla exprimiert; sowie in Nebennierenvorläuferzellen in Kulturen, deren Wachstum von Hes3-stimulierende Behandlungen verstärkt wird. Zusammenfassend betrachtet führt dies uns zu der Annahme, dass der STAT3-Ser/Hes3 Signalweg eine bedeutende Rolle bei Problemen auf organweiter Ebene spielt, welche von einer metabolischen Dysfunktion ausgelöst wird.

Diabetes Typ 1 und Typ 2 (Insulinresistenz) sind zwei Krankheitsbilder des metabloschen Sydroms bei denen eNSZs in diesem Zustand vermutlich betroffen sind. Es gibt Beweise, dass inflammatorische Reaktionen im Hypothalamus zum Verlust der NSC führen können.

Ob das Absterben der NSCs jedoch als eine direkte Konsequenz der gestörten Insulin-Kaskade gilt, bleibt derzeit ungeklärt.

Materialen und Methoden

***In vivo* Tiermodelle.** Alle Mäuse der Alterungsexperimente erhielten normal-kalorisches Standardfutter und wurden zu verschiedenen Zeitpunkten aufgegeben. Typ 1 Diabetes wurde durch eine einzige, hochdosierte Injektion von Streptozotocin (STZ) ausgelöst, Typ 2 Diabetes durch Einnahme einer fettreichen Diät (60% Fett, zu Englisch High Fat Diet, kurz HFD). Metformin wurde durch Trinkwasser verabreicht. ***In vitro* NSZ Kulturen**: Fetale neurale Stammzellen wurden von Mausembryonen am Tag 13.5 isoliert und unter bereits etablierten Bedingungen zur NSC-Erhaltung kultiviert. Um den direkten Effekt klassischer Pharmazeutika zur Behandlung metabolischer Erkrankungen auf NSZs zu analysieren wurden die so gewonnen Zellkulturen mit Metformin und Exenatid stimuliert. Die Proliferationsrate wurde dabei mittels EdU- Inkorporation festgestellt. Die Zellen wurden in ihrem eigenen Erneuerungszustand untersucht. Weiterhin wurde ihre differenzierungspotential in anderen Zelltypen auch durch Entnahme von Mitogenen vom der mittleren Zellkultur beurteilt. **Immunzytochemie/Biochemische Methoden:** Standardmethoden der Immunfluoreszenz, X-Gal Färbung und Western Blot Analyse wurden festellung den Änderungen der Proteinexpression des Zielgens verwendet. **Molekularbiologische Methoden**: Die Transkriptionsrate des Zielgens auf mRNA Level wurde mittels PCR und qPCR bestimmt. **Phänotypische Analysen:** Phänotypische Charakterisierungen wurden von uns in Zusammenarbeit mit unserer Kollegen des German Mouse Clinic (GMC) in München durchgeführt.

Ergebnisse

Aus unseren Daten geht hervor dass die Hes3- Level im Gehirn von diversen Parametern beeinflusst werden konnten. So führte Altern allein zu einer reduzierten Hes3-Expression auf mRNA Ebene, Typ 1 Diabetes jedoch zu einem Anstieg. Typ II Diabetes vermindert Hes3 Expression auf mRNA. Folglich schließen wir dass die eNSZs durch Diabetes zeitabhängig moduliert werden.

Zusätzlich beantworten wir die Frage inwieweit traditionell verschriebene Medikamente zur Behandlung metabolischer Dysfunktionen einen Einfluss auf die He3s-Expressionsrate im Hirn eine Rolle spielt. Tatsächlich konnte es gezeigt werden, dass die Verabreichung von Metformin im Mausmodell zur einer Reduktion der Hes3-Expressionsrate im Hypothalamus führte. Darauf aufbauend untersuchten wir den direkten Einfluss von Metformin auf primären

Maus-fNSZs in Zellkulturen. Die Gabe von Metformin reduzierte dabei die Zellanzahl, die Proliferation und führte zu einem veränderten Zellerscheinungsbild: die Zellen zeigten eine generell differenzierte Morphologie mit einem breiteren und flacheren Zellkörper mit weiteren Zellvorsprüngen. Die Hes3-Expressionsrate stieg jedoch signifikant nach 72h.

Die Ergebnisse unserer Metformin-Experimente führten uns zu der Fragestellung ob die gesteigerte Hes3-Expressionrate ein direkter Effekt der durch Metformin aktivierten Signaltransduktionwege oder eher eine Stressreaktion darstellte. Daraufhin inkubierten wir NSZs mit Exenatid, eine andere Routine Medikament zur Behandlung von Diabetes. In de Vorangegangenen Studie unserer Arbeitsgruppe mit MIN6, einer Maus-Insulinomazelllinie, konnte es gezeigt werden dass Exenatid zu einem Anstieg in Zellzahl und Hes3-Expressionsrate führte. Die Proliferationsrate in NSZ-Kulturen war unverändert und die Hes3-Expression stieg signifikant nach 72h. Die Erhöhung von Exenatid vermehrt die Zellzahl, jedoch ohne Einfluss auf die Morphologie. Dies weist darauf hin dass beide Medikamente auf verschiedenen Wegen die STAT3-Ser/Hes3-Achse wirken. Insbesondere unterschiedet sich Hes3-Ebenen von anderen zellulären Parametern. Vor allem betont dies jedoch welchen unterschiedlichen Effekt zwei herkömmlich verwendete Diabetesmedikamente auf NSZs ausüben können.

Aufgrund den Hinweise auf Hes3 Regulation von metabolischen Parametern und Medikation, untersuchten wir abschließend die Rolle von Hes3 mittels einer etablierten Hes3-Null-Mauslinie. Unter normalen Bedingungen zeigen sich Hes3-Null-Mäuse keinerlei offensichtliche phänotypische Veränderungen. Frühere Arbeiten unserer Gruppe konnten jedoch zeigen dass diese Mäuse unter chemischen-induzierten Stress ein geringeres Regenerationspotential im Pankreas und Gehirn aufweisen. Um weitere Auffälligkeiten zu Nachweisen führten wir eine phänotypische Analyse von Hes3-Null-Mäusen unter Normaldiät und HFD (als Typ 2 Diabetes–Modell) durch. Dabei fanden wir milde Phänotypen in Verbindung mit dem Nervensystem, dem Immunsystem und Metabolismus unter Normaldiät. Auf molekularer Ebene übte die Hes3-Deletion Einfluss insbesondere auf andere Gene der Hes-Superfamilie im Maushirn aus. Interessanterweise konnten wir diese Unterschiede nicht mehr nach Fütterung der HFD nachweisen, was ein Zusammenspiel metabolischer Faktoren (möglicherweise zirkulierendes Insulin) und der Regulierung der Hes/Hey Gene im Hirn vermuten lässt. Zusammenfassend zeigen unsere Daten ein breites Rollenspektrum von Hes3, insbesondere unter pathologischen Bedingungen.

Fazit

Unsere Arbeit stellt fest, dass mehrere Parameter des metabolischen Zustands sowie Diabetes Medikamente Hes3-Expression im Gehirn beeinflussen. Im Vordergrund steht das

metabolische Syndrom einen als die Ursache für neurologische Erkrankungen wie Alzheimer-Krankheit, Parkinson-Krankheit und Multiple Sklerose. Daher ist ein tieferes Verständnis davon, wie metabolische Erkrankungen sowohl auf der molekularen wie auch zellulären Ebene unser Gehirn beeinflussen von absoluter Dringlichkeit notwendig. In dieser Studie etablieren wir einen neuen zellulären Biomarker und eine Signalkomponente, die durch metabolische Dysfunktion reguliert wird.

List of Scientific Papers

Publications Under Submission

(1) **Nikolakopoulou P**. et al., *Metabolic dysfunction regulates Hes3 expression in the adult mouse brain. (Original data paper – outcome of the work presented in the PhD thesis)*

(2) Poser S. **…Nikolakopoulou P**….et al**.,** *Controlling distinct signaling states in cultured cancer cells provides a new platform for drug discovery. (Original data paper – not included in the PhD thesis)*

Publications (Included in the thesis)

(3) Toutouna L, **Nikolakopoulou P**, Poser SW, Masjkur J, Arps-Forker C, Troullinaki M, Grossklaus S, Bosak V, Friedrich U, Ziemssen T, Bornstein SR, Chavakis T, Androutsellis-Theotokis A. *Hes3 expression in the adult mouse brain is regulated during demyelination and remyelination*. Brain Res. 2016 PubMed PMID: 27018293

(4) **Nikolakopoulou P**, Poser SW, Masjkur J, Fernandez Rubin de Celis M, Toutouna L, Andoniadou CL, McKay RD, Chrousos G, Ehrhart-Bornstein M, Bornstein SR, Androutsellis-Theotokis A. *STAT3-Ser/Hes3 Signaling: A New Molecular Component of the Neuroendocrine System?* Horm Metab Res. 2016 PubMed PMID: 26783739

(5) Masjkur J, Poser SW, **Nikolakopoulou P**, Chrousos G, McKay RD, Bornstein SR, Jones PM, Androutsellis-Theotokis A. *Endocrine Pancreas Development and Regeneration: Noncanonical Ideas From Neural Stem Cell Biology.* Diabetes. 2016 PubMed PMID: 26798118

(6) Masjkur J, Arps-Forker C, Poser SW, **Nikolakopoulou P**, Toutouna L, Chenna R, Chavakis T, Chatzigeorgiou A, Chen LS, Dubrovska A, Choudhary P, Uphues I, Mark M, Bornstein SR, Androutsellis-Theotokis A. *Hes3 is expressed in the adult pancreatic islet and regulates gene expression, cell growth, and insulin release.* J Biol Chem. 2014 PubMed PMID: 25371201

Publications (Not included in the thesis)

(7) Chatzigeorgiou A, Chung KJ, Garcia-Martin R, Alexaki VI, Klotzsche-von Ameln A, Phieler J, Sprott D, Kanczkowski W, Tzanavari T, Bdeir M, Bergmann S, Cartellieri M, Bachmann M, **Nikolakopoulou P**, Androutsellis-Theotokis A, Siegert G, Bornstein SR, Muders MH, Boon L, Karalis KP, Lutgens E, Chavakis T. *Dual role of B7 costimulation in obesity-related nonalcoholic steatohepatitis and metabolic dysregulation.* Hepatology. 2014 PubMed PMID: 24845056

List of Abbreviations

AD	Alzheimer's disease
AKT	Protein kinase B (PKB)
ALS	Amyotrophic lateral sclerosis
AMPK	5' AMP-activated protein kinase
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
AUC	Area Under Curve
bFGF	Basic fibroblast growth factor
bFib	Bovine Fibronectin
bHLH	Basic helix-loop-helix
BMI	Body mass lindex
BW	Body Weight
cAMP	Cyclic adenosine monophosphate
CBL	Cerebellum
CD4	Cluster of differentiation 4
CD44	CD44 antigen
CD8	Cluster of differentiation 4
Chol T	Cholera Toxin
CNS	Central nervous system
CNTFR	Ciliary neurotrophic factor receptor
CTRL	Control
CTX	Cortex
DAPI	4'-6-Diamidino-2-Phenylindole
EAE	Experimental autoimmune encephalomyelitis
ECG	electrocardiography
EdU	5-ethynyl-2´-deoxyuridine
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNSCs	Endogenous Neural Stem Cells
Ex-4	Exendin-4
FGRF	Fibroblast growth factor receptor
fNSCs	fetal Neural Stem Cells
GDNF	Glial-derived neurotrophic factor
GFP	Green fluorescent protein
GLP-1	Glucagon-like Peptide 1
GMC	German Mouse Clinic
Hes	Hairy and Enhancer of Split

Hey	Hairy/enhancer-of-split related with YRPW motif protein
HFD	High Fat Diet
HPA	Hypothalamic-Pituitary-Adrenal
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPT	Hypothalamus
ICEMED	Imaging and Curing Environmental Metabolic Diseases
IF	Immunofluorescence
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
Insulin R	Insulin receptor
ipGTT	Intraperitoneal glucose tolerance test
ipITT	Intraperitoneal insulin tolerance test
Jak	Janus kinase
KO	Knock-out
LIB	Laser interference biometry
MBP	Myelin basic protein
MET	Metformin
MicroCT	Micro Computer Tomography
MIF	Microphage Migration Inhibitory Factor
MIN6	Mouse insulinoma cells
Mins	Minutes
Mot	Motor
MPI-CBG	Max Planck Institute of Molecular Cell Biology and Genetics
MS	Multiple Sclerosis
mTOR	Mechanistic target of rapamycin
ND	Normal Diet
NF-kB	Nuclear factor k-light-chain-enhancer of activated B cells
NgN3	Neurogenin 3
NMR	Nuclear magnetic Resonance
NP40	Nonidet P40
NSCs	Neural Stem Cells
O.C.T.	Optimum cutting temperature
OCT	Optical Coherence Tomography
p38 MAPK	P38 mitogen-activated protein kinases
PBCs	Peripheral Blood Leukocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDX1	Pancreatic and duodenal homeobox 1

Pen-Strep	Penicillin streptomycin
PFA	Paraformaldehyde
PI3	Phosphatidylinositol-4, 5-bisphosphate 3
Pir	Piriform
PLO	Poly-L-ornithine
PPI	Prepulse Inhibition
qPCR	Real-Time quantitative PCR
RB	Remaining brain
Rbpj	Recombining binding protein suppressor of hairless
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room Temperature
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
Sens	Sensory
SGZ	Subgranular zone
SHH	Sonic hedgehog
STAT3	Signal transducer and activator of transcription 3
STAT3-Ser	STAT3-Serine
STAT3-Tyr	STAT3-Tyrosine
STZ	Streptozotocin
SVZ	Subventricular zone
SVZ	Subventricular zone
TEWL	Trans-Epidermal Water Loss
TEWL	Transepidermal water loss
Tu	Tubercle
WB	Western Blot
WT	Wild-type

Table of Contents

1 Introduction

The adult brain exhibits low regenerative ability. Stem cell-based transplantation approaches have been largely unsuccessful, due to the difficulty to recapitulate the complex cytoarchitecture of the central nervous system (CNS). Endogenous neural stem cells (eNSCs) are a new therapeutic option as pharmacological activation and increase of their number *in vivo* is accompanied by powerful neuroprotection in various disease models (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2009; Dietrich and Kempermann, 2006; Emsley et al., 2005; Sun, 2014). This avoids the problems inherent with grafting, the problem of regenerating complex neuronal circuits, and reveals a new function of eNSCs, that of neuroprotection.

Direct injection of insulin into the brain increases the number of eNSCs and promotes the rescue of injured dopamine neurons in the adult brain through a novel signaling pathway called the STAT3-Ser/Hes3 signaling axis (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008). STAT3-Ser regulates Hes3, and together they form a convergence point for several signals, including Notch, Tie2, and insulin receptor activation (Poser et al., 2013). Hes3 is expressed in both proliferating and quiescent NSCs, which makes it an exceptional biomarker for NSC identification.

Metabolic syndrome often results in diabetes (Type I, Type II) and insulin resistance, suggesting that eNSCs may be affected by the condition. There is evidence that obesity induces inflammatory reactions in the hypothalamus, leading to neural stem cell loss (Livesey, 2012).This may be independent of the insulin dysregulation but it may also involve insulin effects.

In this part of my thesis, an overview of the literature relevant to the work is presented. Initially we introduce adult neurogenesis (historically, the observation that lead neuroscientists realize that there must be an adult eNSC population) followed by a comparison of therapeutic strategies involving cell replacement through cell grafting and those that involve the activation of endogenous NSCs. Then, the STAT3-Ser/Hes3 signaling

axis is presented in greater detail and in the context of inducing the activation of eNSC and neuroprotection. Lastly, we introduce the interconnection between metabolic syndrome and neurological disease, which is at the heart of the main aim of this work.

1.1 The "plastic brain": Neural Stem Cells, progenitors and precursors

The adult brain contains a variety of high plasticity cell types, including neural stem cells (NSCs) that generate new neurons, as well as neural progenitors, that provide replacement glia and neuroprotective factors. However, these terms are often confused. NSCs exhibit two main features: (1) they can self-renew indefinitely and (2) they are multipotent for the various neuronal and glial cell types. Multipotent progenitors of the adult brain are a type of cell that is similar to the neural stem cells. They can self renew but only a limited number of times and they can differentiate into at least two different types of cell lineage. On the other hand, lineage-specific precursors or progenitors are cells that are still undifferentiated but committed to a certain lineage (e.g. neuronal, astroglial or oligodendrocytic) (Emsley et al., 2005). There is controversy about the biology behind each term and the exact definition as the field is still evolving. Thus, often neural stem cells and the different progenitors/precursors are all referred as "precursors".

Neural precursor cells are vital for brain tissue health since they are able to: (1) maintain their undifferentiated state, and thus, a pool of replacement cells (2) exhibit high mobility (3) resist hypoxia and injury (4) proliferate and (5) produce mature neurons and glia. The heterogeneity of these cells along with their specialization and differentiation capabilities gives great opportunities for medical applications (Emsley et al., 2005; Mitchell et al., 2004). Adult neural precursors, with limited differentiation potential towards certain lineages can be of great use for the production of certain cell types. This can be of great importance in the case of neurodegenerative disease, where depending on the insult different neural cell types are affected.

1.2 Functional adult neurogenesis

The adult brain exhibits extraordinary stability, which enables the long-term memory storage combined with the required amount of plasticity, which is required for adaptation to new circumstances as well as for new memory formation. One way that the brain exhibits its plasticity as well as its ability to repair itself is with the generation of new cells.

The existence of NSCs in the adult mammalian brain was inferred in 1962 by Joseph Altman who demonstrated the incorporation of tritiated thymidine in adult neurons (Altman, 1962).

Later, Kaplan, Gould, and colleagues provided supporting evidence (Eriksson et al., 1998; Gage, 2002; Gould et al., 1999; Kaplan and Hinds, 1977; Kaplan, 1981). Goldman and Nottebohm reported similar phenomena in the adult songbird brain, demonstrating massive seasonal neuronal turnover as male birds develop new songs (Chen et al., 2015). Hockfield and McKay identified nestin as a biomarker of fetal NSCs (Hockfield and McKay, 1985), while Cattaneo and McKay developed cell culture techniques that allowed the maintenance of these cells *in vitro* (Cattaneo and McKay, 1991). Subsequently, Reynolds and Weiss were able to isolate and culture NSCs from the adult mammalian brain, which exhibited proliferation capability, as well as the potential of differentiation into neurons and glia after treatment with appropriate growth factors *in vitro* (Sato et al., 2004). These and other studies led to efforts aimed at promoting brain repair by either transplanting newly formed neurons into the brain, therefore replacing lost cells or by stimulating endogenous NSCs (eNSCs) to facilitate their neuroprotective role following brain injury or against degenerative disease (Dimou and Gotz, 2014; Hermann et al., 2014).

1.3 NSCs in conventional and nonconventional regions of the adult brain

Over the last years stem cell research labs have focused mostly on the investigation of the cellular source for the *de novo* generated neurons in the adult mammalian brain. These efforts resulted in the discovery of the so called "stem cell niches". The two most recognized NSC niches of the adult mammalian brain are: (1) the subventricular zone (SVZ) and the sub granular zone of the hippocampus (SGZ) (Alvarez-Buylla and Lim, 2004; Luskin, 1993; Reynolds and Weiss, 1992). Cells isolated from those areas exhibit self-renewal and differentiation potential similar to that of embryonic and fetal derived SCs and they can be differentiated into the neurons, astrocytes and oligodentrocytes. Additionally, they express traditional NSC markers like the intermediate filament protein nestin (Lendahl et al., 1990). More recently, there has been evidence that NSCs reside throughout the brain; they mostly maintain their quiescent state and they are in close proximity to the blood vessels as is described in ***Figure 1.1***.

NSCs can be cultured in the form of aggregate cultures (neurospheres) or in the form of a monolayer culture. These techniques have been widely used by a variety of labs, which led to the establishment of defined culture conditions and protocols. For example a basic characteristic of most of the methods is the addition of Epidermal Growth Factor (EGF) or basic Fibroblast Growth Factor (bFGF) as a mitogen source, that provide for the expansion of multipotent precursors. Neurosphere cultures, which were used traditionally, may be better for studying clonal propagation capabilities of an isolated cell type (as it is simple to assess

the generation of neurospheres from single cells), however, they comprised a very heterogeneous mixture of cell types. In the sphere, depending on their exact location, different cells have different accessibility to drugs and proteins added to the culture medium, making pharmacological studies more difficult. Likewise, the microenvironment around each cell in the sphere may differ, further confounding data interpretation.

In contrast, monolayer cultures offer a more homogeneous and easy to use system, which can be employed for drug screening experiments or signal transduction studies in order to study the mechanisms that govern NSC growth and differentiation.

Androutsellis-Theotokis and Poser presented an established protocol in 2013 about how to grow adult NSCs from conventional and non-conventional areas of the brain. This protocol enables the generation of adult NSC cultures that can be used for a variety of applications from developmental studies to medical application and drug screening (Poser and Androutsellis-Theotokis, 2013). This work establishes a technique to further assess the differentiation potential of NSCs isolated from the adult rodent brain.

1.4 Neurodegenerative diseases, cell replacement and endogenous NSCs

Recent findings by various research groups have shown the existence of stem cells in various adult tissues. These primary cells are thought to be playing a major role in the regeneration of the tissue or the organ they reside in as a response to injury or natural turnover of differentiated cells. In the adult mammalian brain, stem cells residing in the subventricular zone and the dentate gyrus are part of the mechanism underlying neurogenesis for replacement of neurons. However, it is worth mentioning that cell replacement is not the only way to respond to an injury. Recent findings show that some populations of eNSCs in the CNS may have a neuroprotective function, which could be an alternative way to treat brain injury (Kittappa et al., 2012). Thus, activation of eNSCs would be another way to treat brain injury. There is great controversy concerning which approach is the most effective. However, nowadays we know that the choice between cell replacement and endogenous activation of NSCs depends on a variety of factors including the nature of the injury and the nature of the tissue.

Cell replacement strategies through exogenous sources have recently received an enormous boost by reprogramming technologies that allow patient-specific cell sources to be converted into immature cells (somatic cells into embryonic stem cell-like cells), which can then be directed to differentiate towards specific cell fates and then can be grafted into the patient (Sanchez Alvarado and Yamanaka, 2014). However, a fundamental hindrance towards

achieving functional cell replacement therapy is the complex cytoarchitecture of the CNS. Graft-derived neurons need to be precisely placed and they oftentimes need to connect brain areas that are centimeters away from each other. In the case of Parkinson's disease (PD) for example, dopamine neurons, which extend from the substantia nigra to the striatum, die over long periods of time. Following the transplantation approach, injured neurons are replaced in one specific area (which usually is the striatum) and the new cells promote dopamine production. It has been impossible, however, thus far for those new grafted cells to recapitulate the former functional complex neuronal network (Kittappa et al., 2012; Nikolakopoulou et al., 2016). Nevertheless, in less complex tissues (e.g. liver) or in cases that the stem cell niche is easily accessible the cell replacement approach may be quite effective.

There are several other recent findings that oppose the cell grafting as an effective treatment for neurodegenerative disease. There has been evidence from research related to PD and amyotrophic lateral sclerosis (ALS) manifesting the fact that neurodegeneration is not restricted to certain cell types. When grafted cells were transplanted into the brains of PD patients, many acquired the disease themselves (Kordower et al., 2008; Li et al., 2008; McKay and Kittappa, 2008; Yamanaka et al., 2008). This supports the idea that cell grafting may help with reducing the symptoms of the disease but not in eliminating it. Last but not least, a donor-derived brain tumor following neural stem cell transplantation in a boy suffering from ataxia telangiectasia was reported in 2009 (Amariglio et al., 2009). This finding was the first to suggest that grafted stem/progenitor cells may be involved in gliomagenesis and implies the urgent need for further research concerning the safety of such methods.

In parallel to these efforts, scientists have been exploring the possibility to pharmacologically target eNSCs, to increase their number and putative neuroprotective functions. These cells are already naturally incorporated into the tissue, within specific microenvironments (the neurovascular niche) and by understanding the signals that these cells respond to, it is possible to increase their number several fold via direct local injection of a number of soluble factors (Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009). Two evidently contradictory facts about eNSC raise the question of their natural function: (1) eNSCs reside in many areas of the adult mammalian brain and they are plentiful and (2) Adult Neurogenesis is a rare event (even in the case of injury) and it is confined to certain brain areas (the subventricular zone and the subgranular zone of the hippocampus) (Kittappa et al., 2012). So, what is the purpose of eNSCs outside the two traditional stem cell niches?

Recent findings suggest the role of eNSCs in neuroprotection via the production of neuroprotective factors such as glial-derived neurotrophic factor (GDNF) and sonic hedgehog (SHH) *in vivo* and *in vitro* (Androutsellis-Theotokis et al., 2006; Ourednik et al.,

2002). The neuroprotective function of eNSCs is further supported by the fact that they are located close to neurons and blood vessels (Androutsellis-Theotokis et al., 2009) making them ideals candidates for trophic support in both the healthy neuronal state and upon neuronal injury. The latter phenomenon was observed with the use of novel biomarkers and a schematic representing this "blood vessel-neuron-stem cell association" is shown in ***Figure 1.1***.

eNSCs that differentiate into neurons are also capable of extending axons in significant distances throughout the adult brain. Recent experiments have proven their ability to respond to growth factors and to differentiate into neurons with long axonal projections that extend in large distances and survive for big time periods in areas of the brain that are not traditionally neurogenic (Emsley et al., 2005).

Taken together, these findings suggest that eNSCs are broadly distributed in the adult brain and that there are molecular mechanisms that can be used to alter their number, the expression of neuroprotective cytokines and that can also be used to control their differentiation potential so as to potentially replace injured and lost neurons.

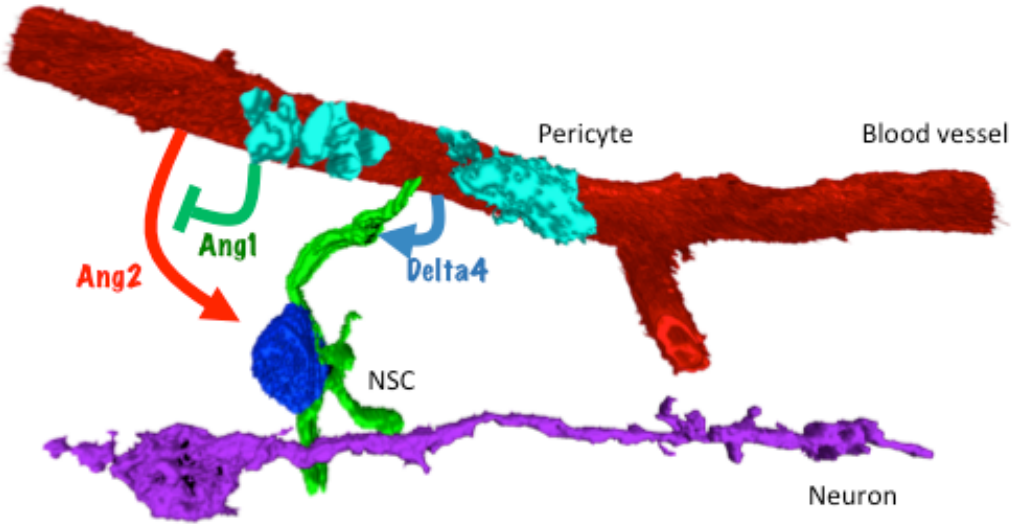

Figure 1.1. eNSCs reside in close proximity to the blood vessels and to the neurons. *Different signals from components of the vascular system (Ang2 and Delta4 from vascular endothelial cells, Ang1 from pericytes) regulate the STAT3-Ser/Hes3 Signaling Axis and, therefore, eNSCs.*

1.5 The STAT3-Ser/Hes3 signaling axis in NSCs

NSCs interpret signaling pathways in their own distinct ways. For example, whereas many cell types in the body utilize the JAK/STAT pathway for growth and survival, the same pathway leads to the differentiation of NSCs (Bonni et al., 1997; Levy and Darnell, 2002; Rajan and McKay, 1998). Specifically, tyrosine phosphorylation of STAT3 is a key component of the survival pathway of most cell types, but NSCs avoid this phosphorylation in order to avoid differentiating into glia. Instead, NSCs utilize the serine phosphorylation of STAT3 for their survival, which is largely redundant for many other cell types (Androutsellis-Theotokis et al., 2006). Several inputs into STAT3-serine phosphorylation have been identified, including a non-canonical branch of Notch signaling (activated by Notch receptor ligands such as Delta4 and Jagged1), angiopoietin 2 (a ligand of the Tie2 receptor), basic fibroblast growth factor (bFGF), and insulin (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009; Androutsellis-Theotokis et al., 2010a; Androutsellis-Theotokis et al., 2010b). These treatments lead to the transcriptional induction of Hes3, a transcription factor/passive repressor belonging to the Hes/Hey family of basic helix-loop-helix genes (Imayoshi and Kageyama, 2014; Lobe, 1997). This pathway is opposed by JAK and p38 MAP kinases and, therefore, inhibitors of these kinases promote its activation and NSC survival. Hes3 itself mediates many of these functions, as adult cultured NSCs show a reduced response to Delta4 and insulin stimulation, and to JAK inhibition (Androutsellis-Theotokis et al., 2008). A schematic diagram of the STAT3-Ser/Hes3 signaling axis is given in ***Figure 1.2***.

As an extension of these observations, carcinogenesis is sometimes viewed as the manifestation of aberrant regenerative mechanisms. The cells that carry the regenerative ability of a tumor, termed cancer stem cells, express genes common to their non-cancerous stem cell counterparts (Hanahan and Weinberg, 2011; Poser et al., 2013). In line with this notion, putative cancer stem cells from the aggressive brain tumor glioblastoma multiforme express Hes3 both in the patient and in culture. Hes3 RNA interference also opposes the growth of cultured cancer stem cells from human glioblastoma multiforme biopsies (Park et al., 2013). This is a manifestation of how different cell types utilize the same pathway in specific ways. A schematic diagram showing the differential use of the STAT3-Ser/Hes3 signaling axis is shown in ***Figure 1.3***.

Pharmacological activation of this pathway has powerful consequences as it directly promotes the survival of cultured NSCs from many sources. *In vivo*, the same treatments increase the number of putative eNSCs and induce long-lasting neuroprotection in models of ischemic stroke and Parkinson's disease (Androutsellis-Theotokis et al., 2006). One of these treatments is the direct injection of insulin into the lateral ventricle of adult rats, suggesting

that circulating insulin may also regulate eNSC number and function, and rendering the endocrine pancreas a key regulator of the eNSC population in both established neurogenic zones, such as the subventricular zone and the subgranular zone of the dentate gyrus of the hippocampus, but also in the brain parenchyma (Androutsellis-Theotokis et al., 2008). The disease-modifying functions of this pathway may involve, in part, cell replacement but more likely, the increased release of neuroprotective factors (Ohta et al., 2012). Hes3, in particular, may also be involved in the reprogramming of induced pluripotency cells and adult somatic cells to the NSC fate (Cassady et al., 2014; Salewski et al., 2012).

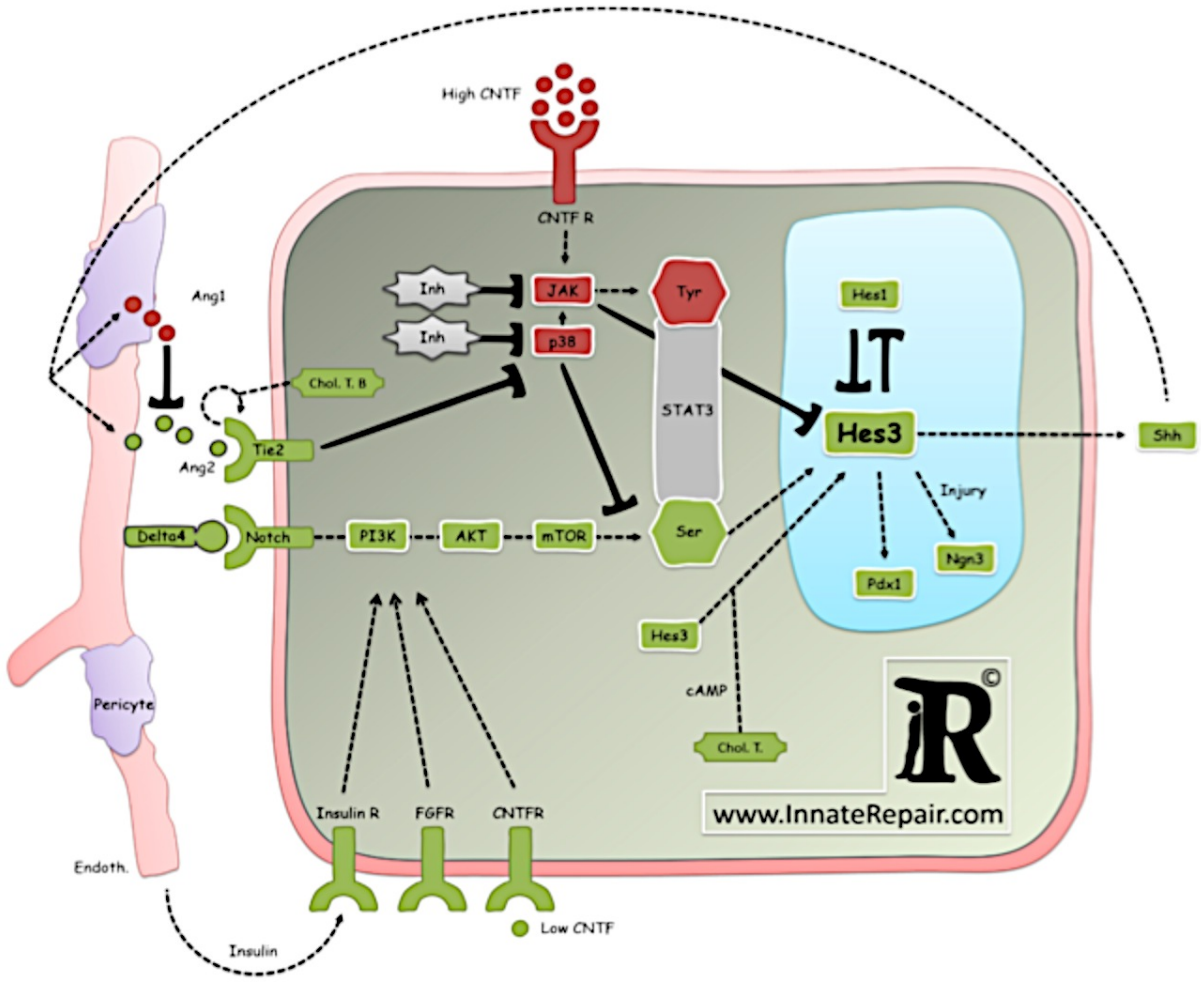

Figure 1.2. The Hes3 signaling pathway. *In NSCs STAT3-Tyr phosphorylation leads to NSC differentiation, while STAT3-Ser phosphorylation promotes self-renewal. This molecular pathway with the STAT3-Ser phosphorylation at its core regulates Hes3 and together they form a merging point for several signals including insulin receptor activation (Androutsellis-Theotokis et al., 2006; Masjkur et al., 2012; Poser et al., 2013)*

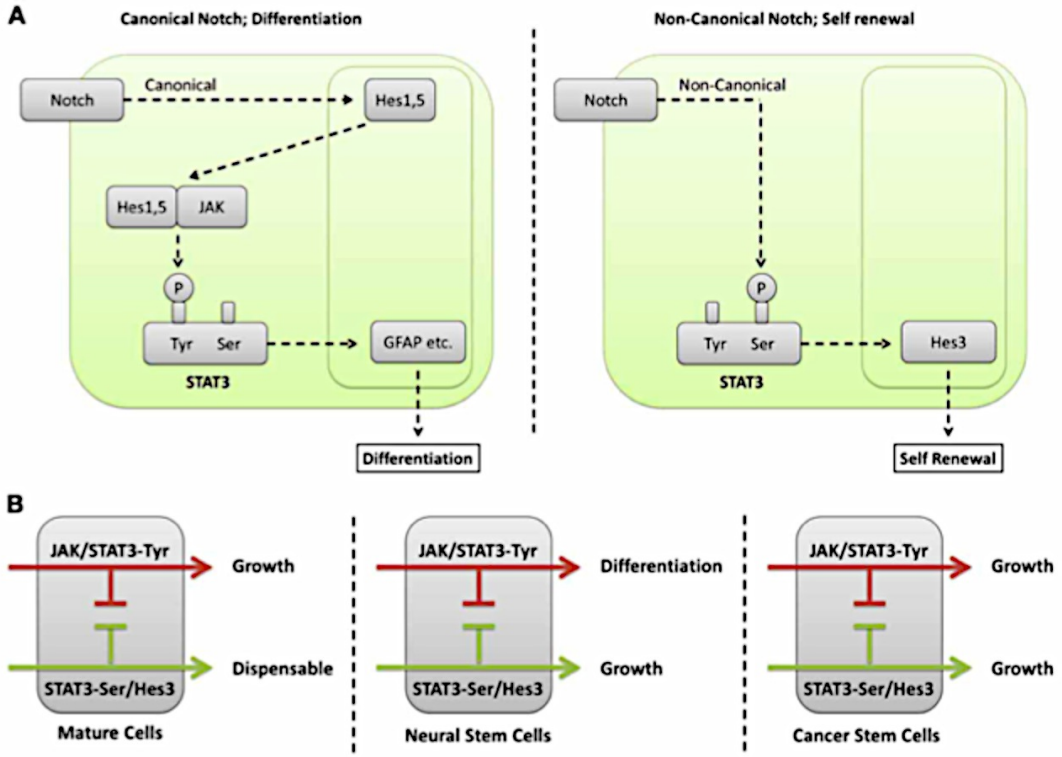

Figure 1.3. Notch signaling in SCs. *(A) Simplified diagram showing the canonical and non-canonical function of the Notch signaling pathway in NSCs. In canonical Notch signaling, the cleaved intracellular domain of the Notch receptor translocates to the nucleus where it regulates transcription of target genes, including Hes1 and Hes5. A non-canonical branch of Notch signaling leads to STAT3-Ser phosphorylation in the absence of detectable STAT3-Tyr phosphorylation. This is followed by increased transcription of Hes3. Hes3, in turn, promotes growth through poorly understood mechanisms. (B) STA3-Ser/Hes3 signaling promotes cancer stem cell (CSC) growth. This exhibits the potential of different cell types to use molecular signaling pathways in specific ways (e.g. mature cells like neurons use the JAK/STAT pathway leading to STAT3-Tyr phosphorylation for growth as opposed to immature cells that the utilize the same pathway to differentiate) (Poser et al., 2013).*

Taken together, the regulation of the STAT3-Ser/Hes3 signaling axis is very important in regeneration and carcinogenesis. Activation of eNSCs is achievable through a non-canonical branch of Notch with STAT3-Ser phosphorylation at its core; multiple treatments that induce this modification lead to increased Hes3 expression. For this reason, our lab has studied STAT3 and Hes3 in the context of regeneration, cancer and diabetes.

1.6 Beyond the brain: The STAT3-Ser/Hes3 signaling axis operates in plastic cells

To take full advantage of the therapeutic potential of eNSCs, many aspects of their biology need to be further understood, including the molecular mechanisms that regulate their number and fate specification, to allow the direction of their fate to appropriate cell types for replacement, neuroprotection, or immunomodulation. Throughout this work, we focus on a recently identified signaling pathway that regulates the number of NSCs *in vitro* and *in vivo* (Poser et al., 2013). A major input into the pathway is insulin, providing a new molecular mechanism via which the pancreas may regulate aspects of brain function. Thus, we hypothesized that the STAT3-Ser/Hes3 signaling axis may be relevant to other parts of the neuroendocrine system. Indeed, recent work shows the operation of this pathway in the pancreas, the adrenal gland and the hypothalamus (Masjkur et al., 2014a; Masjkur et al., 2014b; Nikolakopoulou et al., 2016), suggesting that it deserves further study with relevance to metabolism and the neuroendocrine system.

1.6.1 STAT3-Ser/Hes3 Signaling Axis in the pancreatic islet

It appears highly likely, then, that the endocrine pancreas can regulate aspects of brain functions, such as neuroprotection and regeneration, via the release of insulin and its actions on endogenous NSCs. Molecular mechanisms that regulate insulin production/release and pancreatic islet beta cell properties may thus be directly relevant to brain function (Ziegler et al., 2015). Remarkably, the same signaling pathway also regulates key functions of pancreatic islets, as well as their resistance to damage. These observations suggest that the STAT3-Ser/Hes3 Signaling Axis in the pancreas regulates the same signaling pathway in eNSCs and with it, the function of both of these cell types (Nikolakopoulou et al., 2016).

Specifically, Hes3 is expressed in pancreatic islets and Hes3 null mice exhibit more pronounced damage to the pancreatic islet beta cell population when treated with the toxin streptozotocin (STZ), relative to wild type controls (Masjkur et al., 2014a), resulting in a greater loss of beta cells and an earlier diabetic phenotype, as assessed by blood glucose levels. Following STZ-induced damage, Hes3 promoter activity (in this study the activity of only one of the two promoters, Hes3a, was assessed) is elevated, suggesting an involvement in pancreatic islet regeneration (***Figure 1.4***).

1.6.2 STAT3-Ser/Hes3 Signaling Axis in the adrenal cortex and medulla

We recently reported Hes3 expression in the bovine adrenal medulla and in cultures of chromaffin progenitor cells from this tissue (Masjkur et al., 2014c). Using a reporter mouse strain where the Hes3 gene has been replaced by the lacZ gene under the control of the Hes3a promoter (one of the two known promoters of Hes3) (Hirata et al., 2000), we also showed the expression of Hes3 in the mouse medulla, corroborating the bovine data (Fig. 1). In addition, we reported Hes3a promoter activity in the adrenal cortex. In the cortex, Hes3 expression was most prominent in the *zona glomerulosa,* where sonic hedgehog (Shh) – expressing progenitor cells reside (Laufer et al., 2012). Because in NSCs, Hes3 is a positive regulator of Shh expression (Androutsellis-Theotokis et al., 2006), this observation raises the question of whether Hes3 is expressed in adrenocortical progenitors and whether it functions to induce Shh expression. Copper chelation by a cuprizone-rich diet induces massive upregulation of Hes3a promoter activity further suggesting that different insult paradigms should be investigated to unveil roles of the STAT3-Ser/Hes3 Signaling Axis (Nikolakopoulou et al., 2016) (***Figure 1.4***).

1.6.3 STAT3-Ser/Hes3 Signaling Axis in tanycytes of the hypothalamus?

The STAT3-Ser/Hes3 Signaling Axis regulates pancreatic beta cells as well as neural and neuroendocrine stem cells and progenitor cells in the adult brain and adrenal gland. Given this broad range of cells that utilize the pathway, it is reasonable to speculate that hypothalamic tanycytes may also be subject to regulation by this pathway. Indeed, tanycytes of the median eminence express Hes3 (***Figure 1.4***). It remains to be determined how this pathway may affect the stem/progenitor cell properties of tanycytes and, as a consequence, metabolic regulatory functions of the hypothalamus.

The question of how the stem cell/progenitor properties of tanycytes come into play in hypothalamic function is still under intense investigation. Tanycytes may be non-terminally specified immature cells tasked with the ability for metabolic sensing (a complex biochemical function) and, at the same time, the capacity to contribute to tissue remodeling by generating new neurons. In this way, tanycytes may be able to dynamically contribute to tissue plasticity, in response to dietary and other changes (Bolborea and Dale, 2013) .

1.6.4 STAT3-Ser/Hes3 Signaling: A new molecular component of the neuroendocrine system?

Hes3 is expressed in pancreatic islet cells where it regulates insulin release (at least *in vitro*) and regenerative responses (*in vivo*). Insulin (direct injections into the lateral ventricle) regulates the number of endogenous neural progenitor cells in the adult brain. Hes3-expressing cells in the adult brain are found in a number of areas, including the SVZ, cerebral cortex, hippocampus, and hypothalamus (***Figure 1.4*** green arrows and red box). In the hypothalamus, Hes3+ cells are found in the median eminence where a fraction of them co-localizes with vimentin-expressing β2 tanycytes. Hes3 expression in these cells raises the question of possible roles of Hes3 in the neuroendocrine system, for example, through the regulation of adrenal function via the HPA Axis. The adrenal gland also contains Hes3-expressing cells. In the adult mouse adrenal medulla it co-localizes with a subpopulation of nestin positive cells (nestin-GFP). The magnification in the right side of the panel shows a perinuclear localization of Hes3 in nestin-GFP positive cells (yellow arrow), whereas cells negative for nestin-GFP displayed a nuclear localization of Hes3 (blue arrow; Scale 50 μm). Hes3+ cells in the adrenal medulla exhibit a scattered distribution and may represent adrenomedullary chromaffin progenitors, as suggested by *in vitro* studies showing the expression of Hes3 in isolated cultured progenitors. In the adrenal cortex, Hes3+ cells are located in the general area where progenitor cells are also thought to reside. Copper chelation by a cuprizone-containing diet induces massive activation of Hes3a promoter activity in the adrenal (***Figure 1.4***).

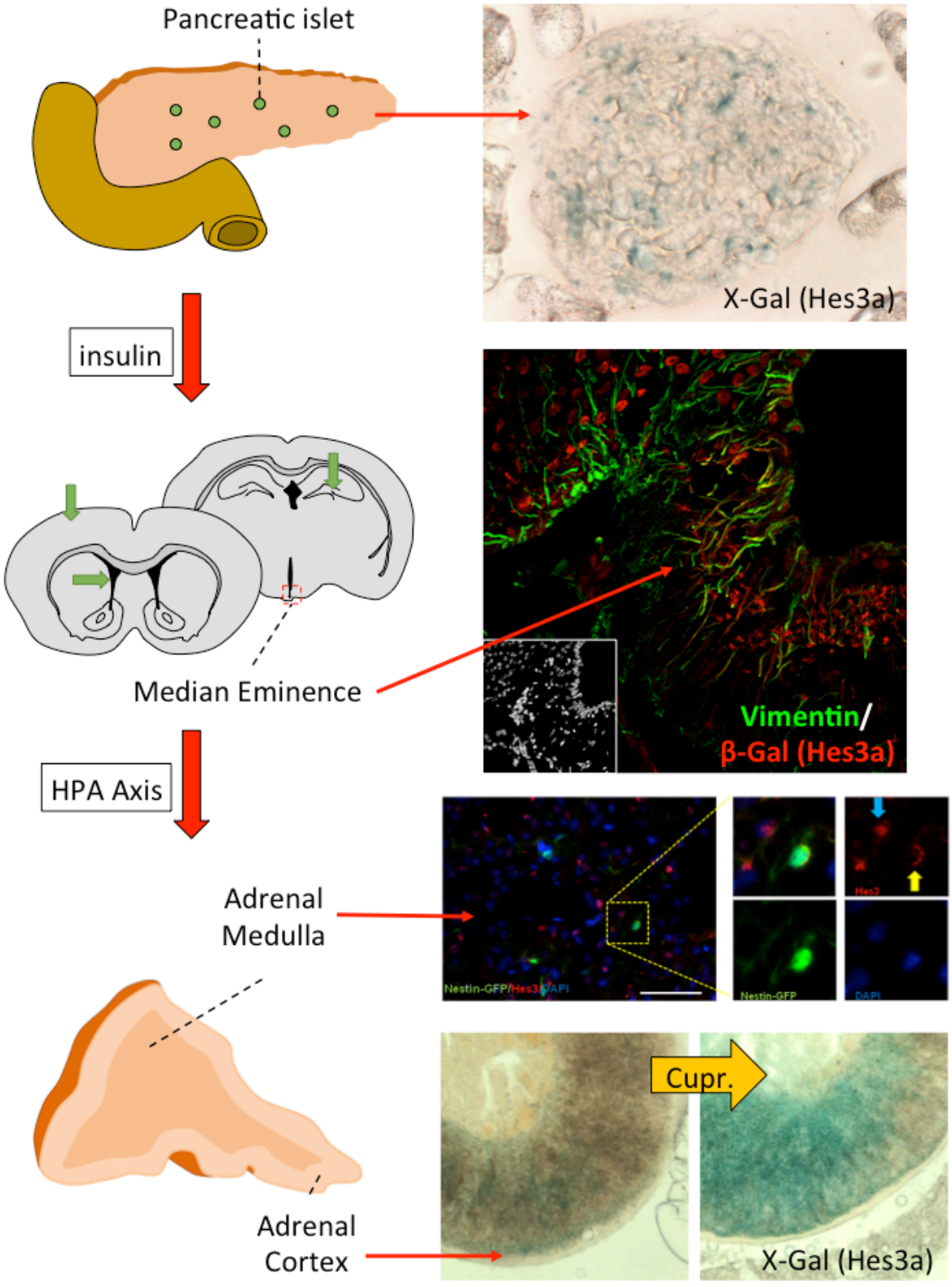

Figure 1.4. The STAT3-Ser/Hes3 signaling axis in the neuroendocrine system. *Hes3 is expressed in the pancreatic islet, in the progenitor cells of the adrenal cortex and medulla and in the tanycytes of the hypothalamus. A detailed description is provided in the text (Nikolakopoulou et al., 2016).*

1.7 Metabolic syndrome and neurological disease

Recent evidence manifests a clear connection between the occurrence and progression of neurodegenerative disease and metabolic dysfunction, manifesting how impaired metabolism may intensify neurological symptoms (Farooqui et al., 2012; Procaccini et al., 2016). Here, the evidence relevant to Alzheimer's disease, Parkinson's disease and Multiple Sclerosis is reviewed.

1.7.1 Metabolic dysfunction and Alzheimer's disease

Alzheimer's disease (AD) is the most recognized form of dementia and it typically begins with subtle memory loss. With age, cognitive decline and memory loss become severe resulting in poor quality of life and eventually death (Lee et al., 2001). Recent findings support the idea of a link between impaired metabolism and AD progression. Specifically it was shown that: (1) High Body Mass Index (BMI) and Type 2 diabetes increase the risk for AD, (2) Leptin, insulin and IGF-1 resistance correlate with AD, (3) Insulin signaling is altered in AD, (4) Treatment with diabetes treatments like several agonists of insulin, IGF-1 and GLP-1 shows beneficial effects on AD, (5) Leptin administration decreases serum levels of Aβ and improves cognitive performance, (6) Ghrelin has neuroprotective function and reduces reactive oxygen species (ROS) production and cytochrome c release and (7) Hyper/Hypo-activation of the mTOR pathway is a risk for AD and associates with reduction in autophagy (Procaccini et al., 2016).

1.7.2 Metabolic dysfunction and Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, which mainly affects the motor system and has as main outcome the " Parkinsonism", a syndrome characterized by intense disabilities (Rocha et al.). Recent findings support the idea of a link between impaired metabolism and the progression of PD. Those are: (1) Insulin/IGF-1 resistance and Type 2 diabetes worsen PD (2) The reduction of insulin relates to α-synuclein aggregation (3) GLP-1 agonists exhibit beneficial effects on PD (4) High or low IGF-1 levels associate with negative effects on PD (4) Leptin and ghrelin play a role in PD development (5) Impaired mTOR signaling is associated with PD and (6) Prolonged mTOR activity can lead to dyskinesia in PD patients (Procaccini et al., 2016).

1.7.3 Metabolic dysfunction and Multiple Sclerosis

Multiple Sclerosis (MS) is the most common chronic autoimmune inflammatory degenerating disease of the CNS, characterized by confined areas of inflammation, demyelination, axonal loss, and gliosis in both the brain and the spinal cord. In long term, MS leads to severe neurological symptoms (Milo and Miller, 2014). For many years, MS was considered as a strictly autoimmune disease; nevertheless, with the recognition that the neurological deficits come as a result of neurodegeneration the whole perception changed. Specifically, while the primary pathological cause of MS is the immune mediated destruction of myelin in the CNS, it is now understood that the neurological disability observed in MS patient is a result of the progressive axonal loss (Stadelmann et al., 2008; Trapp and Nave, 2008).

As described above for the case of AD and PD, recent evidence has suggested a direct crosstalk between metabolic alterations and the neurodegeneration observed in MS. Specifically: (1) Obesity is a risk factor for MS; (2) Leptin-deficient ob/ob mice are resistant to experimental autoimmune encephalomyelitis (EAE) (the most widely used model of MS); (3) Low calorie intake increases EAE lifespan; (4) IGF1 prevents the progression of MS; (4) Hyper activation of mTOR signaling relates to defective mechanisms of autophagy; (5) Leptin acts as neurotrophic factor and (6) Ghrelin decreases the severity of the EAE symptoms (Procaccini et al., 2016).

1.7.4 Metabolism and neurodegenerative disease: Are they connected?

Taken together, recent findings of the scientific community suggest a strong connection between metabolism and neurodegeneration. According to this scenario, hormones like insulin, leptin and ghrelin may have a far more important role than as feeding and appetite regulators. They exhibit neuroprotective functions and further research in this field could result in better outcomes for neurodegenerative disease and potential novel drug discovery possibilities.

During the last years a connection between metabolic disorders and neurodegeneration has been established. However, the mechanisms underlying the pathogenesis of the neurodegeneration due to alterations in metabolism remain largely unknown. Therefore, it is crucial to develop tools that detect tissue alterations due to metabolic disorders. Such an approach may lead to better therapeutic strategy and prevention.

1.8 Main Aim – Hypothesis

STAT3-Ser/Hes3 signaling regulates various plastic cell populations in multiple organs of the endocrine/neuroendocrine system.

Insulin is a master regulator of the pathway and, as a result, we hypothesized that the STAT3-Ser/Hes3 Signaling may be involved in tissue problems that result from metabolic dysfunction. Metabolic syndrome often results in diabetes (Type I, Type II) and insulin resistance, suggesting that eNSCs may be affected by the condition. There is evidence that obesity induces inflammatory reactions in the hypothalamus, leading to NSC loss (Livesey, 2012). However, it is not clear if damage to NSCs is also directly linked to insulin signaling disruption. Recent findings suggest an interconnection between metabolic disorders and neurological disease. Thus, it is crucial to maintain homeostasis of the eNSCs of the brain in such conditions. It is therefore mandatory to develop tools and biomarkers capable of detecting the altered eNSC state in the brain.

The main aim of my study is to investigate whether the STAT3-Ser/Hes3 signaling axis and, specifically, Hes3 expression can be utilized as a tool to assess the brain state in metabolic syndrome and aging. Thus, this work has three main objectives: (a) to confirm that Hes3 is expressed in specific mouse brain areas (b) to investigate if eNSCs are affected by metabolic syndrome and aging with the use of Hes3 as a biomarker and (c) to assess the importance and the functionality of the Hes3 gene in metabolic syndrome.

2 Materials and Methods

2.1 Animal experiments

2.1.1 Animal use authorization

Adult C57Bl/6J mice or Hes3 null mice in the C57Bl/6J background were used throughout the experiments. The Hes3 null mouse line was kindly provided by R. Kageyama (Hirata et al., 2001). 6 week-old male mice were either obtained from Janvier or bred in MPI-CBG or the Medical Faculty's animal house in Dresden, Germany. Mice were used according to our experimental needs and in accordance with the approved guidelines from the Landesdirektion Sachsen. Mice were kept under normal conditions [Food (Normal diet unless stated differently) and water ab libitum, 12:12h light-dark cycle, temperature controlled room (20-22°C)]. Bedding was changed in the cages once per week. At the GMC mice are housed according to the GMC housing conditions and German laws. All tests performed at the GMC were approved by the responsible authority of the district government of Upper Bavaria, Germany.

2.1.2 Genotyping

Genotyping of the mice for the experiments among WT and Hes3 null mice was performed either in house or in the MPI-CBG facility using the primers and the following protocols:

Hes3 Genotyping

Hes3 +: FW 5' GGCGGGCTGCACGCTTTAATGGGACACATG-3

Hes3 KO (-): REV 5'-ATTACGCCAGCTGGCGAAAGGGGGATGTGC-3

Hes3 WT (-): REV 5'-CACTATGTCTGCTTGCCAAGTCCTGGCTGC-3'

The Reverse Transcriptase PCR (PCR) reaction has three primers and not two. It is designed this way to allow us to identify heterozygotes from homozygotes in a single

reaction. Homozygotes can be Hes3+, Hes3+ or they can be LacZ+, LacZ+. The Hes3+ band is approximately 441bp and the band from the LacZ is around 838bp. So, the homozygote Hes3 will give only one band size 441bp, the homozygote for LacZ will give only one band 838bp and the heterozygote will give both bands (441bp and 838bp). The DNA region that we wanted to amplify is GC rich and therefore we added betaine enhancer solution (Sigma, B0300). PCR reactions were performed using 5-50ngr of genomic DNA, 10x buffer, 2.5mM dNTPs, 10x PCR betaine, 10 μM primer Hes3+, 10 μM primer Hes3 WT- , 10 μM primer Hes3 KO, Taq DNA polymerase (5U/μl) and ddH_2O to the desired final volume. The PCR reactions were performed with the following protocol:

Step	Temperature (°C)	Time	# Cycles
Initial Denaturation	95	2 min	1
Denaturation	95	30 sec	35
Annealing	63	1 min	
Extension	72	2 min + 5sec/cycle	
Final extension	72	5 min	1

LacZ detection was used as an extra positive control. The primers that we used detect only the presence of the LacZ gene and they are the following: LacZ FW: 5'-CTGCGATGTCGGTTTCCG-3 LacZ RV: 5'-GGATGGTTCGGATAATGCG-3'. PCR reaction for LacZ primer pair is:

Step	Temperature (°C)	Time	# Cycles
Initial Denaturation	94	5 min	1
Denaturation	94	45 sec	30
Annealing	63	45 min	
Extension	72	1 min 30sec	
Final extension	72	10 min	1

2.1.3 *In vivo* models

2.1.3.1 Aging

For the aging experiments mice were divided into several groups and kept under normal conditions until certain time points. We investigated aging effects on Hes3 expression on young mice (4 months old) versus older mice (8 months and 10 months old).

2.1.3.2 Diabetes mouse models

Prior to the experiments all mice were subjected to a one to two week acclimatization period to the housing conditions. *Type I diabetes-Single High Dose STZ:* 8 week-old mice were injected intraperitoneally [phosphate-buffered saline (PBS) vehicle control or STZ (180 mg/kg, Sigma Aldrich, S0130)] and were euthanized 8 weeks later.

Type II diabetes-HFD: 8 week old mice were fed Normal Diet (ND, 10% kcal % fat, D12450B, OpenSource Diets – Research Diets) or High Fat Diet (HFD, 60% kcal % fat, D12450B, OpenSource Diets – Research Diets) and euthanized after ~10 weeks ("Short Feeding"), or ~30 weeks ("Long Feeding").

2.1.3.3 Metformin administration

24 mice were divided into two groups, one served as control group and one as experimental group. Metformin was administered in the drinking water (2g/l, Sigma, D-150959) starting at the eight weeks of age. Water was changed 2 times per week and mice were euthanized 8 weeks later.

2.1.4 *In vivo* metabolic Analyses

In all the *in vivo* experiments body weight (BW) was measured weekly at the same time of the day.

Intraperitoneal glucose tolerance test (ipGTT): Mice were used for the glucose tolerance test after a 16-18 hours-lasting overnight food-withdrawal. In the beginning of the test, the body weight of mice was determined. For the determination of the fasting blood glucose level, the tip of the tail was scored using a sterilized scalpel blade and a small drop of blood was analyzed with the Accu-Chek Aviva glucose analyzer (Roche/Mannheim). Thereafter mice were injected intraperitoneally with 2 g of glucose/kg body weight using a 20 % glucose solution, a 25-gauge needle, and a 1-ml syringe. 15, 30, 60 and 120 minutes after glucose injection, additional blood samples (one drop each) were collected and used to determine blood glucose levels as described before. After the experiment was finished, mice were placed in a cage with plentiful supply of water and food.

Intraperitoneal insulin tolerance test (ipITT): Prior to the ipITT mice were fasted for 6h. We then followed the procedure described above for the ipGTT. After fasting, BW and glucose levels were determined (fasting values, time point 0) and then the mice were injected with insulin (Lilly, Bad Homburg, Germany) (0.75U/kg of body weight). Blood glucose levels were determined 15, 30, 60 and 120 minutes post injection. After the experiment was finished, mice were placed in a cage with plentiful supply of water and food.

Fasting BW, Fasting Blood Insulin, and Fasting Blood Glucose levels: Prior to euthanization mice were fasted overnight. The next morning and prior to euthanization, fasting values of BW and glucose levels were determined. Mice were euthanized and blood samples were collected for serum insulin determination. Serum insulin concentration was determined with an ultra-sensitive mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (90080, Crystal Chem) and according to the manufacturer's instructions.

2.1.5 Nociception

Pain is the perception of an aversive or unpleasant sensation that originates from a specific region of the body. The highly subjective nature of pain is one of the factors that make it difficult to define and to treat clinically. Pain is more than a conspicuous sensory experience that warns of danger.

Nociceptors are activated by tissue injury but also by mechanical, thermal, or chemical stimuli. Harmful stimuli applied to the skin or to subcutaneous tissue, activate nociceptors, the peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root or in the trigeminal ganglia.

The hot plate test

The hot plate test was used in order to assess the responsiveness of the somatosensory system of the Hes3 null mice to thermal pain (nociceptive pain). The mice were placed on a metal surface maintained at 52 ± 0.2 C (Hot plate system was made by TSE GMBH, Germany. Locomotion of the mouse on the hot plate was constrained by 20 cm high Plexiglas wall to a circular area with a diameter of 28 cm. Mice remained on the plate until they performed one of three behaviors regarded as indicative of nociception: hind paw lick (h.p. licking), hind paw shake/flutter (h.p. shaking) or jumping.

We evaluated only hind paw but not the front paw responses, because fore paw licking and lifting are components of normal grooming behavior. Each mouse was tested only once since repeated testing leads to profound changes in response latencies. The latency was recorded to the nearest 0.1 s. To avoid tissue injury 30 s cut-off time was used. The data values are given in seconds.

2.1.6 Histology

2.1.6.1 Organ isolation and Tissue Preparation

Brains were dissected, fixed overnight and cryoprotected in 30% sucrose solution phosphate buffered saline at 4°C until sinking. Brains were then embedded in Tissue-Tek optimal cutting temperature (O.C.T.) compound (VWR 25608-930). We identified the mouse brain areas that we were interested in with the use the Mouse Brain Atlas (Franklin and Paxinos, 2008). Tissue slices were prepared in the cryostat and mounted on glass slides. Slides were either processed directly of frozen (minus 80 degrees) until further use.

2.1.6.2 Immunofluorescence

For immunofluorescence (IF) staining the following protocol was followed. Slides were allowed to dry in room temperature (RT) for 10-15 minutes prior to staining. Then, they were washed with PBS (1 fast, 2 x 5min washes) while shaking. Slides were then incubated for 1h in permeabilization solution (0.3% Triton X in PBS). Washes with PBS followed (3x10min) and then slides were incubated for 1h in blocking solution [5% BSA-0.1% Tween-20 in PBS (PBS-T)]. Primary antibodies were diluted in blocking solution and overnight incubation in RT followed. The next day, sections were washed thoroughly (3x15min with PBS-T, RT) and then secondary antibody incubation (diluted in blocking solution) for 1h in RT followed. Nuclei were stained with 4′, 6-Diamidin-2-phenylindol (DAPI) diluted in PBS (1:10000). 2 last washes with PBS followed and slides were mounted (Aqua-Poly/Mount, 18606, Polysciences). Slides were directly imaged or stored at 4°C for further processing. A complete list of the antibodies and the respective dilutions is provided in the form of a table in the end of the section 1.1.6.

2.1.6.3 Whole mount brain staining for β-Galactosidase using X-gal

Whole mount X-gal staining: Brains were dissected and fixed in 2% PFA. They were then washed 3 times with washing solution [0.02% Nonidet P40 (NP40) in PB (0.1M phosphate buffer pH7.2); (3x20mins, RT)]. 1mgr/ml 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal, ThermoFisher, R0404) was diluted in working stain solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$ and 5mM EGTA in PB), and brains were stained in the dark overnight in RT or 37°C depending on the strength of the staining. Washes with cold PBS followed and images of the brains were acquired with a standard camera. (β-Galactosidase detection in certain brain areas was performed with antibody staining as described above).

2.1.6.4 Imaging and Analysis

Images were acquired with a Zeiss Axiovison Apotome fluorescent microscope or a Zeiss LSM780 confocal system. Image analysis was performed using the Fiji software (Schindelin et al., 2012).

Primary Antibodies				
Antibody	**Company**	**Catalogue #**	**Host species**	**Dilution**
Hes3	Santa Cruz	sc-25393	rabbit	1/100
Hes3 (B-6)	Santa Cruz	sc-398654	mouse	1/100
Hes3 (B-12)	Santa Cruz	sc-55587	mouse	1/100
LacZ	MP Biomedicals	8559762	rabbit	1/500
Vimentin	Abcam	ab24525	chicken	1/2000
SecondaryAntibodies				
Type	**Company**	**Catalogue #**	**Host species**	**Dilution**
a-rabbit IgG 594	Jackson ImmunoResearch	711-515-152	donkey	1/500
a-mouse IgG 488	Jackson ImmunoResearch	715-485-151	donkey	1/500
a-chicken IgY 488	Jackson ImmunoResearch	703-546-155	goat	1/500

2.1.7 PCR and Real-Time quantitative PCR (qPCR)

2.1.7.1 Tissue Collection and RNA Isolation

Brain tissues were dissected on ice. Depending on the experimental plan brain without cerebellum and olfactory bulb (indicated as BRAIN), brain without cerebellum, olfactory bulb and hypothalamus (indicated as Remaining Brain ‘’RB’’), hypothalamus (HPT) and cerebellum (CBL) were dissected. RB was split into two hemispheres and one was directly processed for RNA extraction. CBL was also divided in two parts and the same procedure was followed. Full HPT was used for the RNA extraction. Total RNA was isolated with the High Pure RNA isolation kit (Roche, 11828665001) and 1 µg of total RNA per sample was reverse transcribed using Promega M-MLV reverse transcriptase (Promega, M170B).

2.1.7.2 PCR and qPCR experiments

PCR experiments were performed with DreamTaq Green DNA Polymerase (EP0712, ThermoScientific and the respective protocol) consisting of 3-5 ul cDNA (always synthesized from 1μgr RNA), 0.2uM of each primer, 0.2uM dNTPs and 0.75U Taq Polymerase per reaction.with addition of betaine (5M, Sigma, B0300) and ddH_2O to a final 30ul reaction volume. qPCR experiments were performed with SsoFast EvaGreen Supermix (172-5201, Biorad) in a CFX384 Real time PCR Detection System. Relative gene expression was evaluated with the ΔΔCt method upon normalization to hypoxanthine-guanine phosphoribosyltransferase (HPRT). The primer sets and reaction protocols are listed below.

Primer Sets used for PCR and qPCR experiments:

Gene	Seq5'—>3' FW	Seq5'—>3' REV	Method
mHes1	AAGATAGCTCCCGGCATTCCAAGC	AGCGCGGCGGTCATCTGC	PCR; qPCR
mHes3	AAAGCTGGAGAAGGCCGATA	TCCTTGCCTACGTCTCACCA	PCR
mHes3a	GTGATCTCCAAGCCTCTGATGGAGAA	CAGCTTTCGTTTCCGTATCTGATGTGA	PCR; qPCR
mHes3b	CCAGCAGCTTCCGAAAGATCTCCA	TCTCCAGCTTTCGTTTCCGTATCTGA	PCR; qPCR
mHes5	CAACAGCAGCATAGAGCAGC	AGGCTTTGCTGTGTTTCAGG	PCR; qPCR
mHes6	GGTGCAGGCCAAGCTAGAG	TGAAAGCTGCTACCCTCACG	PCR; qPCR
mHes7	CCCAAGATGCTGAAGCCGTTGGT	AGCTTCGGGTTCCGGAGGTTCT	PCR; qPCR
mHey1	AGGCATCATCGAGAAGCGCC	AGCTTAGCAGATCCCTGCTTCTCA	PCR; qPCR
mHey2	TGAGAAGACTAGTGCCAACAGC	TGGGCATCAAAGTAGCCTTTA	PCR; qPCR
mHeyL	CAGCCCTTCGCAGATGCAA	CCAATCGTCGCAATTCAGAAAG	PCR; qPCR
mHPRT	AAGCTTGCTGGTGAAAAGGA	TTGCGCTCATCTTAGGCTTT	PCR; qPCR

PCR Protocol steps:

Step	Temperature (°C)	Time	# Cycles
Initial Denaturation	95	10 min	1
Denaturation	95	4 min	35
Annealing	60	30s	
Extension	72	1 min	
Final extension	72	10 min	1

qPCR Protocol steps:

Cycling Step	Temp (°C)	Time	# Cycles
Enzyme activation	95	1 min	1
Denaturation	95	5 sec	40
Annealing/Extension	60	15 sec	
Melt Curve	65-95°C (in 0.5 °C increment)	5 sec/step	1

2.1.8 Western Blot

2.1.8.1 Protein Isolation and Western Blot

Mice were euthanized and brain tissues were dissected on ice. Whole brains or brain tissue parts (as indicated) were dissected and tissue was lysed in 300-500μl (depending on the tissue part) lysis buffer using an IKA® T10 basic ULTRA-TURRRAX® mechanical tissue homogenizer. Lysis buffer contained RIPA lysis buffer with addition of 7x cOmplete, Mini protease inhibitor cocktail (Roche, 11836153001) and 10x PhosSTOP phosphatase inhibitor cocktail (Roche, 04906845001). Tissue was either processed directly for WB experiments or stored in -80°C for further use. Protein concentration was determined in Qubit® 2.0 Fluorometer (Invitrogen) with the Qubit® Protein Assay Kit (Q33211, Life Technologies) according to the manufacturer's instructions. Protein was loaded equally (30-60μgr) on each lane of a 4%-12% gradient Nu-PAGE Mini Tris-Base gel and run in an XCell *Sure-Lock*® Mini-Cell System (Invitrogen) with NuPAGE® MOPS SDS Running Buffer (Invitrogen NP0001). The XCell II™ Blot Module (Invitrogen) was used for the transfer of proteins to nitrocellulose membrane. Throughout the procedure the instructions of the manufacturer were followed accompanying the WB system. The successful protein transfer was verified with ponceau staining (P7170, Sigma). Membranes were washed 3 times with TBS-T and blocked for 1h in blocking buffer in RT. Primary antibody was diluted in blocking buffer and with no washing step, overnight incubation of the membranes in the primary antibody solution followed (4°C, shaking). The next day, the membranes were washed thoroughly

(3x15mins in TBS-T). Peroxidase conjugated secondary antibodies were diluted in 5% fat-free milk in TBS-T and then incubation with the secondary antibody followed (1h, RT, shaking). Membranes were washed again with TBS-T (3x15mins, RT). Lastly, membranes were incubated in SuperSignal™ West Pico or Femto Chemiluminescent Substrate (34080 or 34095, Invitrogen) and imaging followed with a LAS4000 system. A complete list of the antibodies and the respective dilutions is provided below.

Primary Antibodies				
Antibody	**Company**	**Catalogue #**	**Host species**	**Dilution**
Hes3	Santa Cruz	sc-25393	rabbit	1/100
Hes3 (B-6)	Santa Cruz	sc-398654	mouse	1/100
Hes3 (B-12)	Santa Cruz	sc-55587	mouse	1/100
STAT3 (pan)	Santa Cruz	sc-293151	mouse	1/200
Phospho-STAT3 (Ser 727)	Cell Signaling	9134	rabbit	1/1000
Phospho-STAT3 (Tyr 705)	Cell Signaling	9145	rabbit	1/2000
AKT (pan)	Cell Signaling	4691	rabbit	1/1000
Phospho-AKT (Ser 473)	Cell Signaling	4060	rabbit	1/2000
GAPDH	Abcam	ab8245	mouse	1/10000
Secondary Antibodies				
Type	**Company**		**Catalogue #**	**Dilution**
a-mouse IgG HRP-conj	Jackson ImmunoResearch		115-035-003	1/2500
a-rabbit IgG HRP-conj	R&D systems		HAF008	1/2500

2.2 Mouse phenotyping

Comprehensive phenotypical characterization of Hes3 null mice was done in collaboration with the German Mouse Clinic (GMC), Munich, Germany (Gailus-Durner et al., 2005). Beginning with the age of 9 weeks, a total of 64 mice (16 males, 16 females, 16 control males and 16 control females) were subjected to various non-invasive tests including dysmorphology, cardiovascular health, energy metabolism, clinical chemistry, eye, behavior, neurology, nociception, immunology, allergy, and pathology. Tests were conducted using the protocols described before (Fuchs et al., 2011) and referenced at https://www.mouseclinic.de (Click on "VIEW RESULTS OF MUTANT LINES" or "phenomap" and search for project "Hes3_KO").

In a second cohort (11 males, 11 females, 11 control males and 11 control females) we conducted a 24-weeks high fat challenge feeding a purified diet [E15741-347 (D12492 mod.) Ssniff Spezialdiäten GmbH, Soest, Germany, containing 60 energy-% from beef tallow]. Mice were subjected to the following tests beginning at the age of 12-13 weeks: Body composition analysis (qNMR, Minispec LF 50, Bruker, Ettlingen, Germany), 21 hours indirect calorimetry (Phenomaster, TSE Systems Gmbh, Bad Homburg, Germany), rectal body temperature,

body surface temperature (thermosensor: Almemo ZA 9040, data logger: Almemo 2290-8, Ahlborn, Holzkirchen, Germany), and ipGTT after overnight food deprivation.

2.3 Neural stem cell cultures

2.3.1 Preparation – Coatings

Fetal Neural Stem Cells (fNSCs) were grown throughout the experiments on Poly-L-ornithine [PLO (Sigma, P3655)] – bovine Fibronectin [bFib (R&D Systems, 1030-FN)] coated flasks, dishes and plates depending on our experimental needs. Plastic bottom dishes: PLO was used at concentration of 75μg/ml in ddH_2O (5x) and bFib at a concentration of 1μg/ml in PBS. Glass bottom dishes: PLO was used at concentration of 495μg/ml in ddH_2O (5x) and bFib at a concentration of 10μg/ml in PBS. Culture dishes were coated with PLO 2 days before plating the cells and for at least 24 hours. Next day dishes were washed with PBS (1fast, 2x5min) and then they were coated with bFib for at least 24 hours as well. Before plating the cells the culture dishes were washed twice with PBS (1fast, 2x5min).

2.3.2 Cell Isolation and Cell Culture

fNSCs were dissected and cultured from mouse embryos at embryonic day 13.5 (E13.5) following previously described and established protocols (Johe et al., 1996). Cells were grown in serum-free N2 medium [DMEM-F12 (Sigma Aldrich, D8062), 100 μg/mL Apo-Transferrin (Sigma Aldrich, T2036), 10 mg/ml Insulin (I9278, Sigma Aldrich) 20 nM Progesterone (Sigma Aldrich, P8783), 100 μM Putrescine (Sigma Aldrich, P5780) and 30 nM Selenite (Sigma Aldrich, S5261), 100 μg/mL Pen-Strep (Life Technologies, 15140122)]. For NSC maintenance basic fibroblast growth factor [bFGF (233-FB-01M, R&D Systems)] was added to the cells at a concentration of 20ng/ml daily as described before (Poser and Androutsellis-Theotokis, 2013). After dissection cells were plated and they were grown for 3-4 days (bFGF every day, media change every 2 days). After 4-5 days in culture cells were passaged to new precoated dishes. At this point the culture is relatively homogeneous and it is mostly comprised of Nestin (a marker of NSCs (Hockfield and McKay, 1985)) positive multipotent precursors. To induce differentiation to neurons and glia the N2+bFGF medium was replaced with N2-bFGF. Cells were fed for time periods above 5 days and medium was changed every 2 days.

2.3.3 Pharmacological Manipulation (Metformin – Exendin-4)

***Dose dependence experiments – Cell Number*:** Cells were seeded at 10,000 cells per well in a 12 well plate and treated with different concentrations of metformin (Sigma, D-150959)

or exendin-4 [Ex-4 (Biotrend, BP0111)] beginning 24 hours after plating. Cells were fixed after 72h with 4% PFA for 20 minutes and nuclei were stained with 4',6-Diamidino-2 Phenylindole (DAPI).

Cell Proliferation: fNSCs proliferation was determined after 72h by incubating the cells with 10 µM 5-ethynyl-2´-deoxyuridine (EdU) for 5h and followed by visualization using the Click-IT EdU Alexa Fluor 594 Imaging Kit (Invitrogen C10339) following the manufacturer's instructions. Five images (using a 20x objective) from each well were acquired (3 wells per plate) with a standard Zeiss structured illumination microscope (Zeiss – Axio Observer Z1, inverted) and cells were counted using the Fiji software. The same procedure was followed for both cell number and proliferation experiments.

PCR/qPCR*:* For the time-course experiments cells were seeded at 500,000 cells per T25 flask and treatments (0 or 500 µM metformin and 0 or 200nM Ex-4) were added 24hours after plating. Cells were collected at 6, 24, 48, and 72h and processed directly for PCR/qPCR experiments. Cell culture medium (including bFGF and the respective treatment was changed every 24hours.

Western Blot**:** Cells were washed quickly on ice with cold PBS. 30 µl of lysis buffer was added to the cells and they were detached using a cell scrubber. Samples were either processed directly for WB experiments or stored in -80°C for further use. Western blot experiments were performed as described above (see section 2.1.8.1.)

2.4 Heat maps

Heat maps were generated in Morpheus https://software.broadinstitute.org/morpheus/.

2.5 Statistical analyses

Data are expressed as means ± SEM. Statistical analyses were performed in Graphpad Prism (GraphPad Software, Inc., San Diego, CA). The Student's t test, a Mann- Whitney U test, or one-way ANOVA were used and significance was set at $p < 0.05$ (denoted as an asterisk in the figures). A detailed account of the statistical methods used in the phenotypic analysis of the Hes3 null mice is provided in the GMC Report (https://www.mouseclinic.de).

3 Results

Notch receptor activation can lead to canonical downstream signaling that involves the association of the cleaved intracellular domain of the Notch receptor with other proteins and the direct binding of the complex to target gene promoter regions such as Hes1 and Hes5 (Artavanis-Tsakonas et al., 1999). It can also lead to a non-canonical branch that involves the indirect activation of Hes3 (Androutsellis-Theotokis et al., 2006). This non-canonical branch of Notch or " STAT3-Ser/Hes3" was initially associated with neural stem cells. It was later proven to be a master regulator of cancer stem cells as well (Park et al., 2013). More recently, it was also found that Hes3 is expressed in the adult mouse pancreatic islet and regulates gene expression, cell growth and insulin release (Masjkur et al., 2014a). Insulin regulates Hes3 expression (Androutsellis-Theotokis et al., 2008); impaired insulin signaling is potentially linked to neurodegeneration and brain insulin resistance has been associated with Alzheimer's disease (de la Monte and Wands, 2005).

My thesis project aims to further investigate the STAT3-Ser/Hes3 signaling axis as a tool to characterize the brain state in metabolic syndrome and aging. To achieve this, my experimental plan had three main objectives: (a) to show that Hes3 is expressed in specific mouse brain areas (b) to investigate if eNSCs are affected by metabolic syndrome and aging with the use of Hes3 as a biomarker and (c) to assess the importance and the functionality of the Hes3 gene in metabolic syndrome. To achieve these aims, a combination of *in vivo* and *in vitro* tools were utilized.

Initially, we investigated Hes3 expression in the adult mouse brain. We used PCR to measure Hes3 at the mRNA expression level and immunofluorescence to detect Hes3 at the protein level. However, throughout this thesis, quantification of our data is mainly based on qPCR analysis (due to the limited specificity of the available polyclonal anti-Hes3 antibodies). We utilized *in vivo* models of aging and diabetes in an effort to address whether the brain is affected by metabolic syndrome. To obtain clues to the functions of Hes3, we performed a general phenotypic analysis of Hes3 null mice. Since Hes3 null mice revealed stress-induced

phenotypes before (Masjkur et al., 2016; Toutouna et al., 2016) we performed the phenotypic analysis under both normal and stressed conditions.

3.1 Hes3 is expressed in the mouse brain

To investigate Hes3 expression in the mouse brain we utilized a combination of PCR, qPCR, and immunofluorescence (IF) experiments. To measure Hes3 expression, we prepared RNA extracts from mouse brains where the olfactory bulb and all parts hind to the cortex were removed unless stated differently. PCR analysis showed expression of Hes3 in the 17 week old mice tested (***Figure 3.1*** *B, C*). We used primer sets that detect total Hes3 as well as primer sets that detect each of the two isoforms (Hirata et al., 2000) of Hes3 (Hes3a and Hes3b), demonstrating that both isoforms are expressed.

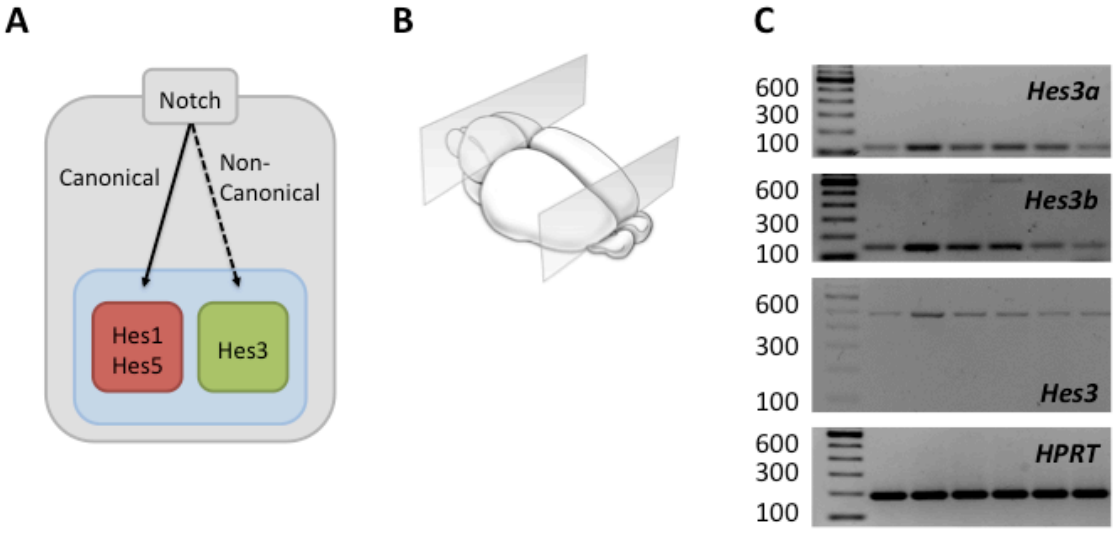

Figure 3.1. Hes3 is expressed in the mouse brain. *(A) Canonical and non-canonical branches of Notch signaling induce the expression of different members of the Hes/Hey gene family. (B) Diagram showing the brain area used for PCR, qPCR, and Western Blot experiments (unless otherwise noted). PCR blots for Hes3, Hes3a, and Hes3b showing mRNA expression in the 17 week old mouse brain. HPRT was used as a housekeeping control (N=6. Each lane corresponds to a different mouse).*

We performed the immunofluorescence study in the area of the septo-diencephalon. We observed that in this area of the brain Hes3 is expressed in the outer layer of the cortex forming a characteristic dense layer. We defined this layer as the “Hes3 zone”. The antibody staining against Hes3 further confirmed the Hes3 expression in the mouse brain at the protein level (***Figure 3.2***).

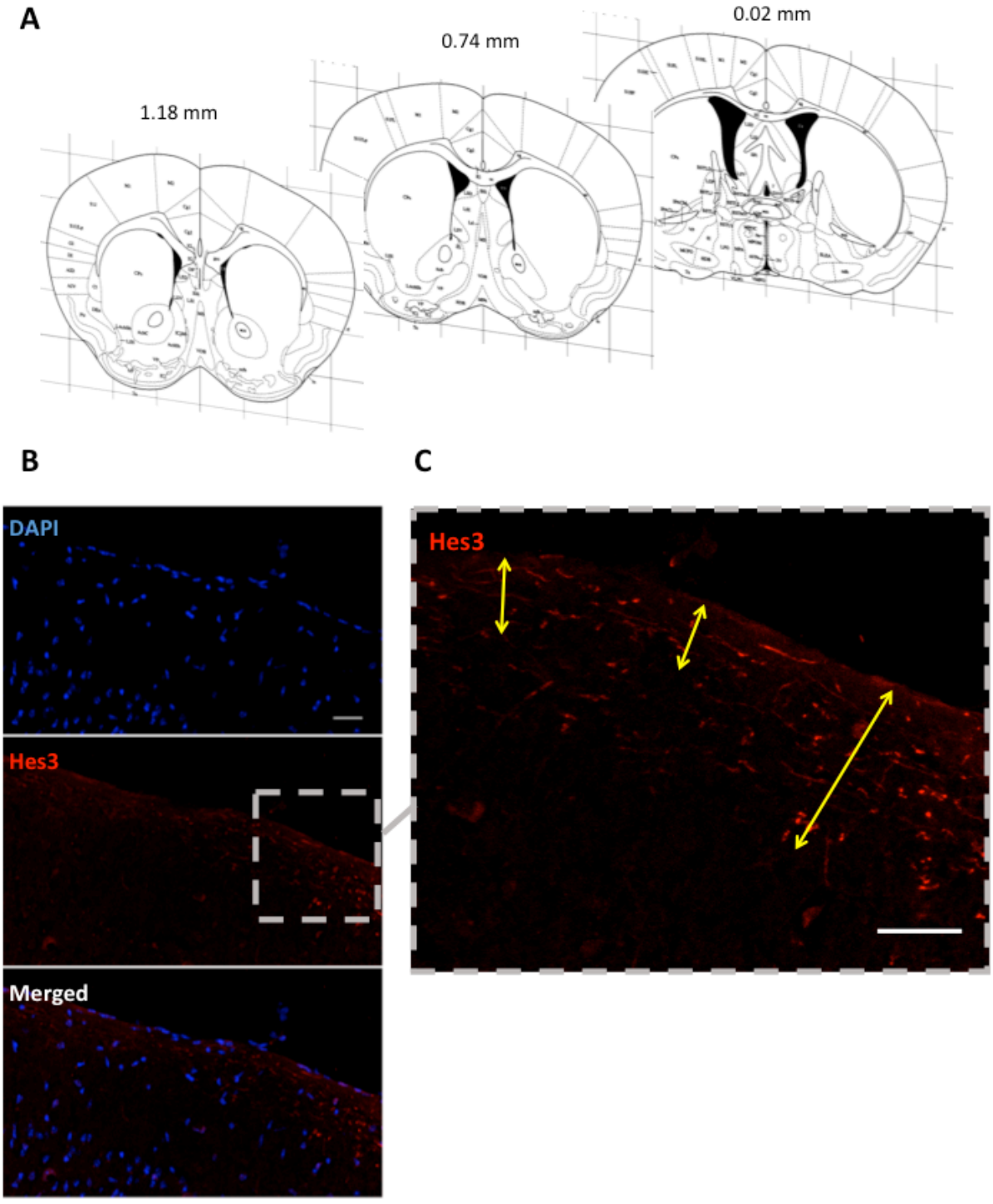

Figure 3.2. Hes3 expression in the mouse brain cortex. *(A) Immunofluorescence study for Hes3 expression in the septodiencephalic area of the mouse brain (Bregma 1.18 to 0.02mm). (B) Hes3 expression in the motor cortex of the mouse brain. (C) Magnified imaged of the Hes3 staining in the motor cortex. To measure the ''Hes3 zone'' we performed 3 measurements per image (yellow arrows). Scale bar 30μm.*

3.2 Aging and diabetes models alter Hes3 in the brain

Insulin is a powerful inducer of Hes3 expression. Impairment in insulin signaling has been widely associated with neurodegenerative disease. Aging results in lower amount of insulin in the CNS, which may be associated with cognitive impairment and neurodegeneration (Laron, 2009). We therefore addressed whether Hes3 expression is altered in type 1 and type 2 diabetes mouse models and aging. In the pancreas, the toxin streptozotocin (STZ) induces a powerful increase in Hes3 expression, possibly in an effort to promote regeneration of pancreatic islet cells (Masjkur et al., 2014a; Masjkur et al., 2016; Poser et al., 2015). Here we addressed whether similar effects can also be observed in the brain. STZ is used to damage pancreatic islet cells and produce animal models for the study of type 1 diabetes that exhibit reduced production and systemic circulation of insulin (Al-Awar et al., 2016). HFD-induced type 2 diabetes is a complex condition that involves elevated circulating insulin (an activator of Hes3) as well as complex inflammatory responses (some of which promote and others oppose Hes3 expression) (Masjkur et al., 2014a; Ohta et al., 2012). It is therefore difficult to predict the effect of particular HFD paradigms on brain Hes3; here we investigated this question using established HFD protocols.

3.2.1 Hes3 expression decreases with age

To assess the effect of aging on Hes3 expression we compared two groups of mice referred to as "young" (~17 weeks old) and "old mice" (~34 weeks old). To measure Hes3 expression, we prepared RNA samples from brain tissue as described above (***Figure 3.1*** *B*). The data revealed significant reduction of Hes3 expression from 16 to 34 weeks of age. An additional cohort of animals confirmed the reduction in Hes3 mRNA levels from 16 to 42 weeks of age (Reduction: Hes3a 0.12±0.04 fold, $p<0.05$; N=5,4 and Hes3b:0.66±0.05 fold, $p<0.05$; N=6,4). A polyclonal antibody against Hes3 further confirmed the reduction in Hes3 expression from 16 to 34 weeks of age (***Figure 3.3*** *B-D)*

3.2.2 Pancreatic islet damage by streptozotocin increases Hes3 expression in the brain

Consistent with published studies, mice treated with STZ exhibited increased glucose levels, and reduced insulin levels, demonstrating successful implementation of this animal model. In these mice, Hes3a and Hes3b mRNA levels in the brain were significantly increased; in contrast, the mRNA levels of the canonical Notch signaling targets Hes1 and Hes5 were not

altered (***Figure 3.4***). These data show that pancreatic damage leads to Hes3 expression changes in the brain**.**

3.2.3 High Fat Diet reduces Hes3 expression in the brain

HFD is a common approach to study aspects of obesity and metabolic dysfunction in mice (Islam and Loots du, 2009). We subjected the mice to HFD for different time periods (indicated as "short feeding', "intermediate feeding", and "long feeding" and we measured changes in the Hes3 mRNA expression levels with qPCR. In both the short and long HFD feeding groups the mice gained BW and exhibited an increase in circulating glucose and insulin levels, as expected in this model of type 2 diabetes **(*Figure 3.5*** *A-C*). In the short HFD feeding group, Hes3a, Hes3b, Hes1, but not Hes5 expression was reduced; in the long HFD feeding group, Hes3a and Hes3b expression showed a tendency to decrease but did not reach statistical significance. Again, Hes1 but not Hes5 levels also dropped (***Figure 3.5*** *D-E*).

Hes3 in the mouse brain is expressed in distinct plastic cell types depending on the area of interest; in the motor cortex Hes3 is expressed in oligodentrocyte precursors (Toutouna et al., 2016) and in the hypothalamus in the tanycytes of the median eminence (Nikolakopoulou et al., 2016). Therefore, we used a separate group of mice kept under HFD conditions for an intermediate time period (16 weeks), to identify particular brain areas where Hes3 mRNA levels are regulated. Mice under HFD gained weight as expected. From this group we prepared RNA samples from distinct brain areas: Hypothalamus (HPT), Cerebellum (CBL), and the Remaining Brain without HPT, CBL, and olfactory bulbs that we denote here as “RB”). In this group of mice we measured significant reductions in both Hes3a and Hes3b levels (***Figure 3.6*** *A-C*).

These results show that HFD induces significant alterations in the expression of Hes3 and Hes1 in the brain.

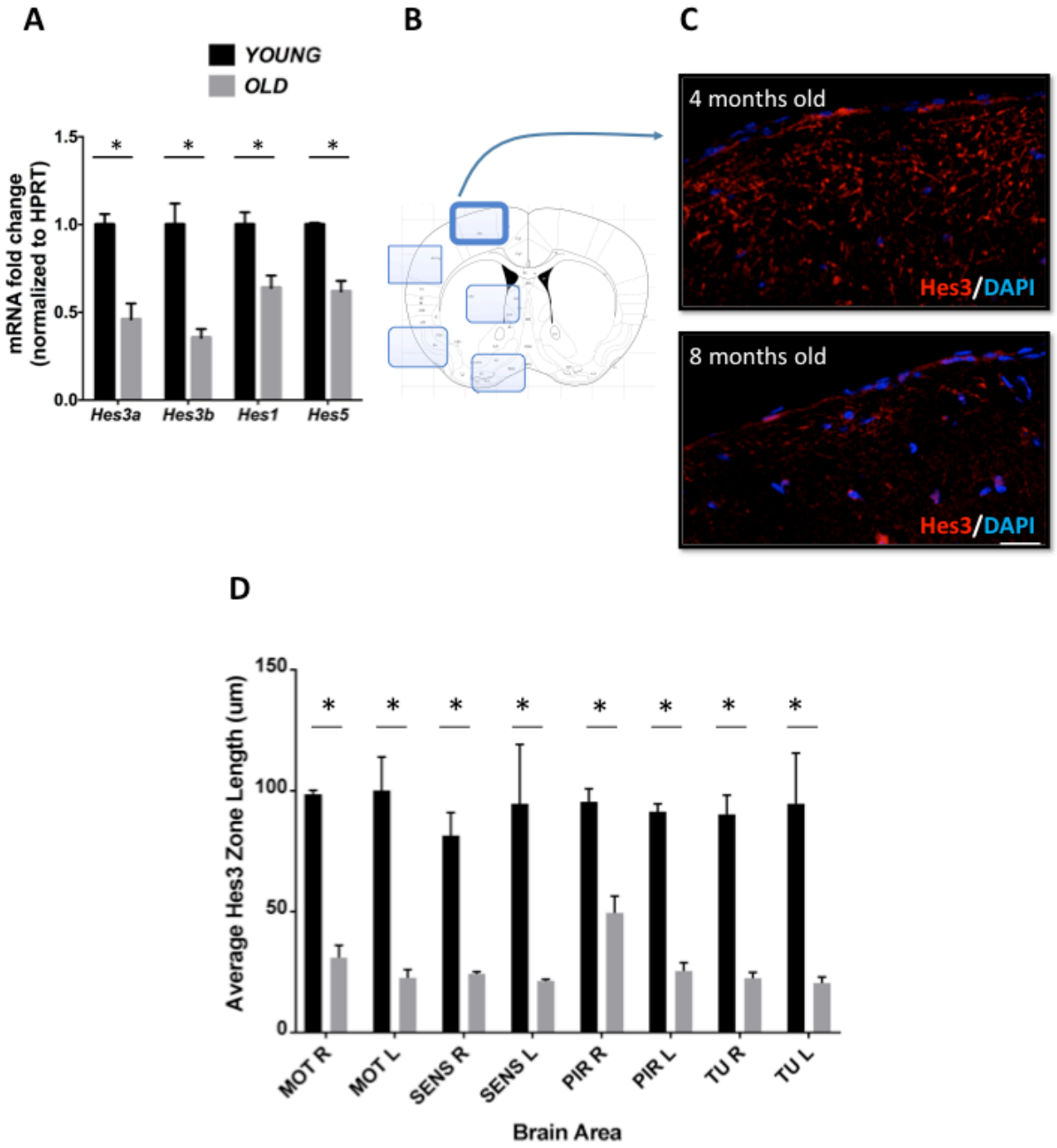

Figure 3.3. Hes3 expression decreases with age. *(A) qPCR analysis of the relative mRNA expression of Hes/Hey genes in young and old mice (N=3-6). (B) Diagram showing the brain areas used for image acquisition and Hes3 signal measurement. (C) Examples of images used for Hes3 quantification. The particular images correspond to the top field marked in (A). Nuclei are stained with DAPI and are shown in blue (Scale bar: 50μm). (D) Quantification of Hes3 immunolabeling signal from (C). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

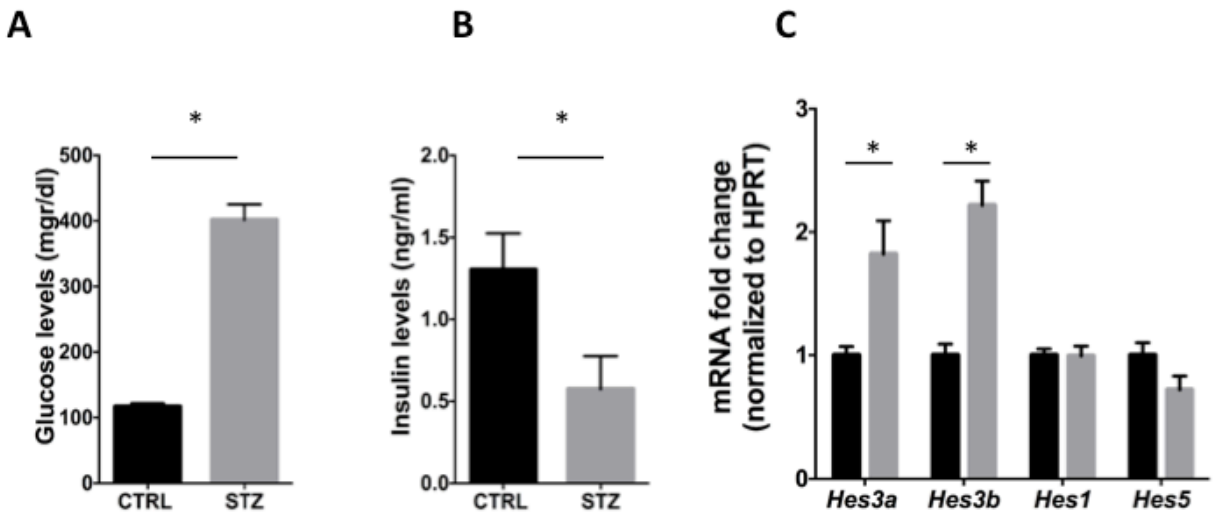

Figure 3.4. Streptozotocin increases Hes3 expression in the mouse brain. *(A,B) A single high dose of STZ increases blood glucose levels, and reduces insulin levels 8 weeks after injection, indicating the establishment of the STZ model of type 1 diabetes (N=8). (C) A single high dose of STZ significantly increases Hes3a and Hes3b levels in the brain ($p<0.05$); Hes1 and Hes5 levels do not significantly change (N=6-7. HPRT was used as a reference gene). Data are presented as mean ± SEM. Mann-Whitney U test; *$p<0.05$.*

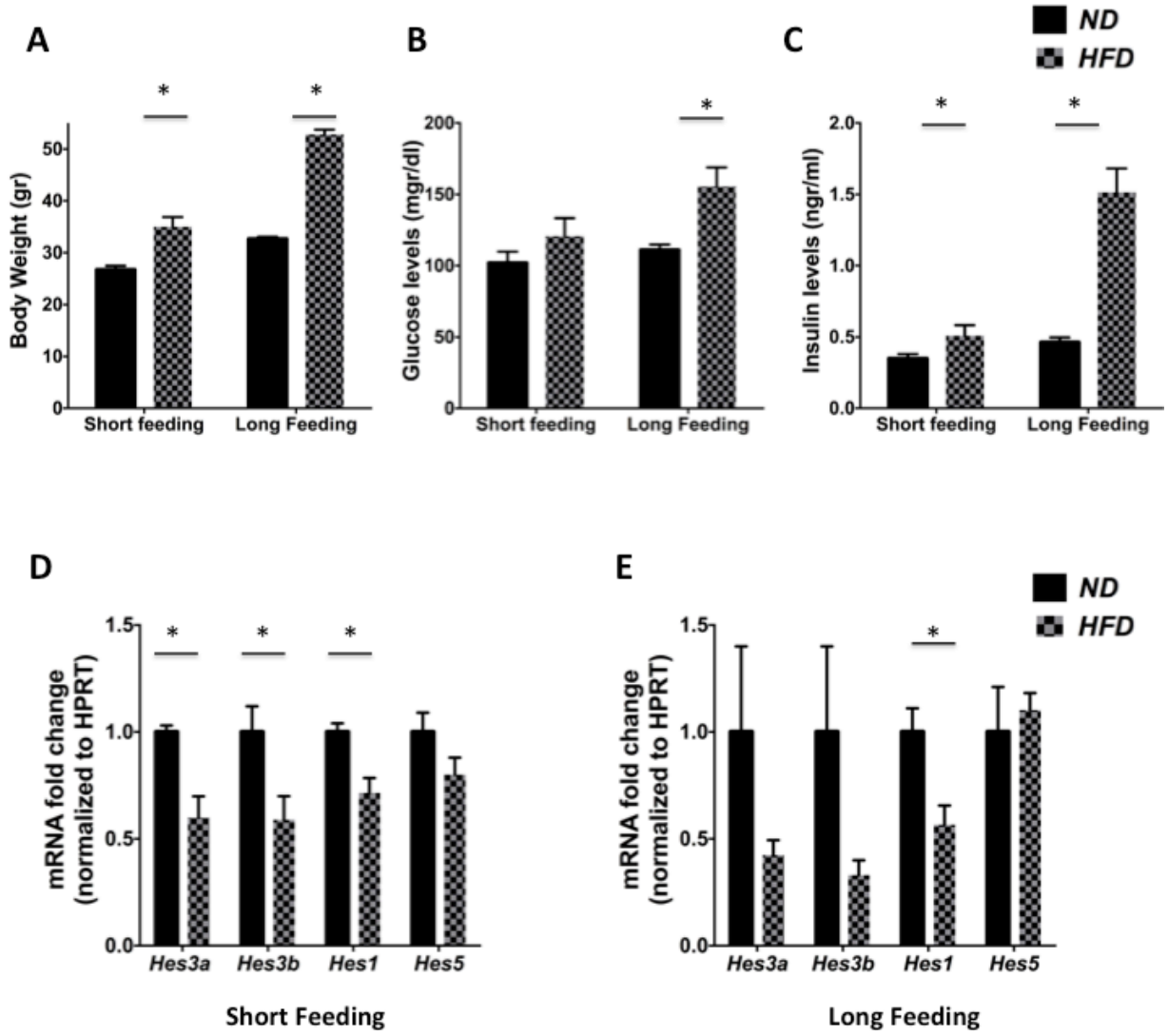

Figure 3.5. HFD decreases Hes3 expression in the mouse brain. *(A-C) HFD increases BW, blood glucose levels, and insulin levels at different time points, indicating the establishment of the Type II diabetes model (N=4-10; measurements were performed after an overnight fasting). (D) HFD (short and long feeding) regulates Hes/Hey gene expression (N=4-7. HPRT was used as a reference gene). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

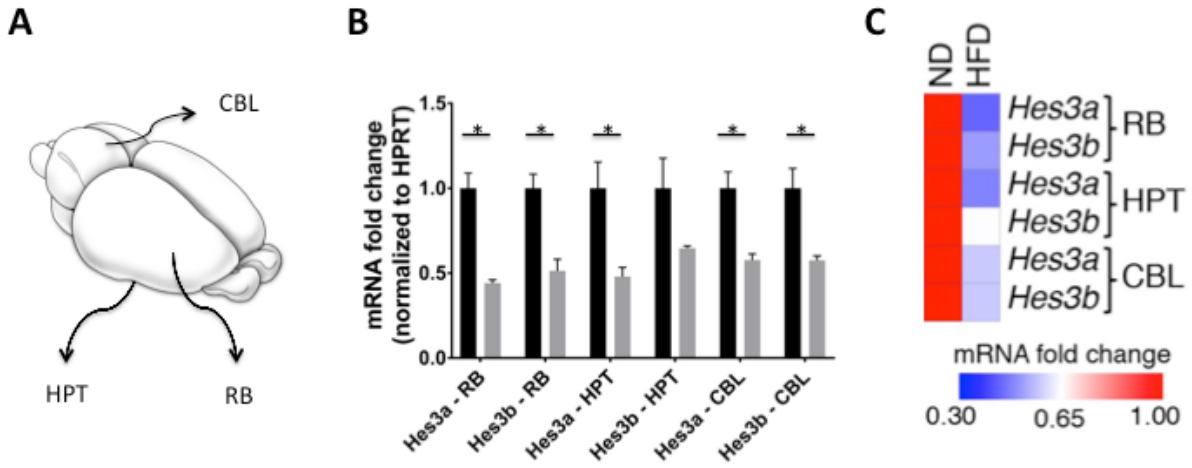

Figure 3.6. HFD regulates Hes3 expression in different brain areas. *(A) Diagram showing the dissected brain areas. (B) Average relative gene expression for Hes3a and Hes3b in different brain areas. (N=5-8; BR, HPT, CBL; intermediate HFD feeding; HPRT was used as a reference gene). (C) Heatmap of the data shown in (B). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

3.3 Common diabetes medication affect neural stem cells (NSCs) in the brain

We also investigated whether common medication for metabolic related dysfunction also affects Hes3 expression in the adult brain. Metformin is a widely prescribed medication; its effects on cognition and the progression of neurodegenerative disease are not yet fully understood. It regulates signaling pathways that intercept with Hes3; we thus addressed the effect of metformin on Hes3 expression in the living brain; and in cultured NSCs to address the question if metformin acts on the NSCs directly.

3.3.1 Metformin decreases Hes3 expression in the brain

We addressed the effect of metformin on Hes3 expression in the living brain. Mice were given metformin in the drinking water for 8 weeks, starting at the age of 8 weeks. At the end of the experiment, brains were dissected and RNA preparations were made from RB, HPT, and CBL. HPT is involved in metabolic regulation and Hes3 is expressed in the hypothalamic tanycytes (Nikolakopoulou et al., 2016), Hes3 is highly expressed in the CBL although its roles are unknown (Hirata et al., 2001); RB would provide an overview of Hes3 regulation in the remaining brain areas. Metformin did not significantly affect BW gain (not fasted values);

fasting BW was not affected, glucose levels were significantly reduced and fasting insulin levels were slightly but not statistically significantly increased (***Figure 3.7*** *A-D*). Our data show that metformin affects the expression of multiple Hes/Hey genes in the living brain (***Figure 3.7*** *E-H*). Interestingly, Hes3 is significantly decreased in the hypothalamus after metformin treatment.

3.3.2 Metformin opposes growth but increases Hes3 expression in cultured NSCs

Metformin affects the expression of multiple Hes/Hey genes in the adult mouse brain. A summary of the data obtained of our *in-vivo* experiment described above is given in the form of a heat map (***Figure 3.8*** *A*). To address the direct effect of metformin on NSCs, we used established culture systems of primary mouse fetal NSCs (fNSCs). Dose response experiments demonstrated that 500µM metformin reduced cell number, 5-ethynyl-2´-deoxyuridine (EdU) incorporation decreased, and cell morphology changed to a more differentiated appearance with longer processes (***Figure 3.8*** *B-E*).

3.3.3 Exendin-4 promotes growth and increases Hes3 expression in cultured NSCs

Exendin-4 (Ex-4) is another commonly used type-2 diabetes medication. We previously showed that it induces Hes3 expression in a cultured mouse insulinoma cell line (Masjkur et al., 2014a). Thus, we hypothesized that it may affect NSCs as well. Dose dependence experiments revealed a significant increase in the cell growth at concentrations (200nM) while the morphology of the cells and proliferation (as indicated via EdU staining) were not affected (***Figure 3.10*** *A-D*). qPCR analysis revealed increase in the Hes3 mRNA expression level at 72 hours similarly to the result we observed with metformin. This result suggests that the increase in Hes3 expression by metformin may be part of a stress response to the treatment. Hes5 mRNA expression levels increase as well after 72 hours treatment with Ex-4 (***Figure 3.10*** *F*).

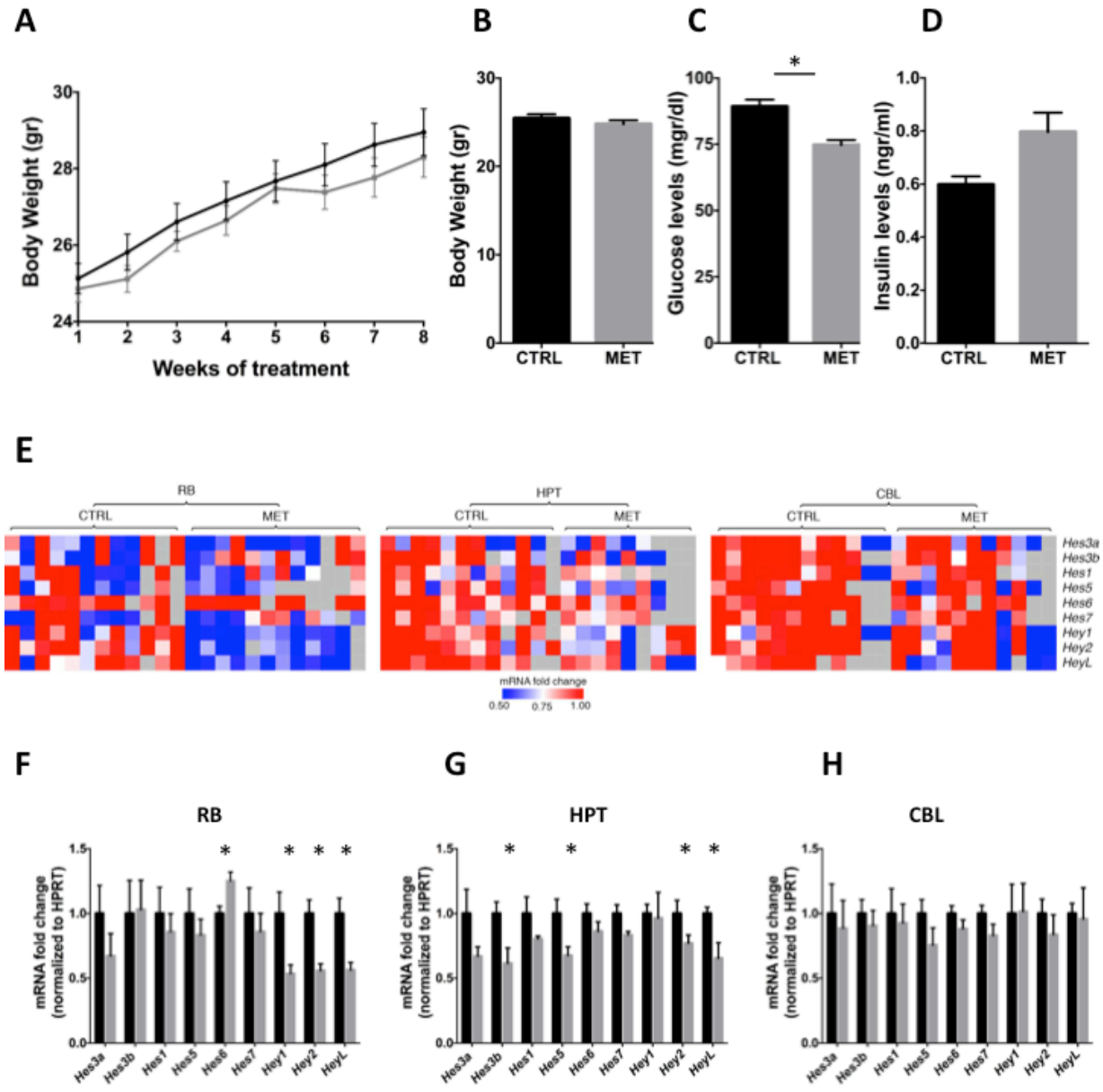

Figure 3.7. Metformin regulates Hes3 expression in vivo. *(A) BW progression in mice treated with metformin (2g/l in the drinking water, continuously for 8 weeks), and control mice (normal drinking water; not fasted BW measurements; N=12). (B) BW, glucose, and insulin measurements in mice treated with metformin, and control mice. (N=12; measurements were performed after an overnight fasting). (E) Heatmap showing gene expression levels of different Hes/Hey genes in different areas of the brain in control and metformin-treated mice. (N=5-12; MET, Metformin; HPRT was used as a reference gene]. (F-H) Bar graph version of the qPCR analysis shown in (E). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

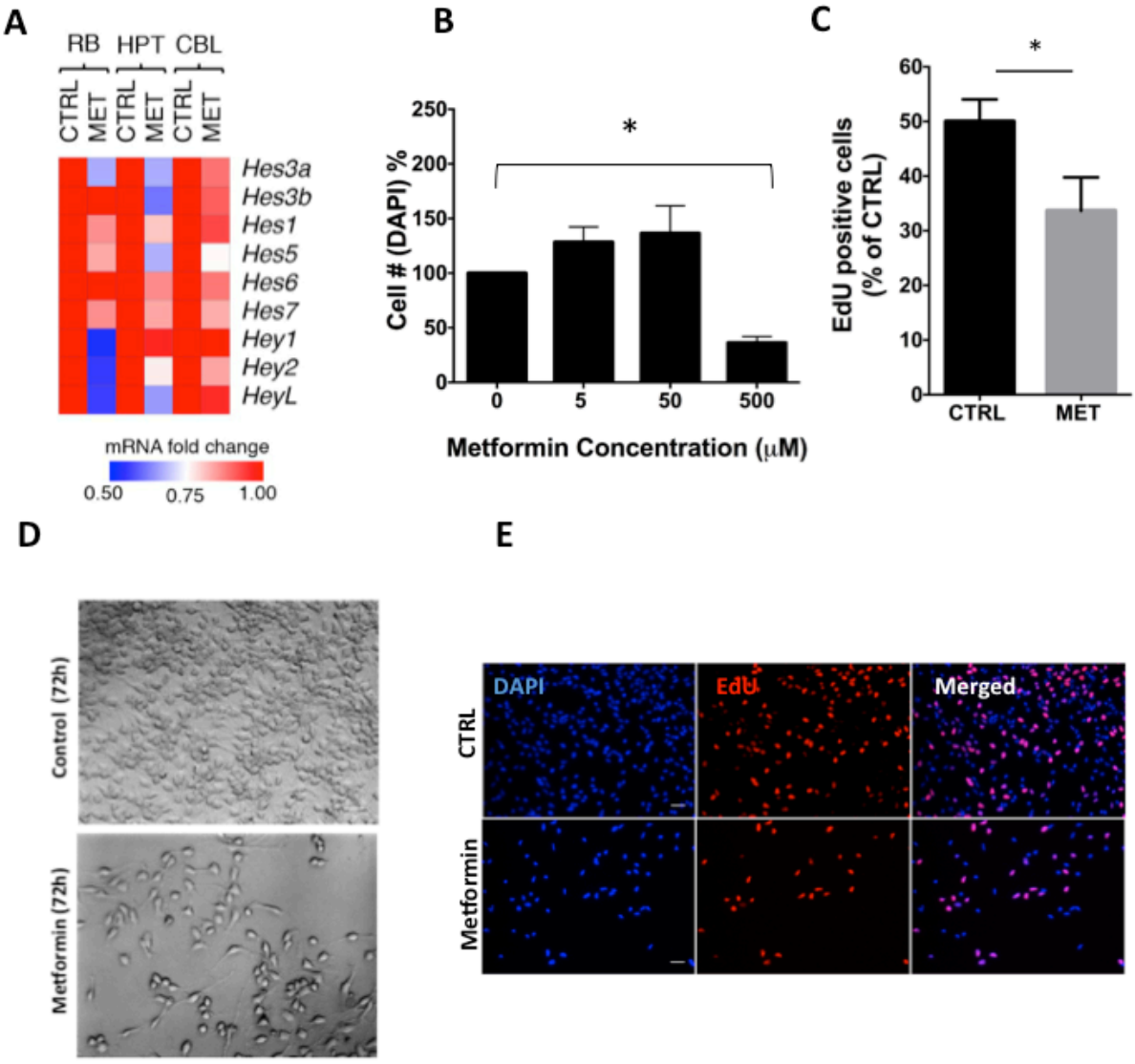

Figure 3.8. Metformin affects cultured NSCs. *(A) The heatmap shows average gene expression levels in different areas of the brain after metformin treatment for 2 months. (N=5-12; MET, Metformin) (B) Metformin reduces cell number (DAPI-stained nuclei counts) in a dose-dependent manner (N=4; 72h, 500μM). (C) Metformin (500μM) reduces EdU incorporation in vitro (N=3; 72h (D, E) Brightfield images of control and metformin-treated (500μM, 72 hours) primary fNSC cultures. Scale bar: 30 μm. Data are presented as mean ± SEM. Unpaired t-test; *p<0.05. HPRT was used as a reference gene.*

Time course experiments revealed a time-dependent increase in Hes3a and Hes3b mRNA levels; at 72 hours we observed increased mRNA levels of Hes3a and Hes3b but not *Hes1* or Hes5 (***Figure 3.9*** *A, B*). These data show that metformin regulates Hes3 levels in cultured NSCs.

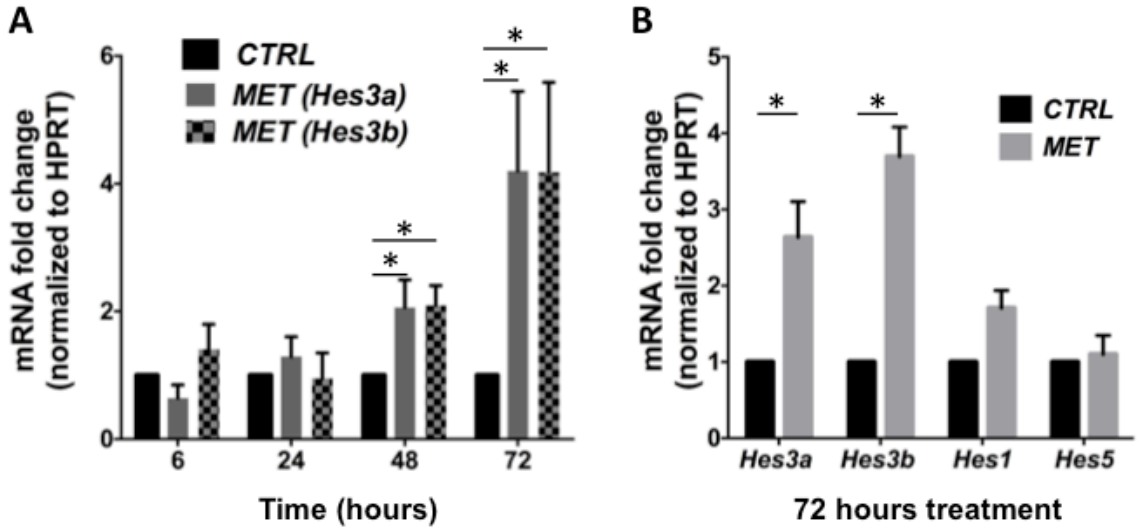

Figure 3.9. Metformin treatment increases Hes3 mRNA expression levels. *(A) Metformin (500μM) regulates Hes3a and Hes3b mRNA levels in vitro a time dependent manner (N=3). (B) Metformin (500μM) regulates Hes3a and Hes3b but not Hes1 and Hes5 mRNA levels in vitro (N=3, 72h). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05. HPRT was used as a reference gene.*

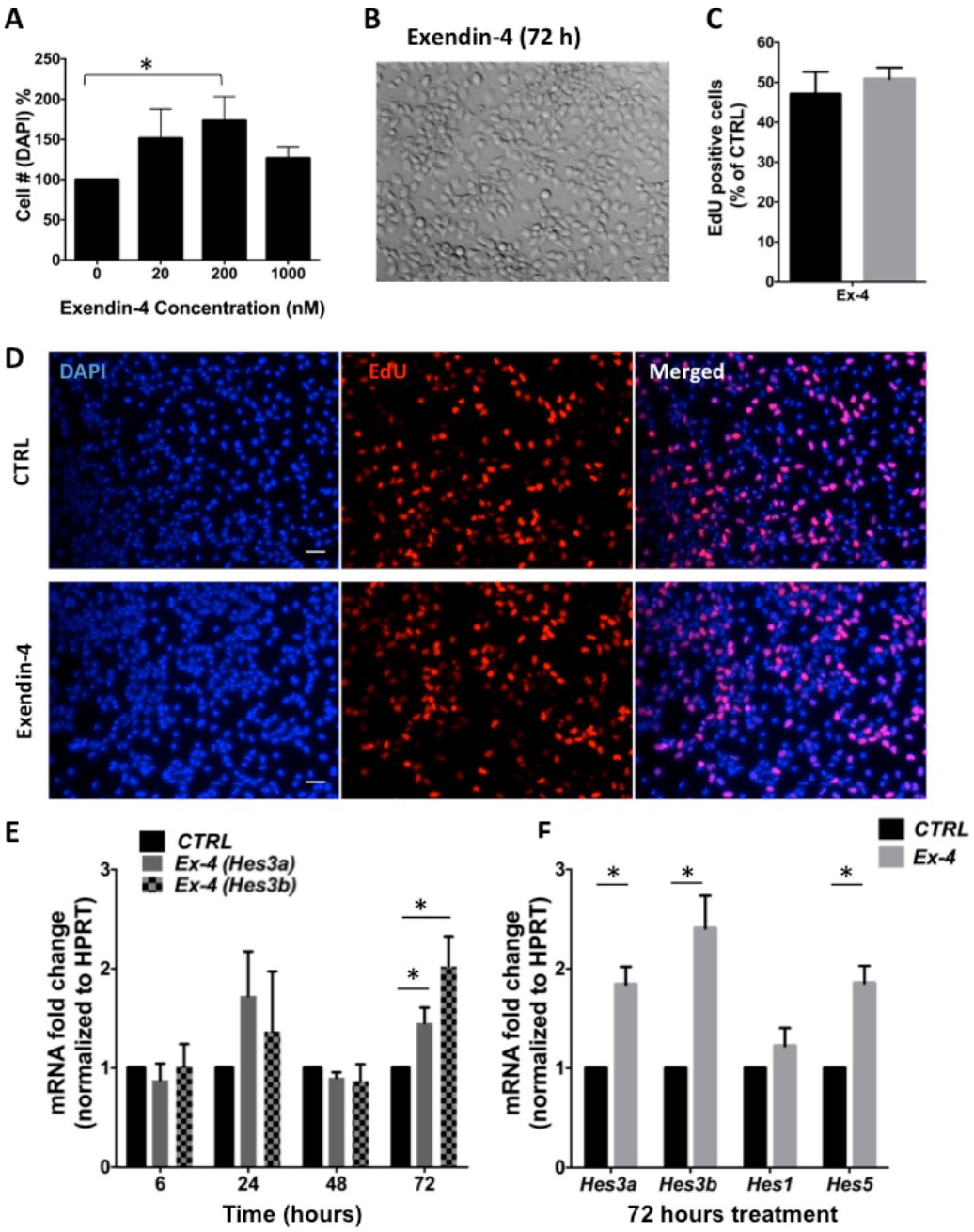

Figure 3.10. Exendin-4 regulates Hes3 expression in vitro. *(A) Ex-4 increases cell number (DAPI-stained nuclei counts) in a dose-dependent manner (N=7; 72h, 200nM). (B) Brightfield image of primary mouse fNSC cultures treated with Ex-4 (200nM) for 72 hours (to compare with Figure 1.8 D). (C) Ex-4 (200nM) does not significantly alter EdU incorporation (N=3; 72h treatment). (D) Image examples of EdU incorporation 72 hours after treatment with Ex-4 (200nM). EdU was added 5 hours before cell fixation. Scale bar: 30 µm. (E) Ex-4 (200nM) regulates Hes3a and Hes3b mRNA levels in vitro a time dependent manner (N=4). (F) Ex-4 (200nM, 72h) regulates Hes/Hey gene mRNA levels in cultured fNSCs (N=4). Data* are presented as mean ± *SEM. Unpaired t-test; *p<0.05. HPRT was used as a reference gene.*

3.3.4 Metformin and Exendin-4 affect the STAT3-Ser/Hes3 signaling axis

We performed Western Blot analysis to compare the effect of both treatments on components of the STAT3-Ser/Hes3 signaling axis. The results are shown in ***Figure 3.11***.

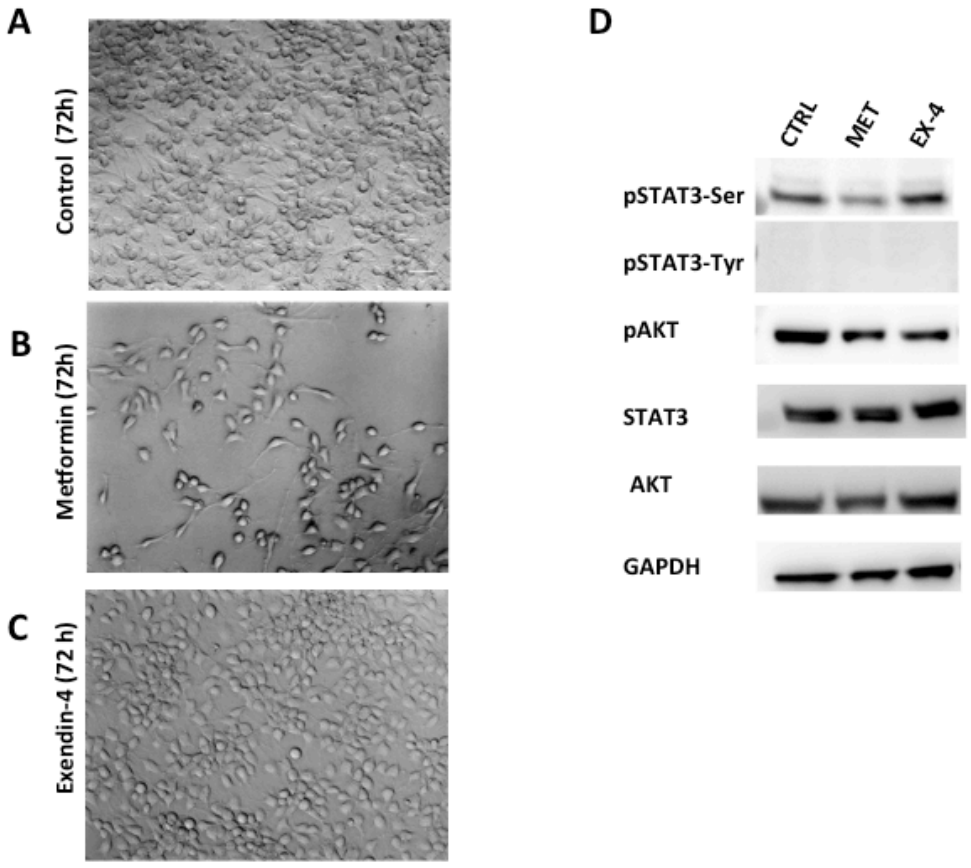

Figure 3.11. Metformin and Exendin-4 affect the STAT3-Ser/Hes3 signaling pathway in mouse NSC cultures. *(A-C) Brightfield images of the NSC cultures in the control condition; after 72 hours treatment with Metformin (500 μM) and after 72 hours treatment with Exendin-4 (200nM). Scale bar 30 μm. (D) Metformin (500 μM, 72 hours) decreases phosphorylation of the STAT3 on Ser727 and AKT phosphorylation; Ex-4 (200nM, 72 hours stimulates the phosphorylation of the STAT3 on Ser727 and increases AKT phosphorylation.*

These data show that metformin and exendin-4 regulate Hes3 levels in cultured NSCs and dissociate between the effects of these drugs on Hes3 levels and their effects on cell number.

3.4 Hes3 null mice exhibit a quasi-normal phenotype

The expression of Hes3 is significantly affected by aging, diabetes, and diabetes medication. To obtain clues to the functions of Hes3, we performed a general phenotypic analysis of Hes3 null mice. Homozygous *Hes3* null mice are generally healthy and breed normally (Hirata et al., 2001) but also have phenotypes that become obvious under stress (Androutsellis-Theotokis et al., 2008; Masjkur et al., 2014a; Masjkur et al., 2016; Toutouna et al., 2016). We performed a phenotypic analysis of the Hes3 null mice fed a normal diet (ND) and following a high fat diet (HFD) (***Figure 3.12*** *A, B*). We utilized a range of assays as a first screening test and the data are summarized in **Table 1**. The full report is provided at https://www.mouseclinic.de (Click on "phenomap" and search for project "Hes3_KO").

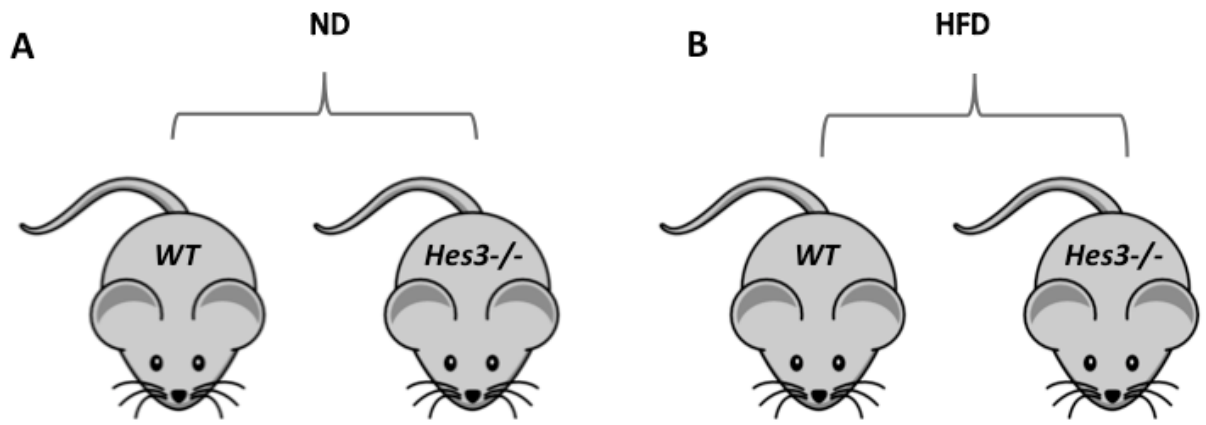

Figure 3.12. Hes3 phenotypic analysis in WT and Hes3 null mice. *(A) Phenotypic analysis in WT and Hes3 null mice under normal conditions [Normal diet (ND): Males N=15,15 Females N=15,15]. (B) Phenotypic analysis in WT and Hes3 null mice under stressed conditions [High fat diet (HFD); N=11,9 Females N=11,11].*

3.4.1 Phenotypic Analysis - Normal Diet (ND)

3.4.1.1 Neurology and Nociception

Primary phenotypic analysis revealed no major phenotypes in the Hes3 null mice. A summary of the data obtained in ND can be found in the upper part of the **Table1.** The data revealed slight phenotypes relevant to brain function such as a trend towards improved muscle function (assessed by grip strength; extensive analysis in the https://www.mouseclinic.de and a trend towards hyperalgesia in the Hes3 null mice [(assessed by the hot plate test (***Figure 3.13***)].

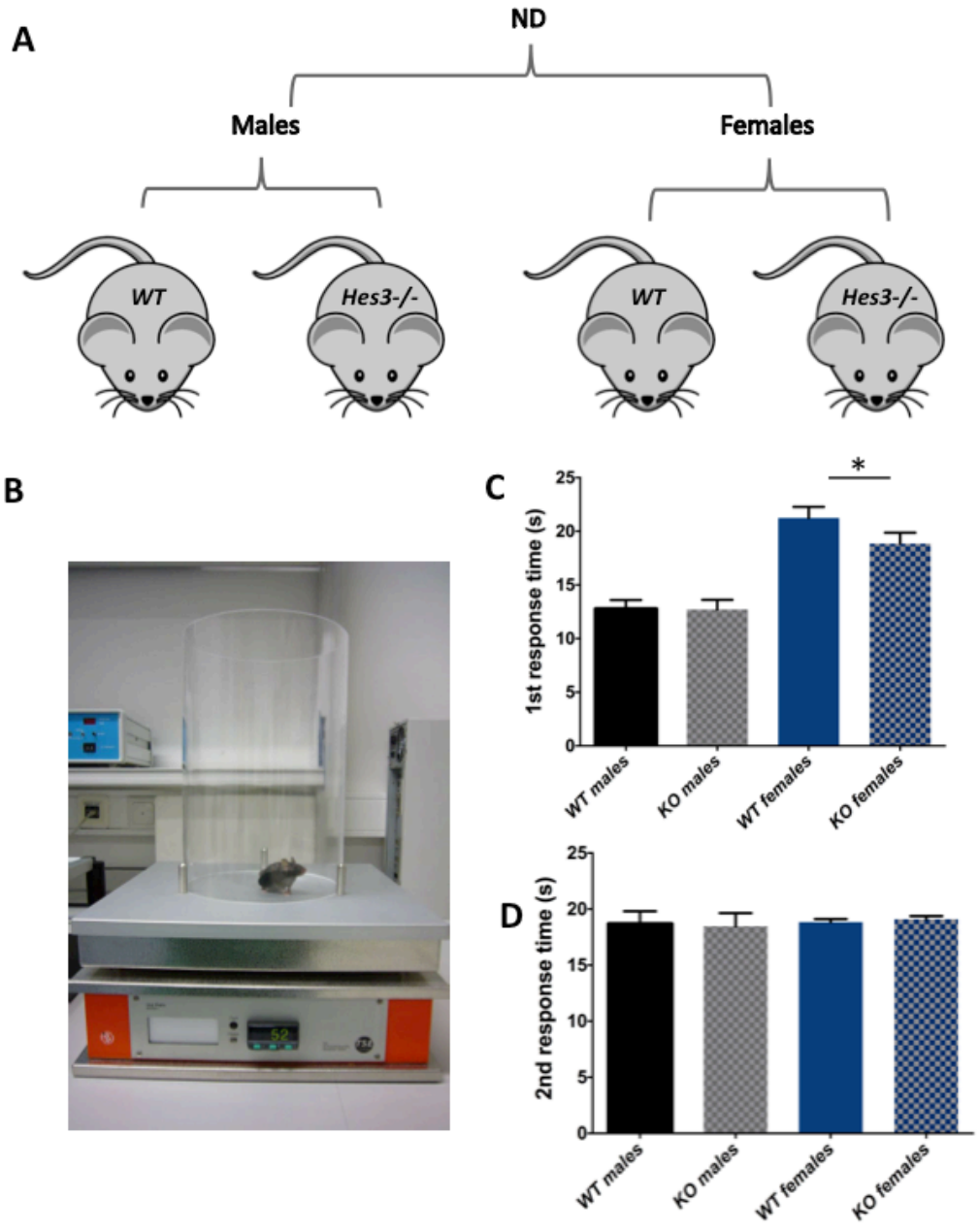

Figure 3.13. Female Hes3 null mice exhibit trend towards hyperalgesia. *(A) WT and Hes3 null mice under ND were tested for nociception with the hot plate test (N=15). (B) The hotplate system. (C) Hes3 null females exhibit shorter first response time. (D) All mice had similar second reaction time. Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

3.4.1.2 Age - Metabolism

Age decreases Hes3 in the mouse brain. During the phenotypic analysis we would subject the mice to long-term experiments leading to "aged" condition. Thus, we performed glucose

tolerance test in young and old mice in order to assess the effect of age on metabolism in the Hes3 null mice. Young Hes3 null mice (9 weeks old) showed higher GTT scores than the WT indicating impaired glucose tolerance. Interestingly, older mice (27 weeks old) showed no big differences in terms of GTT scores (***Figure 3.14***).

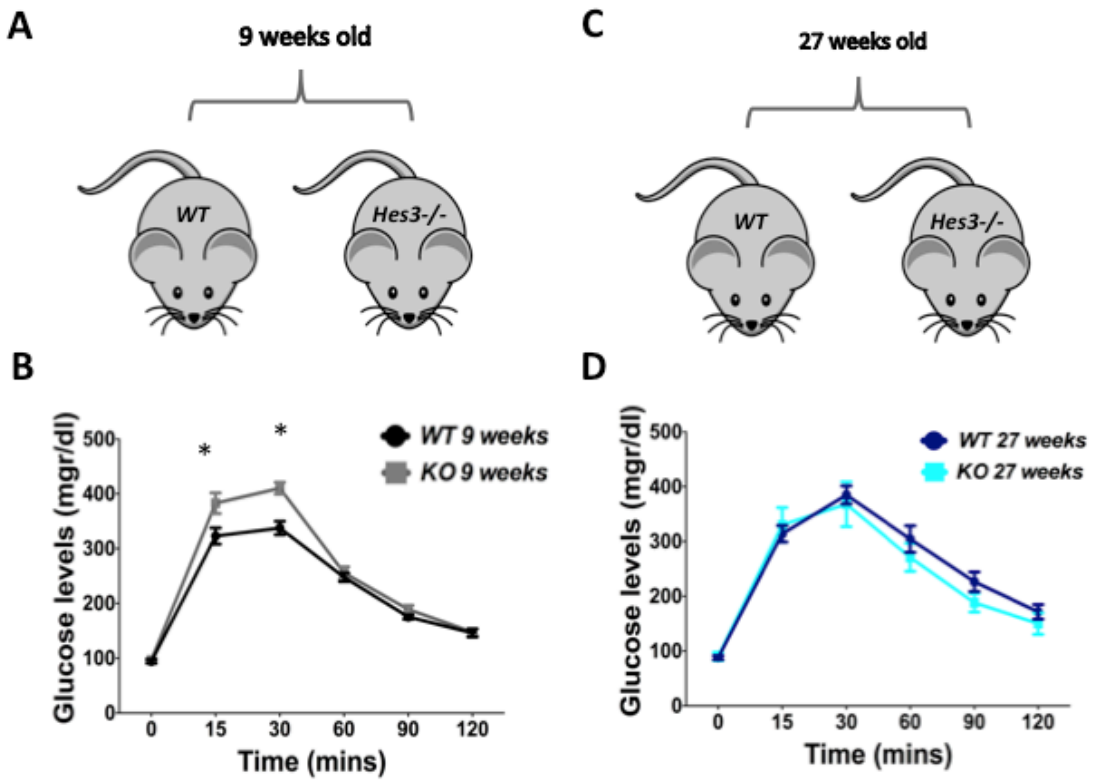

Figure 3.14. Hes3 null mouse phenotyping – Age related phenotypes. *(A, B) Young Hes3 null mice showed higher GTT scores compared to WT control mice (N=12,12) in the glucose tolerance test. (C, D) Older mice showed no difference in the GTT scores (N=7,6). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05*

However, ipITT in the same mice showed lower normalized glucose levels for the Hes3 null mice 15 minutes after insulin injection (***Figure 3.15***). Insulin action in the mouse is mostly characterized from the first fall and till the 30 minutes measurement, since the half time of insulin in the mouse organism is ~10minutes (Ayala et al., 2010). Thus, we consider this result as an indication of higher insulin sensitivity in the Hes3 null mice. In a group of mice analyzed at the 16 weeks of age (intermediate period of time) we observed that Hes3 null mice showed lower GTT scores (refer to GMC report in https://www.mouseclinic.de).

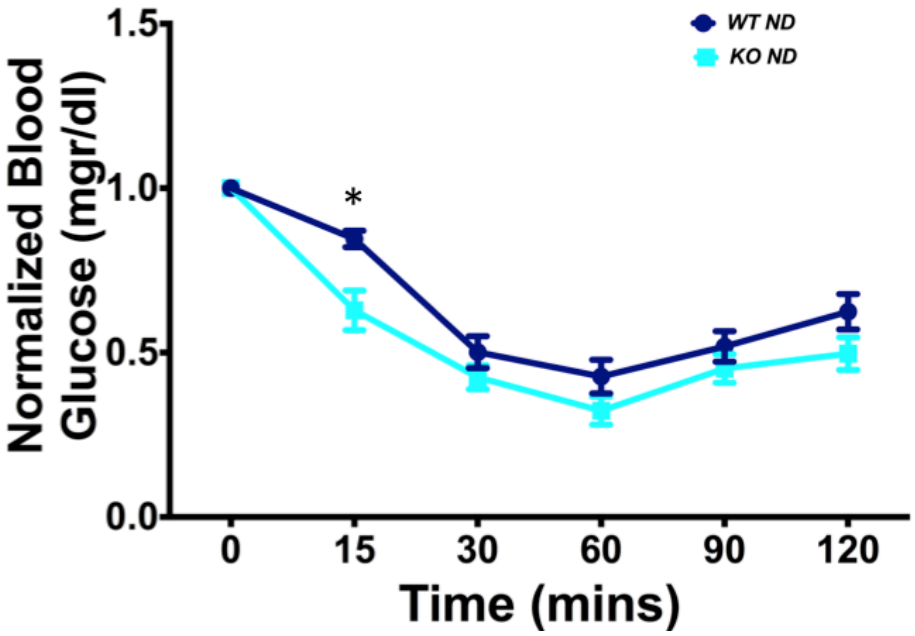

Figure 3.15. Insulin tolerance test. *Hes3 null mice showed lower normalized blood glucose levels 15 minutes after insulin injection. Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

These data suggest interplay among Hes3, glucose metabolism, and compensatory mechanisms of the mouse due to the Hes3 gene deletion. These results suggest that age plays an important role (even at the level of a week difference) in the experimental design and the analysis of the obtained data.

3.4.2 Metabolism Relevant Phenotypes – HFD challenge

We investigated metabolism-relevant phenotypes in more detail, in males. In ND, *Hes3* null mice behaved similarly to wild-type (WT) mice in terms of BW gain (***Figure 1.16*** *A-C*). In both ND and HFD, Hes3 null mice showed lower glucose levels during the course of the ipGTT compared to WT mice, resulting in slightly lower AUC values especially for the second part of the test [AUC (30-120mins)], which might indicate an improved glucose tolerance (HFD: ***Figure 3.17*****;** ND: see https://www.mouseclinic.de). Hes3 null mice in HFD also exhibited a trend towards higher rectal and body surface temperature [For consistency, we show data from males (Mann Whitney U test; p=0.053; N=7,7)]. Two Way ANOVA analysis revealed a significant increase in the body temperature of WT versus KO mice when both male and female mice were analyzed together (p=0.036; N=14,14)] (***Figure 3.17*** *C*).

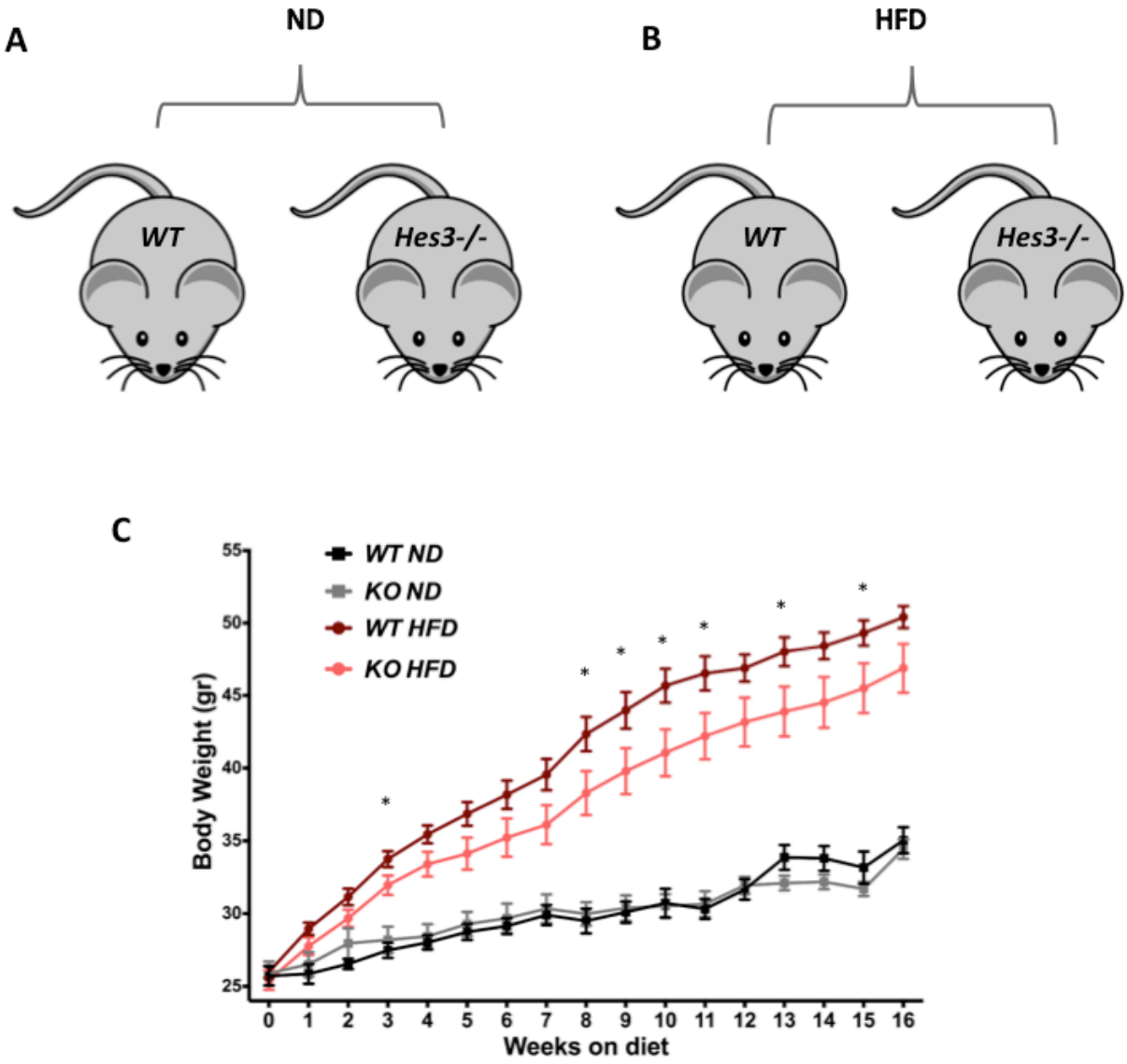

Figure 3.16. Hes3 null mouse phenotyping – Metabolic related phenotypes. *(A, B) The mice groups that were tested for body weight progression in ND and HFD conditions. (C) BW progression in mice fed a ND (N=5, 5), or a HFD (N=11, 9) for 16 weeks. Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

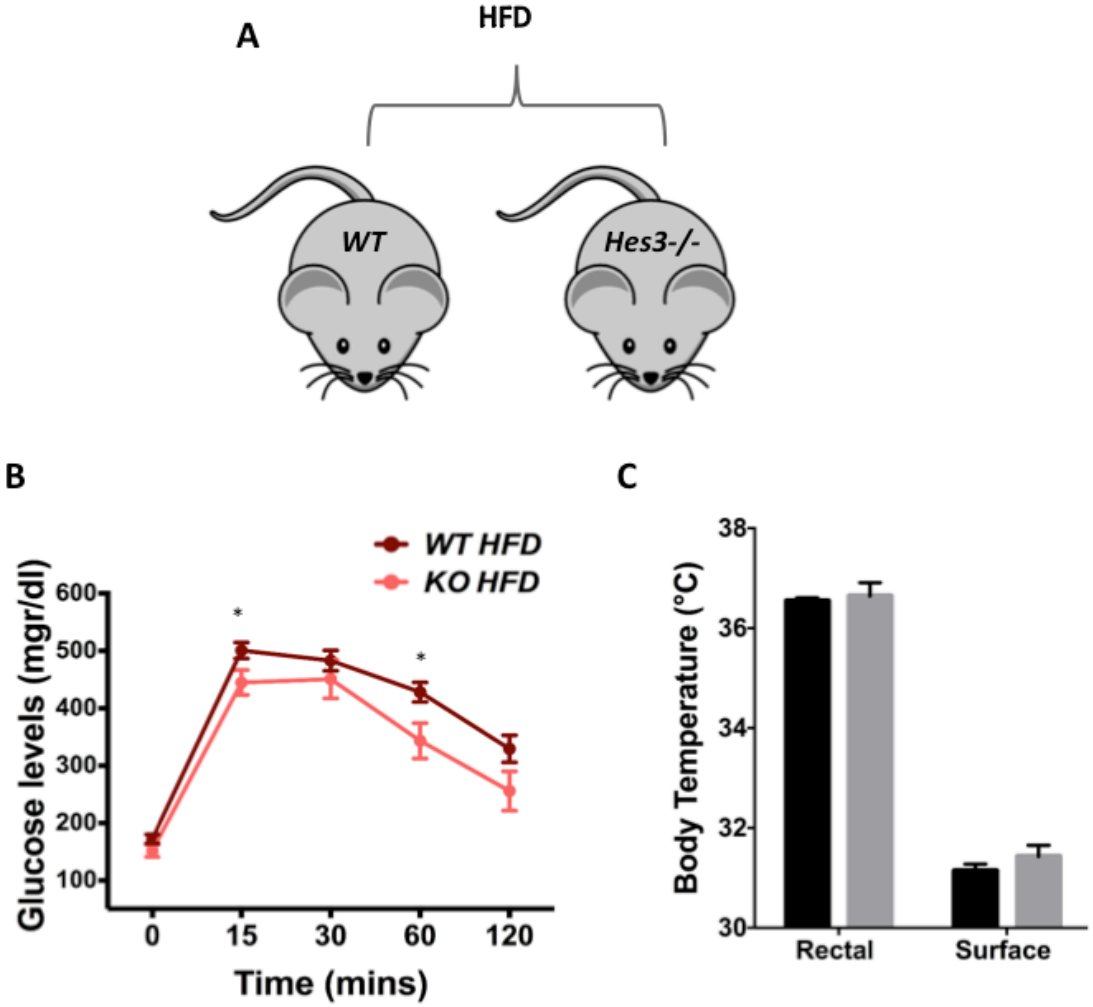

Figure 3.17. Hes3 null mouse phenotyping – Metabolic related phenotypes. *(A) WT and Hes3 null mice were fed a HFD for ~24 weeks. (B) Glucose tolerance test in the HFD challenge reveals lower GTT scores for the Hes3 null mice (N=9,11). (C) Hes3 null mice exhibit a trend towards higher rectal and body surface temperature than controls (N=7,7). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05.*

Table 1. Summary of phenotypic analysis.

Screen	Method	Phenotype summary Hes 3 null
NORMAL DIET		
Energy Metabolism	Indirect calorimetry NMR	-None -Trend towards increased fat content over time mainly in male mutants
Behavior	Open field	-None
	Acoustic startle response, PPI	-None
Neurology	Modified SHIRPA, Auditory brain stem response Rotarod Grip strength	-None -None -None -Small trend towards increased fore paw force
Nociception	Hot plate	- Shorter reaction time for the first pain reaction in females, trend towards hyperalgesia
Dysmorphology	Anatomical observation, X-ray, MicroCT scans (dissected bones)	-None
Cardiovascular	Awake ECG Echocardiography	-No clear phenotype -Very mild increase in septum width in systole (males). -Very mild reduction in heart rate and thus RR interval prolongation -Very mild alterations in QRS, ST and Qtdisp intervals (probably by chance)
Eye	Scheimpflug imaging, OCT, LIB, drum	-Slight decrease in retinal thickness (females)
Clinical Chemistry	IpGTT Insulin levels Clinical chemical analysis Hematology	-IPGTT: Mild trend downwards in males and upwards in females for AUC values. -None -Trend towards changes in creatinine and fructosamine (mainly females) concentrations -Hematology: Slightly lower platelet distribution width
Immunology	Flow cytometry analysis of Peripheral Blood Leukocytes	-Subtle alterations in the leukocyte subpopulations, however no evidence for pathological effects in the immune system: -increased frequency of B cells -decreased frequency of CD4 single positive T cells -increased frequency of CD4 CD8 double positive T cells -increased proportion of CD8 single positive T cells (females) -increased CD44 expression on $CD4^+$ T cells
Allergy	ELISA (IgE concentration) TEWL	-None -None
Pathology	Macro & microscopic analysis	-None
HIGH FAT DIET		
Energy Metabolism	Indirect calorimetry, NMR	-None
	Body temperature	-Slight increase in males and females
	Fat mass	-Mild decrease in females
Allergy	TEWL	-Slight increase in females
	Body surface temperature	-None
Clinical Chemistry	IpGTT	-IPGTT: Mild trend downwards in males for AUC values.

3.4.3 Phenotypic Analysis – Molecular

The function of Hes/Hey genes is largely unknown, as is the manner in which they regulate each other. To obtain information, at the molecular level, on the possible roles of Hes3 in the brain, we measured the expression of Hes/Hey genes in the brains of Hes3 null mice under ND (for 19 weeks, starting at 8 weeks of age) and Hes3 null mice under HFD (for 16 weeks, starting at 8 weeks of age). We prepared RNA samples from distinct areas of the mouse brain for all the different group of mice (WT-ND, KO-ND, WT-HFD and KO-HFD) (***Figure 3.18 A, B***) and we performed qPCR experiments to identify changes in the gene expression levels of the Hes/Hey genes family.

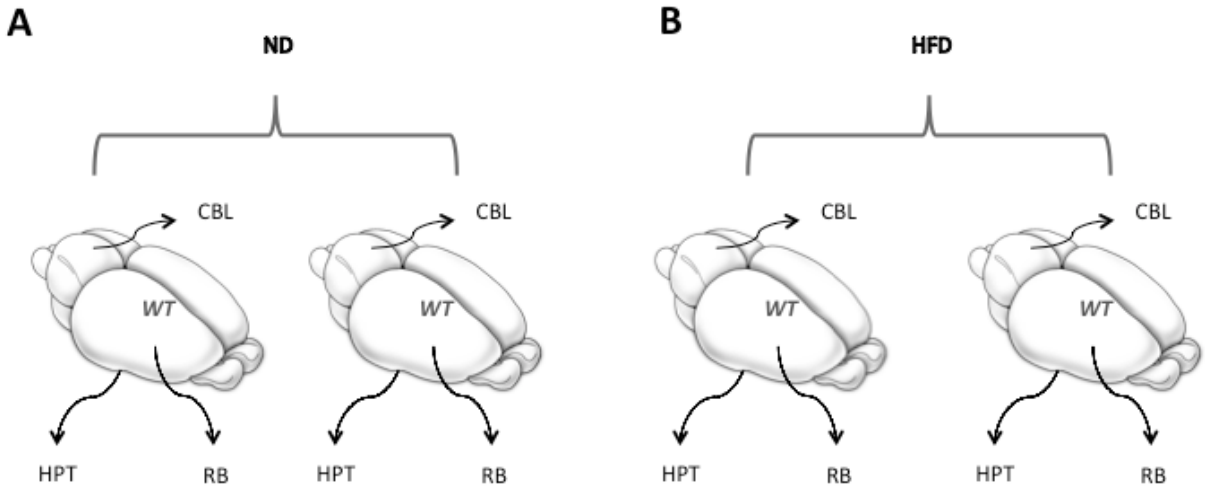

Figure 3.18. RNA isolation from distinct brain areas from the WT and the Hes3 null mouse. *(A, B) We isolated RNA from distinct brain areas (indicated as "RB", HPT, and "CBL" from mice that were fed a ND for 19 weeks or a HFD for 16 weeks.*

qPCR analysis revealed significant differences in the expression levels of several Hes/Hey genes in different areas of the brain. In contrast to the ND groups, in the HFD group we did not observe any significant differences in the expression of the other Hes/Hey genes between Hes3 null mice and wild-type controls, in any of the three brain regions. Data are shown in detail (per mouse, per area and per diet) in the form of a heatmap in ***Figure 3.19***. *A* summary of the data obtained from the qPCR experiments is provided in the form of a heatmap in ***Figure 3.20***.

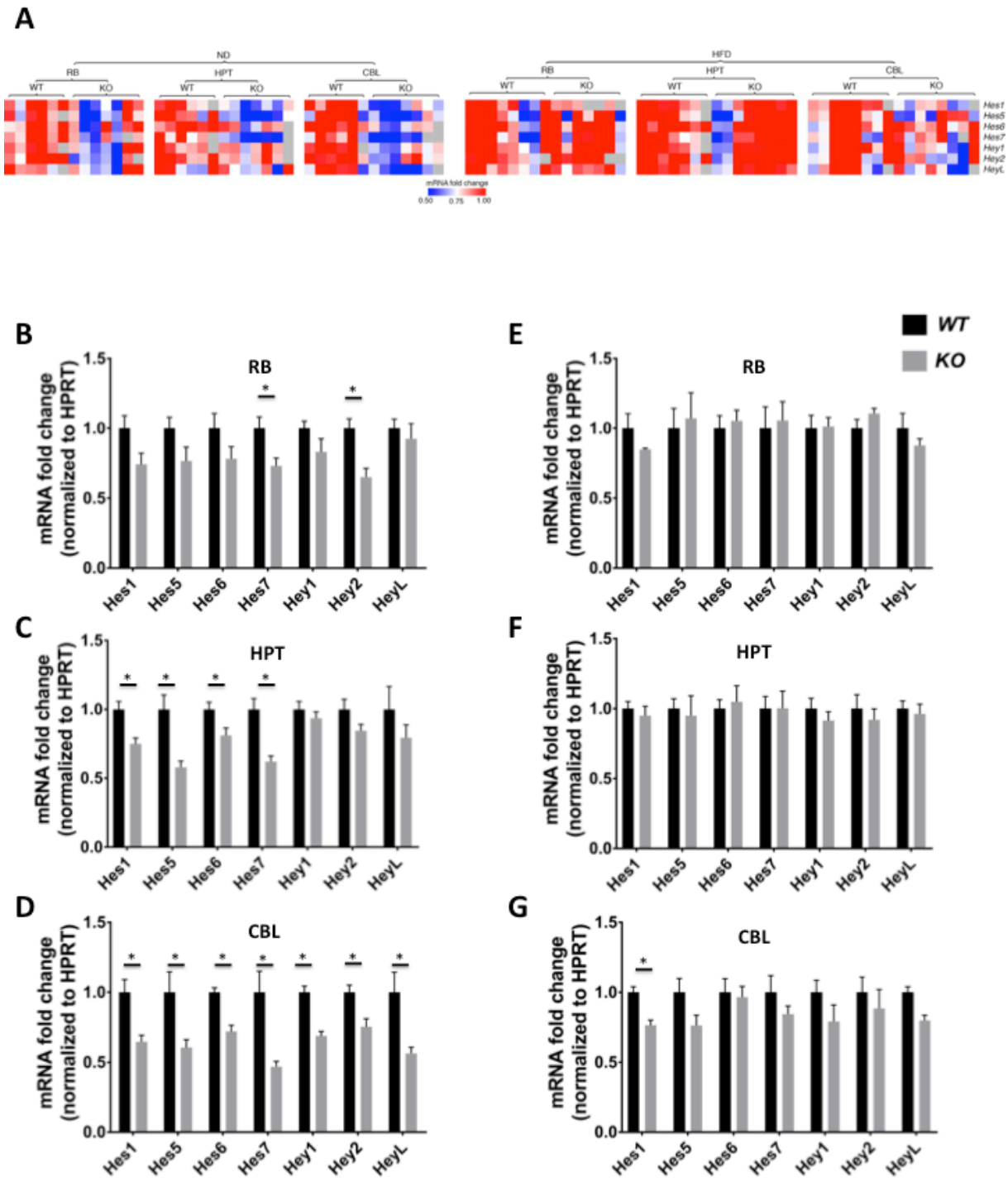

Figure 3.19. Hes3 null mice exhibit altered expression of Hes/Hey genes in the brain. *(A) Heatmap showing gene expression levels of different Hes/Hey genes in different areas of the brain in WT and Hes3 null mice, in ND and HFD conditions. The heat map shows values from individual mice (N=4-8) (B-G) Bar graph version of the qPCR analysis shown in (A). Data are presented as mean ± SEM. Mann-Whitney U test; *p<0.05. HPRT was used as a reference gene.*

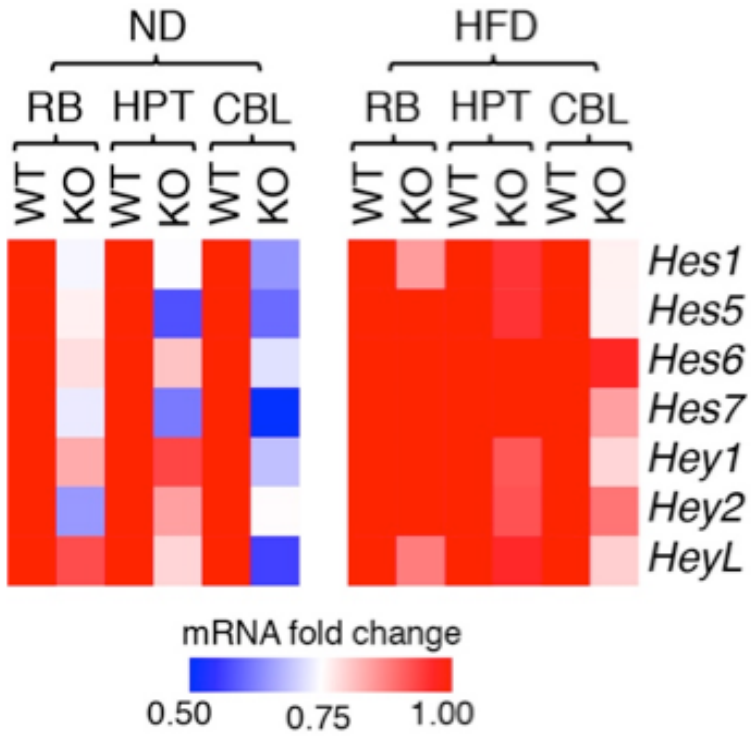

Figure 3.20. Hes/Hey gene expression profiling. *Heatmap of the average qPCR gene expression levels of different Hes/Hey genes in WT and Hes3 null mice in the brain areas indicated under ND and under HFD conditions. (N=4-8).*

These results show that the lack of Hes3 alters the equilibrium of other Hes/Hey genes and that this effect is observed in ND but not HFD. We therefore conclude that Hes3 regulates other Hes/Hey genes in the mouse brain, in a diet-dependent manner.

4 DISCUSSION

Global life expectancy has substantially increased over the last century (World Health Organization, 2017). This comes along with an increase in age-related conditions, such as neurodegenerative disease. Alzheimer's, Parkinson's, Huntington's disease, and Multiple sclerosis are all brain disorders, which progress with age and incorporate neuronal dysfunction/loss in motor, sensory, or cognitive systems. At the same time, the incidence of obesity and metabolic diseases have also increased over the last years, a fact that supports the idea that metabolic syndrome and neurodegenerative disease are highly interconnected (Procaccini et al., 2016). Obesity has been associated with a wide range of diseases such as diabetes, cardiovascular disease and liver disease; however it was recently demonstrated that mid-life obesity is a risk factor for eventual development of neurodegenerative disease such as dementia, Parkinson's and Alzheimer' disease (Abbott et al., 2002; Hassing et al., 2009; Kivipelto et al., 2005). Thus, metabolic dysfunction affects the brain. In the new age of stem cell biology, it is important to address the state of endogenous neural stem cells in the brain and specifically how they are affected by metabolic dysfunction because these cells are known to play important roles in the protection of the brain from insults and its regeneration following damage. But the molecular mechanisms they utilize are complex, unusual, and our understanding of them is incomplete.

Understanding the molecular mechanisms that mediate the effect of metabolic state on neural stem cells in the brain is a subject of intense study as they may hold clues for the progression and treatment of a variety of metabolic and degenerative diseases. The STAT3-Ser/Hes3 Signaling Axis was first identified as a major regulator of neural stem cells and, subsequently, cancer stem cells. Here we discuss how this signaling cascade, with Hes3 at its core is affected in the case of aging and in mouse models of diabetes (Type I and Type II). We also investigated whether common medication (in this work we used Metformin and Exendin-4) for metabolic related dysfunction also affects Hes3 expression in the adult brain.

Our data support the hypothesis that the STAT3-Ser/Hes3 Signaling Axis is a novel tool to characterize brain state in case of metabolic syndrome and deserves further research in the

context of neuroendocrinology. In this part of my thesis, I will provide additional insight into the mechanisms and the potential biological phenomena that may be responsible for our observations.

4.1 Diabetes affects the brain

Type 1 and type 2 diabetes as well as being overweight or obese are risk factors for developing cognitive impairment and dementia (recently reviewed by de la Monde (de la Monte, 2017) and within (Alosco and Gunstad, 2014; Fotuhi et al., 2012; J et al., 2009; Luchsinger et al., 2007; Pedditizi et al., 2016)). In part, this risk is thought to be due to aberrant insulin actions directly to the brain. Beyond stimulating glucose metabolism, insulin (and the related hormone IGF-1) have multiple functions in the brain, supporting the survival of neurons and oligodendrocytes (de la Monte and Wands, 2005), promoting synaptic integrity and plasticity (Chiu et al., 2008), and helping working memory and cognition (de la Monte, 2013). At the signal transduction level, post-mortem analysis of brains from patients with Alzheimer's disease reveals perturbed signaling downstream of the insulin receptor, including reduced insulin and IGF-1 binding to their receptors and impaired PI3K/Akt signaling (Moloney et al., 2010; Rivera et al., 2005; Steen et al., 2005; Talbot et al., 2012). There is interest, therefore, in identifying novel molecular mechanisms downstream of the insulin receptor that may be perturbed in the brain of diabetes patients.

4.2 STAT3-Ser/Hes3: a putative mediator

STAT3-Ser/Hes3 signaling is a recently identified molecular mechanism that integrates a variety of signals, including insulin/PI3K/Akt and regulates NSC number *in vitro* and *in vivo* (Androutsellis-Theotokis et al., 2006). In cultured NSCs, insulin treatment leads to PI3K – dependent Akt phosphorylation and subsequent mTOR and STAT3 phosphorylation on the serine residue. The latter event is critical to the survival of NSCs as transfection with a STAT3 plasmid where the serine residue is mutated to an alanine (and, thus, cannot be phosphorylated) leads to cell death. Following STAT3-serine phosphorylation, transcription of Hes3 is elevated. Hes3 induces the expression of the NSC mitogen and neuroprotective cytokine sonic hedgehog, as revealed by Hes3 overexpression experiments. In vivo, various pharmacological treatments that induce this pathway (as measured by levels of Hes3 expression) promote powerful and long-lasting neuronal rescue and improve motor skills in models of ischemic stroke and Parkinson's disease (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009). Hes3 null mice exhibit reduced pancreatic regeneration (Masjkur et al., 2014a; Masjkur et al., 2016) and

lower levels of myelin basic protein (MBP) in the brain (Toutouna et al., 2016), suggesting deficits in the equilibrium between oligodendrocyte progenitors and oligodendrocytes.

4.3 Hes3 is a special member of the Hes/Hey gene family

Notch receptor activation can lead to canonical downstream signaling that involves the association of the cleaved intracellular domain of the Notch receptor with other proteins and the direct binding to target gene promoter regions such as Hes1 and Hes5 (Artavanis-Tsakonas et al., 1999). It can also lead to a non-canonical branch that involves the indirect activation of Hes3 transcription (Androutsellis-Theotokis et al., 2006). In cultured NSCs, the actions of these two branches are very different. Canonical Notch leads to Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling activation, which promotes STAT3-Tyrosine (STAT3-Tyr) phosphorylation and, thus, gliogenic differentiation (Kamakura et al., 2004). In contrast, non-canonical Notch signaling involving Hes3 transcription maintains self-renewal and promotes cell survival through a STAT3-Ser –dependent mechanism (Androutsellis-Theotokis et al., 2006). The two branches are competing as JAK-STAT activation suppresses Hes3 expression. Treatments that promote Hes3 expression lead to disease modification in animal models of neurological disorder (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009). Therefore, here we focused on the regulation of Hes3 in the context metabolic dysfunction.

The role of Hes3 is largely unknown. However, Hes3 is expressed in several tissues, including the developing and adult brain and pancreas. It has two isoforms (Hes3a and Hes3b), generated by distinct promoters. Hes3b is the larger of the two isoforms and the only one that has DNA-binding properties (Hirata et al., 2000), suggesting the possibility that the two isoforms have distinct functions. Thus, it is crucial to perform experiments that can elucidate distinct functions of Hes3a and Hes3b (e.g. Different regulation via the same stimulus).

4.4 Patterns of Hes3 expression may be specific to cell type and microenvironment

In this work, we assessed Hes3 regulation in different brain areas, including the hypothalamus, an important component of the HPA Axis controlling homeostasis, and which exhibits strong Hes3 expression (Nikolakopoulou et al., 2016). Specific cell types may regulate and utilize the two isoforms differently and future studies may address this aspect.

Both intrinsic (e.g., cell surface receptors leading to particular signaling pathways) and extrinsic (e.g., inflammatory cytokines) factors may affect Hes3 expression in each cell type. It is important to not group all inflammatory cytokines together when addressing their effects on Hes3 as they may differ greatly. Whereas many inflammatory cytokines (e.g., the interleukin family) activate the JAK-STAT signaling pathway and would thus be expected to suppress Hes3 expression, Microphage Migration Inhibitory Factor (MIF), has been shown to promote it via an Akt/mTOR/STAT3-Ser mechanism (Ohta et al., 2012). Therefore, the precise inflammatory responses activated in different disease models may contribute to the exact Hes3 expression patterns that we observed. A better understanding of the interaction between specific inflammatory responses and Hes3 may help inform drug discovery programs aimed at both modulating inflammation and protecting brain tissue.

4.5 Metabolic dysfunction and diabetes medication affect brain Hes3

The insulin signaling branch that leads to Hes3 activation is important for proper brain homeostasis. Here we addressed whether it is affected by metabolic dysfunction. Our data show that in models of aging, type 1 diabetes, and type 2 diabetes, Hes3 expression is significantly regulated in the brain. Metformin is a medication commonly used in patients with diabetes and other indications of metabolic dysfunction (Inzucchi et al., 2012; Wilkin, 2001). It affects a broad range of signaling pathways (Lei et al., 2017), including several effectors of Hes3 in NSCs such as IGF-1 and p38 MAPK (Androutsellis-Theotokis et al., 2006; Sun et al., 2015). Therefore, we hypothesized that it may also regulate Hes3 in the brain. Our data show significant regulation of Hes3 mRNA in the brain of mice given oral metformin.

4.5.1 Age regulates Hes3

Hes3 expression drops significantly with age, which may be expected for a gene expressed in NSC and progenitor cells (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2009). The consequences of this drop are not known, however, increased Hes3 levels correlate with regenerative capability and improved NSC growth (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009; Salewski et al., 2012). The reason behind this drop is also not known; candidate regulators may include cytokines whose expression drops with age and which are known or predicted to stimulate Hes3 expression. There is more direct evidence implicating the inflammatory cytokine MIF. Its levels decrease with age (Mathew et al., 2013; Sauler et al., 2015) and it has been shown to induce Hes3 expression, via Akt and mTOR in cultured NSCs, and to promote their survival (Ohta et al., 2012). MIF has clinical potential as it has been associated

with longevity; particular long-lived mouse breeds exhibit increased levels and caloric restriction (an inducer of longevity) is also characterized by increased MIF levels (Miller et al., 2002).It will be important to identify other Hes3 regulators and to decipher the role of decreasing Hes3 levels in aging.

4.5.2 Diabetes models regulate Hes3 expression in the brain

We used STZ to model type 1 diabetes and a HFD to model type 2 diabetes. We did not observe changes in Hes1 or Hes5 expression, pointing towards important roles of the non-canonical branch of Notch signaling that involves Hes3. One could expect that because in cultured NSCs, insulin induces Hes3 expression (Androutsellis-Theotokis et al., 2008), STZ (which reduces circulating insulin levels) should reduce brain Hes3 expression. However, other factors such as the oxidative stress and inflammatory responses in the brain, which are triggered by STZ and type 1 diabetes may be responsible for the Hes3 increase (Nazem et al., 2015). We previously reported a similar finding in the pancreas where STZ induced powerful upregulation of Hes3 expression in the damaged pancreatic islets (Masjkur et al., 2014a).

HFD-induced type 2 diabetes is a complex condition that involves elevated circulating insulin (an activator of Hes3) as well as complex inflammatory responses (some of which promote and others oppose Hes3 expression) (Masjkur et al., 2014a; Ohta et al., 2012). It is therefore difficult to predict the effect of particular HFD paradigms on brain Hes3; here we investigated this question using established HFD protocols. HFD (short term and long term) decreased Hes3 expression. In addition, we performed a more detailed analysis in a subgroup of mice, where different areas of the brain were dissected and analyzed separately in order to investigate the effects of HFD on Hes3 in each specific area. In all the investigated areas (hypothalamus, cerebellum and rest brain) we observed a reduction in Hes3 expression with HFD.

Additionally, future studies may address whether Hes3+ cells become insulin resistant after prolonged HFD. Taken together, these data further support the hypothesis that Hes3 is modulated in response to stress.

4.5.3 Metformin regulates Hes3 expression in the brain

Metformin is a widely used medication for type 2 diabetes, although its mechanism of action remains unclear. It affects a wide range of signaling pathways, making it difficult to predict off-target effects. In triple-negative breast cancer cell lines, metformin was shown to oppose

both JAK/STAT3-Tyr activity and STAT3-Ser phosphorylation (Deng et al., 2012). It is possible that, depending on which of the two branches of STAT3 is mostly affected, the outcome may either favor or oppose Hes3 expression. In addition, metformin opposes mTOR activation, via 5' AMP-activated protein kinase (AMPK) stimulation (Hawley et al., 2010; Owen et al., 2000; Rozengurt, 2014; Sinnett-Smith et al., 2013), a function that should also oppose Hes3 expression. Inflammatory responses triggered by metformin (as well as by type 2 diabetes itself) (Bloom and Al-Abed, 2014; Dandona et al., 2004; Oliveira et al., 2016) further complicate the predictability of its effects on Hes3 expression.

Our data with cultured NSCs show increased Hes3 expression following metformin treatment for 72 hours. We hypothesize that this may be, in part, due to responses to cellular stress as evidenced by reduced growth and enhanced differentiation.

Ex-4 is a drug commonly prescribed to patients with type 2 diabetes. It promotes cell survival in the presence of toxic immunosuppressive drugs, proliferation, and even pancreatic islet neogenesis in various in vitro and in vivo systems (D'Amico et al., 2005; Li et al., 2005b; Park et al., 2006; Perfetti et al., 2000; Xu et al., 1999). We previously showed that Ex-4 also increases Hes3 expression in MIN6 cells (Masjkur et al., 2014a), suggesting that it may increase Hes3 expression in cultured NSCs without reducing cell number. Indeed, Ex-4 increased Hes3 expression without opposing growth or inducing differentiation. This was expected based on the fact that Ex-4 activates signaling pathways such as Akt that lead to Hes3 induction (Gonzalez et al., 2005; Li et al., 2005a; Masjkur et al., 2014a; Park et al., 2006; Song et al., 2008). Taken together, our results show that both metformin and Ex-4 increase Hes3 expression in cultured NSCs but only metformin opposes their growth. Therefore, Hes3 likely does not mediate the reduced cell growth effects of metformin on NSCs. The observed decrease in the cell proliferation due to the metformin treatment indicates reduced cell growth. Thus, we hypothesize that increased Hes3 expression following metformin treatment may be a consequence of increased cell stress.

The complex manner in which metformin affects NSCs directly or via inflammatory and stress responses may contribute to the lack of consensus regarding its potential role as a therapeutic agent in neurodegenerative disease (Kaneb et al., 2011; Moreira, 2014). It is conceivable that patients on metformin will benefit from concomitant treatments that modulate the inflammatory response in the brain such that Hes3 levels are maintained within an appropriate range. For example, promoting MIF responses while suppressing interleukin responses may have beneficial effects on the neural stem/progenitor population, on neurons, and on cognitive function.

Metformin is currently being considered as a potential anti-aging medication (Barzilai et al., 2016). It may be of value to assess the consequences of metformin treatments on Hes3

levels in different tissues and at different time points as (a) Hes3 is regulated by aging, (b) metformin regulates Hes3 levels, and (c) Hes3 is involved in a variety of regeneration paradigms, at least in the brain and pancreas.

4.6 Hes3 phenotyping provides clues to Hes3 functions

Hes3 null mice are healthy (Hatakeyama et al., 2004; Hirata et al., 2001), however close inspection revealed phenotypes such as reduced levels of MBP in the brain (Toutouna et al., 2016). More strikingly, Hes3 null mice exhibit increased sensitivity to toxic stress and impaired regeneration (Masjkur et al., 2014a; Masjkur et al., 2016; Toutouna et al., 2016). A comprehensive phenotypic analysis confirmed the broad lack of obvious phenotypes but revealed several mild effects, which may prove more significant in experiments involving different stresses. For example, we report mild differences between wild type and Hes3 null mice in ipGTT, ipITT and nociception tests, under normal conditions. Hes3 null mice undergoing HFD exhibit a slightly higher temperature than wild types. Future studies may address the significance of this phenotype by studying body temperature homeostasis in cold room experiments as well as possible roles of Hes3 in adipose (white or brown) tissue.

Our observation that Hes3-/- mice have alterations in the percentage of CD4+ and CD8+ T-cells and on the expression of CD44 suggest that a detailed analysis of the effects of Hes3 on immunological parameters may reveal additional functions of this transcription factor in the immune system.

Notch signaling and, in particular, canonical [Recombining binding protein suppressor of hairless (Rbpj)- and Hes1-dependent] Notch signaling controls browning in the adipose tissue with consequences in thermogenesis (Bi et al., 2014). It will be of interest to assess the possible involvement of non-canonical Notch signaling (Hes3-dependent) in this process as well.

Despite the lack of pronounced phenotypes in the Hes3 null mouse, qPCR analysis reveals widespread differences in the expression of Hes/Hey genes in the brain, under normal conditions. However, when mice were placed on HFD, differences between Hes3 null and wild type mice were not observed. It is possible that because in HFD Hes3 expression is reduced relative to ND, the effect of lacking Hes3 is not as pronounced. It is also possible that HFD regulates other Hes/Hey genes independently from Hes3 in a manner that overrides the genetic lack of Hes3. These results show that the lack of Hes3 alters the equilibrium of the other Hes/Hey genes and that this outcome is affected by diet. Whether this differential role of Hes3 is dependent on particular metabolic or inflammatory parameters is not currently known and certainly requires further investigation.

4.7 Hes3 and metabolic dysfunction: Are they connected?

Our work establishes that multiple parameters of metabolic state as well as diabetes medication affect Hes3 expression in the brain. The scientific literature provides additional clues as to how brain Hes3 may be affected by other systems. Below we provide a conceptual model integrating these findings.

Aging reduces Hes3 expression. The inflammatory cytokine Macrophage Migration Inhibitory Factor (MIF) is a positive regulator of *Hes3* (Ohta et al., 2012) whose expression also drops with age (Mathew et al., 2013; Sauler et al., 2015) and is associated with longevity (Miller et al., 2002). It is possible, therefore, that as MIF drops with age this contributes to the reduction of *Hes3* expression we observed with age.

Insulin is another powerful inducer of Hes3 (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009). Therefore, in type 1 diabetes models, reduced circulating insulin levels may promote a reduction in Hes3 levels in the brain. However, in the STZ models, cell stress caused by the damage to the organism may also promote an increase in brain Hes3 levels. Also, STZ-induced inflammation in the brain may further alter Hes3 expression. Which of these effects prevails may depend on the particular model used and the particular time point of the assessment of Hes3 levels.

HFD involves initial periods of inflammation and subsequent periods of prolonged elevated insulin (Heydemann, 2016). The effect of the HFD-induced inflammatory response on brain Hes3 may depend on the precise response as some inflammatory cytokines such as interleukin 6 (IL-6) are both involved in diabetes mellitus (Kristiansen and Mandrup-Poulsen, 2005) and are predicted to oppose Hes3 expression [because they activate JAK which opposes Hes3 expression (Androutsellis-Theotokis et al., 2006; Masjkur et al., 2014a)] whereas at least one other cytokine, MIF, has been demonstrated to increase Hes3 expression levels (Ohta et al., 2012). Future studies may address how Hes3 expression is specifically altered by oxidative and inflammatory stress components such as oxygen radicals produced by the mitochondria of affected cells and activated, nuclear NF-kB. Cross-talk between NF-kB and JAK-STAT signaling as well as between reactive oxygen species and JAK-STAT increases the complexity by which Hes3 may be regulated (Ahmad et al., 2015; Grivennikov and Karin, 2010; Simon et al., 1998).

The type 2 diabetes medication metformin has multiple effects at the signal transduction level (Dandona et al., 2004; Deng et al., 2012; Hawley et al., 2010; Oliveira et al., 2016; Owen et al., 2000; Rozengurt, 2014; Sinnett-Smith et al., 2013). One of the best described effects is that it opposes mTOR activity. Because mTOR is a potent activator of Hes3 expression, this result may explain our observation that metformin, in vivo, reduces Hes3 levels (in the

hypothalamus of adult mice). Metformin may also induce stress effects on target cells, and this could lead to an increase in Hes3 expression. Our observations using cultured NSCs suggest this as a possibility, as we observed a reduction in cell growth, induced differentiation, and an increase in Hes3 expression.

We therefore suggest that Hes3 integrates various parameters of metabolic state and may serve as a biomarker for brain state in diabetes and other metabolic disorders. A schematic diagram summarizing how Hes3 could be integrated in research programs investigating the relationship metabolic dysfunction and brain condition is provided in ***Fig.4.1***.

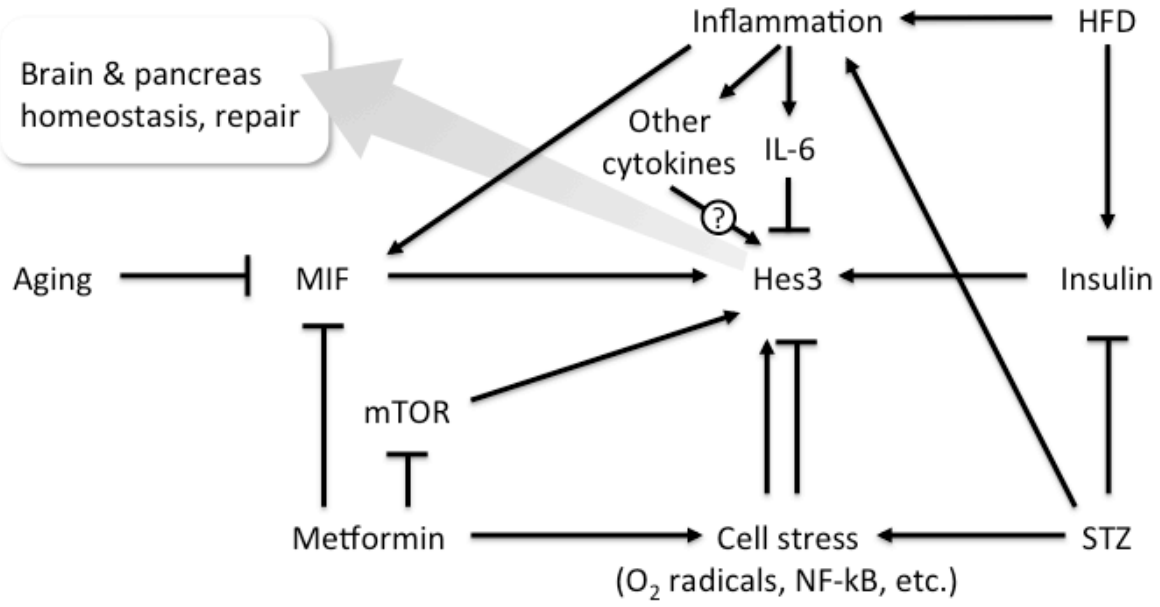

Figure 4.1. Conceptual diagram of how Hes3 may be integrating multiple biological parameters. *We present a hypothesis-driven schematic diagram with potential ideas as to how Hes3 may be integrated in research programs studying the effects of metabolic dysfunction to the brain.*

5 Conclusions and Future Remarks

Extensive research over the last years has established neural stem and progenitor cell populations in the adult brain as major contributors to the functionality of the nervous system in both the healthy and the injured state. Immature cells sense signals from the microenvironment of the tissue they reside in and decide if they should quiesce, proliferate, or differentiate. Such distinct populations of primary cells that have the potential to decide for their fate add to the plasticity potential of the tissue they reside in as well as its regenerative potential upon injury or disease. Therefore it is crucial: a) to understand the mechanisms which regulate the decisions of those cells and b) develop biomarkers that mark them in both the activated as well as in the quiescent state.

As we extensively reported in the previous sections of this work, the STAT3-Ser/Hes3 signaling axis regulates a variety of plastic cells such as the neural stem cells in the brain, the tanycytes in the hypothalamus, oligodentrocyte precursors in the motor cortex, adrenal precursors, pancreatic islet beta cells and cancer stem cells (Androutsellis-Theotokis et al., 2006; Masjkur et al., 2014a; Masjkur et al., 2014c; Nikolakopoulou et al., 2016; Park et al., 2013; Toutouna et al., 2016). Insulin was shown to be one of the major regulators of this signaling cascade (Androutsellis-Theotokis et al., 2008). This immediately pointed to the possibility that metabolic dysfunction may affect brain Hes3.

With this work we aim to provide a further understanding of the interconnection between brain state and metabolic disease, which is characterized by insulin signaling impairment. We showed that brain Hes3 levels drop with age and that they are regulated in diabetes mouse models. In addition, we showed that common medication prescribed for metabolic diseases regulates Hes3 *in vivo* and *in vitro*. Full phenotypic analysis of the Hes3 null mouse line revealed phenotypes relevant to the neurological system, the immune system, and metabolism. At the molecular level, we showed that Hes3 deletion results in significant changes in the expression of other Hes/Hey genes under normal diet conditions.

Last but not least, it was evident that Hes3 expression decreases with aging, as it is expected for a biomarker for NSCs. In addition, the results of the phenotypic analysis were

highly dependent on the age of the mice, which stresses the importance of experimental design in order to reveal phenotypes due to Hes3 deletion.

Taken together, we propose Hes3 as a novel biomarker of the state of eNSCs of relevance to metabolic related conditions. This work introduces a new molecular mechanism and cell subpopulation that regulates the state of the brain in metabolic dysfunction.

This PhD thesis project established that metabolic dysfunction, medication, and aging regulate a key component of the STAT3-Ser/Hes3 Signaling Axis, providing a new molecular mechanism by which peripheral systems affect the brain. Because this molecular mechanism is very important to brain homeostasis and repair, the data open up opportunities to understand multiple important medical issues in the fields of diabetes, aging, and neurodegenerative disease. Future experiments may address how perturbation of Hes3 expression (using the heterozygous and homozygous Hes3 null mice, or by pharmacologically increasing Hes3 expression as previously described (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2008; Androutsellis-Theotokis et al., 2009) may affect brain tissue damage in diabetes models, and cognition in the context of aging and metformin administration. Our phenotypic analyses with the Hes3 null mice reveal immune phenotypes and a full characterization of the affected cell populations could shed additional light on how Hes3 may regulate the immune system and how this in turn, via the secretion of specific cytokines, may affect Hes3 itself.

Within the vast complexity of this molecular mechanism, we clearly showed, using extensive *in vitro* and *in vivo* models, that a key component of the STAT3-Ser/Hes3 Signaling Axis is directly relevant to type 1 and type 2 diabetes mellitus, metformin treatment and aging.

References

Abbott, R.D., Ross, G.W., White, L.R., Nelson, J.S., Masaki, K.H., Tanner, C.M., Curb, J.D., Blanchette, P.L., Popper, J.S., Petrovitch, H., 2002. Midlife adiposity and the future risk of Parkinson's disease. Neurology. 59**,** 1051-7.

Ahmad, S.F., Ansari, M.A., Zoheir, K.M., Bakheet, S.A., Korashy, H.M., Nadeem, A., Ashour, A.E., Attia, S.M., 2015. Regulation of TNF-alpha and NF-kappaB activation through the JAK/STAT signaling pathway downstream of histamine 4 receptor in a rat model of LPS-induced joint inflammation. Immunobiology. 220**,** 889-98.

Al-Awar, A., Kupai, K., Veszelka, M., Szucs, G., Attieh, Z., Murlasits, Z., Torok, S., Posa, A., Varga, C., 2016. Experimental Diabetes Mellitus in Different Animal Models. J Diabetes Res. 2016**,** 9051426.

Alosco, M.L., Gunstad, J., 2014. The negative effects of obesity and poor glycemic control on cognitive function: a proposed model for possible mechanisms. Curr Diab Rep. 14**,** 495.

Altman, J., 1962. Are new neurons formed in the brains of adult mammals? Science. 135**,** 1127-8.

Alvarez-Buylla, A., Lim, D.A., 2004. For the long run: maintaining germinal niches in the adult brain. Neuron. 41**,** 683-6.

Amariglio, N., Hirshberg, A., Scheithauer, B.W., Cohen, Y., Loewenthal, R., Trakhtenbrot, L., Paz, N., Koren-Michowitz, M., Waldman, D., Leider-Trejo, L., Toren, A., Constantini, S., Rechavi, G., 2009. Donor-Derived Brain Tumor Following Neural Stem Cell Transplantation in an Ataxia Telangiectasia Patient. PLOS Medicine. 6**,** e1000029.

Androutsellis-Theotokis, A., Leker, R.R., Soldner, F., Hoeppner, D.J., Ravin, R., Poser, S.W., Rueger, M.A., Bae, S.K., Kittappa, R., McKay, R.D., 2006. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature. 442**,** 823-6.

Androutsellis-Theotokis, A., Rueger, M.A., Mkhikian, H., Korb, E., McKay, R.D., 2008. Signaling pathways controlling neural stem cells slow progressive brain disease. Cold Spring Harb Symp Quant Biol. 73**,** 403-10.

Androutsellis-Theotokis, A., Rueger, M.A., Park, D.M., Mkhikian, H., Korb, E., Poser, S.W., Walbridge, S., Munasinghe, J., Koretsky, A.P., Lonser, R.R., McKay, R.D., 2009. Targeting neural precursors in the adult brain rescues injured dopamine neurons. Proc Natl Acad Sci U S A. 106**,** 13570-5.

Androutsellis-Theotokis, A., Rueger, M.A., Park, D.M., Boyd, J.D., Padmanabhan, R., Campanati, L., Stewart, C.V., LeFranc, Y., Plenz, D., Walbridge, S., Lonser, R.R., McKay, R.D., 2010a. Angiogenic factors stimulate growth of adult neural stem cells. PLoS One. 5, e9414.

Androutsellis-Theotokis, A., Walbridge, S., Park, D.M., Lonser, R.R., McKay, R.D., 2010b. Cholera toxin regulates a signaling pathway critical for the expansion of neural stem cell cultures from the fetal and adult rodent brains. PLoS One. 5, e10841.

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., 1999. Notch signaling: cell fate control and signal integration in development. Science. 284, 770-6.

Ayala, J.E., Samuel, V.T., Morton, G.J., Obici, S., Croniger, C.M., Shulman, G.I., Wasserman, D.H., McGuinness, O.P., 2010. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Disease Models & Mechanisms. 3, 525-534.

Barzilai, N., Crandall, J.P., Kritchevsky, S.B., Espeland, M.A., 2016. Metformin as a Tool to Target Aging. Cell Metab. 23, 1060-5.

Bi, P., Shan, T., Liu, W., Yue, F., Yang, X., Liang, X.R., Wang, J., Li, J., Carlesso, N., Liu, X., Kuang, S., 2014. Inhibition of Notch signaling promotes browning of white adipose tissue and ameliorates obesity. Nat Med. 20, 911-8.

Bloom, J., Al-Abed, Y., 2014. MIF: mood improving/inhibiting factor? J Neuroinflammation. 11, 11.

Bolborea, M., Dale, N., 2013. Hypothalamic tanycytes: potential roles in the control of feeding and energy balance. Trends Neurosci. 36, 91-100.

Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D.A., Rozovsky, I., Stahl, N., Yancopoulos, G.D., Greenberg, M.E., 1997. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science. 278, 477-83.

Cassady, J.P., D'Alessio, A.C., Sarkar, S., Dani, V.S., Fan, Z.P., Ganz, K., Roessler, R., Sur, M., Young, R.A., Jaenisch, R., 2014. Direct lineage conversion of adult mouse liver cells and B lymphocytes to neural stem cells. Stem Cell Reports. 3, 948-56.

Cattaneo, E., McKay, R., 1991. Identifying and manipulating neuronal stem cells. Trends Neurosci. 14, 338-40.

Chen, F., Dong, R.R., Zhong, K.L., Ghosh, A., Tang, S.S., Long, Y., Hu, M., Miao, M.X., Liao, J.M., Sun, H.B., Kong, L.Y., Hong, H., 2015. Antidiabetic drugs restore abnormal transport of amyloid-β across the blood-brain barrier and memory impairment in db/db mice. Neuropharmacology.

Chiu, S.L., Chen, C.M., Cline, H.T., 2008. Insulin receptor signaling regulates synapse number, dendritic plasticity, and circuit function in vivo. Neuron. 58, 708-19.

D'Amico, E., Hui, H., Khoury, N., Di Mario, U., Perfetti, R., 2005. Pancreatic beta-cells expressing GLP-1 are resistant to the toxic effects of immunosuppressive drugs. J Mol Endocrinol. 34, 377-90.

Dandona, P., Aljada, A., Ghanim, H., Mohanty, P., Tripathy, C., Hofmeyer, D., Chaudhuri, A., 2004. Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive action of metformin. J Clin Endocrinol Metab. 89, 5043-7.

de la Monte, S.M., Wands, J.R., 2005. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J Alzheimers Dis. 7, 45-61.

de la Monte, S.M., 2013. Intranasal insulin therapy for cognitive impairment and neurodegeneration: current state of the art. Expert Opin Drug Deliv. 10, 1699-709.

de la Monte, S.M., 2017. Insulin Resistance and Neurodegeneration: Progress Towards the Development of New Therapeutics for Alzheimer's Disease. Drugs. 77, 47-65.

Deng, X.S., Wang, S., Deng, A., Liu, B., Edgerton, S.M., Lind, S.E., Wahdan-Alaswad, R., Thor, A.D., 2012. Metformin targets Stat3 to inhibit cell growth and induce apoptosis in triple-negative breast cancers. Cell Cycle. 11, 367-76.

Dietrich, J., Kempermann, G., 2006. Role of endogenous neural stem cells in neurological disease and brain repair. Adv Exp Med Biol. 557, 191-220.

Dimou, L., Gotz, M., 2014. Glial cells as progenitors and stem cells: new roles in the healthy and diseased brain. Physiol Rev. 94, 709-37.

Emsley, J.G., Mitchell, B.D., Kempermann, G., Macklis, J.D., 2005. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. Prog Neurobiol. 75, 321-41.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., Gage, F.H., 1998. Neurogenesis in the adult human hippocampus. Nat Med. 4, 1313-7.

Farooqui, A.A., Farooqui, T., Panza, F., Frisardi, V., 2012. Metabolic syndrome as a risk factor for neurological disorders. Cell Mol Life Sci. 69, 741-62.

Fotuhi, M., Do, D., Jack, C., 2012. Modifiable factors that alter the size of the hippocampus with ageing. Nat Rev Neurol. 8, 189-202.

Franklin, K.B.J., Paxinos, G., 2008. The mouse brain in stereotaxic coordinates. Vol., Elsevier/Academic Press, Amsterdam; Boston.

Fuchs, H., Gailus-Durner, V., Adler, T., Aguilar-Pimentel, J.A., Becker, L., Calzada-Wack, J., Da Silva-Buttkus, P., Neff, F., Gotz, A., Hans, W., Holter, S.M., Horsch, M., Kastenmuller, G., Kemter, E., Lengger, C., Maier, H., Matloka, M., Moller, G., Naton, B., Prehn, C., Puk, O., Racz, I., Rathkolb, B., Romisch-Margl, W., Rozman, J., Wang-Sattler, R., Schrewe, A., Stoger, C., Tost, M., Adamski, J., Aigner, B., Beckers, J., Behrendt, H., Busch, D.H., Esposito, I., Graw, J., Illig, T., Ivandic, B., Klingenspor, M., Klopstock, T., Kremmer, E., Mempel, M., Neschen, S., Ollert, M., Schulz, H., Suhre, K., Wolf, E., Wurst, W., Zimmer, A., Hrabe de Angelis, M., 2011. Mouse phenotyping. Methods. 53, 120-35.

Gage, F.H., 2002. Neurogenesis in the adult brain. J Neurosci. 22, 612-3.

Gailus-Durner, V., Fuchs, H., Becker, L., Bolle, I., Brielmeier, M., Calzada-Wack, J., Elvert, R., Ehrhardt, N., Dalke, C., Franz, T.J., Grundner-Culemann, E., Hammelbacher, S., Holter, S.M., Holzlwimmer, G., Horsch, M., Javaheri, A., Kalaydjiev, S.V., Klempt, M., Kling, E., Kunder, S., Lengger, C., Lisse, T., Mijalski, T., Naton, B., Pedersen, V., Prehn, C., Przemeck, G., Racz, I., Reinhard, C., Reitmeir, P., Schneider, I., Schrewe, A., Steinkamp, R., Zybill, C., Adamski, J., Beckers, J., Behrendt, H., Favor, J., Graw, J., Heldmaier, G., Hofler, H., Ivandic, B., Katus, H., Kirchhof, P., Klingenspor, M., Klopstock, T., Lengeling, A., Muller, W., Ohl, F., Ollert, M., Quintanilla-Martinez, L., Schmidt, J., Schulz, H., Wolf, E., Wurst, W., Zimmer, A., Busch, D.H., de Angelis, M.H., 2005. Introducing the German Mouse Clinic: open access platform for standardized phenotyping. Nat Methods. 2, 403-4.

Gonzalez, N., Acitores, A., Sancho, V., Valverde, I., Villanueva-Penacarrillo, M.L., 2005. Effect of GLP-1 on glucose transport and its cell signalling in human myocytes. Regul Pept. 126, 203-11.

Gould, E., Reeves, A.J., Graziano, M.S., Gross, C.G., 1999. Neurogenesis in the neocortex of adult primates. Science. 286, 548-52.

Grivennikov, S.I., Karin, M., 2010. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev. 21, 11-9.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell. 144, 646-74.

Hassing, L.B., Dahl, A.K., Thorvaldsson, V., Berg, S., Gatz, M., Pedersen, N.L., Johansson, B., 2009. Overweight in midlife and risk of dementia: a 40-year follow-up study. Int J Obes (Lond). 33, 893-8.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., Kageyama, R., 2004. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development. 131, 5539-50.

Hawley, S.A., Ross, F.A., Chevtzoff, C., Green, K.A., Evans, A., Fogarty, S., Towler, M.C., Brown, L.J., Ogunbayo, O.A., Evans, A.M., Hardie, D.G., 2010. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. Cell Metab. 11, 554-65.

Hermann, D.M., Peruzzotti-Jametti, L., Schlechter, J., Bernstock, J.D., Doeppner, T.R., Pluchino, S., 2014. Neural precursor cells in the ischemic brain - integration, cellular crosstalk, and consequences for stroke recovery. Front Cell Neurosci. 8, 291.

Heydemann, A., 2016. An Overview of Murine High Fat Diet as a Model for Type 2 Diabetes Mellitus. J Diabetes Res. 2016, 2902351.

Hirata, H., Ohtsuka, T., Bessho, Y., Kageyama, R., 2000. Generation of structurally and functionally distinct factors from the basic helix-loop-helix gene Hes3 by alternative first exons. J Biol Chem. 275, 19083-9.

Hirata, H., Tomita, K., Bessho, Y., Kageyama, R., 2001. Hes1 and Hes3 regulate maintenance of the isthmic organizer and development of the mid/hindbrain. Embo j. 20, 4454-66.

Hockfield, S., McKay, R.D., 1985. Identification of major cell classes in the developing mammalian nervous system. J Neurosci. 5, 3310-28.

Imayoshi, I., Kageyama, R., 2014. bHLH factors in self-renewal, multipotency, and fate choice of neural progenitor cells. Neuron. 82, 9-23.

Inzucchi, S.E., Bergenstal, R.M., Buse, J.B., Diamant, M., Ferrannini, E., Nauck, M., Peters, A.L., Tsapas, A., Wender, R., Matthews, D.R., American Diabetes, A., European Association for the Study of, D., 2012. Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetes Care. 35, 1364-79.

Islam, M.S., Loots du, T., 2009. Experimental rodent models of type 2 diabetes: a review. Methods Find Exp Clin Pharmacol. 31, 249-61.

J, S.R.-F., Sa-Roriz, T.M., Rosset, I., Camozzato, A.L., Santos, A.C., Chaves, M.L., Moriguti, J.C., Roriz-Cruz, M., 2009. (Pre)diabetes, brain aging, and cognition. Biochim Biophys Acta. 1792, 432-43.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., McKay, R.D., 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. 10, 3129-40.

Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., Gotoh, Y., 2004. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. Nat Cell Biol. 6, 547-54.

Kaneb, H.M., Sharp, P.S., Rahmani-Kondori, N., Wells, D.J., 2011. Metformin treatment has no beneficial effect in a dose-response survival study in the SOD1(G93A) mouse model of ALS and is harmful in female mice. PLoS One. 6, e24189.

Kaplan, M.S., Hinds, J.W., 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 197, 1092-4.

Kaplan, M.S., 1981. Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 195, 323-38.

Kittappa, R., Bornstein, S.R., Androutsellis-Theotokis, A., 2012. The role of eNSCs in neurodegenerative disease. Mol Neurobiol. 46, 555-62.

Kivipelto, M., Ngandu, T., Fratiglioni, L., Viitanen, M., Kareholt, I., Winblad, B., Helkala, E.L., Tuomilehto, J., Soininen, H., Nissinen, A., 2005. Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease. Arch Neurol. 62, 1556-60.

Kordower, J.H., Chu, Y., Hauser, R.A., Freeman, T.B., Olanow, C.W., 2008. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. Nat Med. 14, 504-506.

Kristiansen, O.P., Mandrup-Poulsen, T., 2005. Interleukin-6 and diabetes: the good, the bad, or the indifferent? Diabetes. 54 Suppl 2, S114-24.

Laron, Z., 2009. Insulin and the brain. Arch Physiol Biochem. 115, 112-6.

Laufer, E., Kesper, D., Vortkamp, A., King, P., 2012. Sonic hedgehog signaling during adrenal development. Mol Cell Endocrinol. 351, 19-27.

Lee, V.M., Goedert, M., Trojanowski, J.Q., 2001. Neurodegenerative tauopathies. Annu Rev Neurosci. 24, 1121-59.

Lei, Y., Yi, Y., Liu, Y., Liu, X., Keller, E.T., Qian, C.N., Zhang, J., Lu, Y., 2017. Metformin targets multiple signaling pathways in cancer. Chin J Cancer. 36, 17.

Lendahl, U., Zimmerman, L.B., McKay, R.D.G., 1990. CNS stem cells express a new class of intermediate filament protein. Cell. 60, 585-595.

Levy, D.E., Darnell, J.E., Jr., 2002. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol. 3, 651-62.

Li, J.Y., Englund, E., Holton, J.L., Soulet, D., Hagell, P., Lees, A.J., Lashley, T., Quinn, N.P., Rehncrona, S., Bjorklund, A., Widner, H., Revesz, T., Lindvall, O., Brundin, P., 2008. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat Med. 14, 501-3.

Li, L., El-Kholy, W., Rhodes, C.J., Brubaker, P.L., 2005a. Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. Diabetologia. 48, 1339-49.

Li, Y., Cao, X., Li, L.X., Brubaker, P.L., Edlund, H., Drucker, D.J., 2005b. beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. Diabetes. 54, 482-91.

Livesey, F.J., 2012. A potential link between obesity and neural stem cell dysfunction. Nat Cell Biol. 14, 987-9.

Lobe, C.G., 1997. Expression of the helix-loop-helix factor, Hes3, during embryo development suggests a role in early midbrain-hindbrain patterning. Mech Dev. 62, 227-37.

Luchsinger, J.A., Reitz, C., Patel, B., Tang, M.X., Manly, J.J., Mayeux, R., 2007. Relation of diabetes to mild cognitive impairment. Arch Neurol. 64, 570-5.

Luskin, M.B., 1993. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron. 11, 173-89.

Masjkur, J., Rueger, M.A., Bornstein, S.R., McKay, R., Androutsellis-Theotokis, A., 2012. Neurovascular signals suggest a propagation mechanism for endogenous stem cell activation along blood vessels. CNS Neurol Disord Drug Targets. 11, 805-17.

Masjkur, J., Arps-Forker, C., Poser, S.W., Nikolakopoulou, P., Toutouna, L., Chenna, R., Chavakis, T., Chatzigeorgiou, A., Chen, L.S., Dubrovska, A., Choudhary, P., Uphues, I., Mark, M., Bornstein, S.R., Androutsellis-Theotokis, A., 2014a. Hes3 is expressed in the adult pancreatic islet and regulates gene expression, cell growth, and insulin release. J Biol Chem. 289, 35503-16.

Masjkur, J., Levenfus, I., Lange, S., Arps-Forker, C., Poser, S., Qin, N., Vukicevic, V., Chavakis, T., Eisenhofer, G., Bornstein, S.R., Ehrhart-Bornstein, M., Androutsellis-Theotokis, A., 2014b. A Defined, Controlled Culture System for Primary Bovine Chromaffin Progenitors Reveals Novel Biomarkers and Modulators. Stem Cells Transl Med.

Masjkur, J., Levenfus, I., Lange, S., Arps-Forker, C., Poser, S., Qin, N., Vukicevic, V., Chavakis, T., Eisenhofer, G., Bornstein, S.R., Ehrhart-Bornstein, M., Androutsellis-Theotokis, A., 2014c. A defined, controlled culture system for primary bovine chromaffin progenitors reveals novel biomarkers and modulators. Stem Cells Transl Med. 3, 801-8.

Masjkur, J., Poser, S.W., Nikolakopoulou, P., Chrousos, G., McKay, R.D., Bornstein, S.R., Jones, P.M., Androutsellis-Theotokis, A., 2016. Endocrine Pancreas Development and Regeneration: Noncanonical Ideas From Neural Stem Cell Biology. Diabetes. 65, 314-30.

Mathew, B., Jacobson, J.R., Siegler, J.H., Moitra, J., Blasco, M., Xie, L., Unzueta, C., Zhou, T., Evenoski, C., Al-Sakka, M., Sharma, R., Huey, B., Bulent, A., Smith, B., Jayaraman, S., Reddy, N.M., Reddy, S.P., Fingerle-Rowson, G., Bucala, R., Dudek, S.M., Natarajan, V., Weichselbaum, R.R., Garcia, J.G., 2013. Role of migratory inhibition factor in age-related susceptibility to radiation lung injury via NF-E2-related factor-2 and antioxidant regulation. Am J Respir Cell Mol Biol. 49, 269-78.

McKay, R., Kittappa, R., 2008. Will Stem Cell Biology Generate New Therapies for Parkinson's Disease? Neuron. 58, 659-661.

Miller, R.A., Chang, Y., Galecki, A.T., Al-Regaiey, K., Kopchick, J.J., Bartke, A., 2002. Gene expression patterns in calorically restricted mice: partial overlap with long-lived mutant mice. Mol Endocrinol. 16, 2657-66.

Milo, R., Miller, A., 2014. Revised diagnostic criteria of multiple sclerosis. Autoimmunity Reviews. 13, 518-524.

Mitchell, B.D., Emsley, J.G., Magavi, S.S., Arlotta, P., Macklis, J.D., 2004. Constitutive and induced neurogenesis in the adult mammalian brain: manipulation of endogenous precursors toward CNS repair. Dev Neurosci. 26, 101-17.

Moloney, A.M., Griffin, R.J., Timmons, S., O'Connor, R., Ravid, R., O'Neill, C., 2010. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. Neurobiol Aging. 31, 224-43.

Moreira, P.I., 2014. Metformin in the diabetic brain: friend or foe? Ann Transl Med. 2, 54.

Nazem, A., Sankowski, R., Bacher, M., Al-Abed, Y., 2015. Rodent models of neuroinflammation for Alzheimer's disease. J Neuroinflammation. 12, 74.

Nikolakopoulou, P., Poser, S.W., Masjkur, J., Fernandez Rubin de Celis, M., Toutouna, L., Andoniadou, C.L., McKay, R.D., Chrousos, G., Ehrhart-Bornstein, M., Bornstein, S.R., Androutsellis-Theotokis, A., 2016. STAT3-Ser/Hes3 Signaling: A New Molecular Component of the Neuroendocrine System? Horm Metab Res. 48, 77-82.

Ohta, S., Misawa, A., Fukaya, R., Inoue, S., Kanemura, Y., Okano, H., Kawakami, Y., Toda, M., 2012. Macrophage migration inhibitory factor (MIF) promotes cell survival and proliferation of neural stem/progenitor cells. J Cell Sci. 125, 3210-20.

Oliveira, W.H., Nunes, A.K., Franca, M.E., Santos, L.A., Los, D.B., Rocha, S.W., Barbosa, K.P., Rodrigues, G.B., Peixoto, C.A., 2016. Effects of metformin on inflammation and short-term memory in streptozotocin-induced diabetic mice. Brain Res. 1644, 149-60.

Ourednik, J., Ourednik, V., Lynch, W.P., Schachner, M., Snyder, E.Y., 2002. Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotech. 20, 1103-1110.

Owen, M.R., Doran, E., Halestrap, A.P., 2000. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J. 348 Pt 3, 607-14.

Park, D.M., Jung, J., Masjkur, J., Makrogkikas, S., Ebermann, D., Saha, S., Rogliano, R., Paolillo, N., Pacioni, S., McKay, R.D., Poser, S., Androutsellis-Theotokis, A., 2013. Hes3 regulates cell number in cultures from glioblastoma multiforme with stem cell characteristics. Sci Rep. 3, 1095.

Park, S., Dong, X., Fisher, T.L., Dunn, S., Omer, A.K., Weir, G., White, M.F., 2006. Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. J Biol Chem. 281, 1159-68.

Pedditizi, E., Peters, R., Beckett, N., 2016. The risk of overweight/obesity in mid-life and late life for the development of dementia: a systematic review and meta-analysis of longitudinal studies. Age Ageing. 45, 14-21.

Perfetti, R., Zhou, J., Doyle, M.E., Egan, J.M., 2000. Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. Endocrinology. 141, 4600-5.

Poser, S.W., Androutsellis-Theotokis, A., 2013. Growing neural stem cells from conventional and nonconventional regions of the adult rodent brain. J Vis Exp. e50880.

Poser, S.W., Park, D.M., Androutsellis-Theotokis, A., 2013. The STAT3-Ser/Hes3 signaling axis: an emerging regulator of endogenous regeneration and cancer growth. Front Physiol. 4, 273.

Poser, S.W., Chenoweth, J.G., Colantuoni, C., Masjkur, J., Chrousos, G., Bornstein, S.R., McKay, R.D., Androutsellis-Theotokis, A., 2015. Concise Review: Reprogramming, Behind the Scenes: Noncanonical Neural Stem Cell Signaling Pathways Reveal New,

Unseen Regulators of Tissue Plasticity With Therapeutic Implications. Stem Cells Transl Med. 4, 1251-7.

Procaccini, C., Santopaolo, M., Faicchia, D., Colamatteo, A., Formisano, L., de Candia, P., Galgani, M., De Rosa, V., Matarese, G., 2016. Role of metabolism in neurodegenerative disorders. Metabolism. 65, 1376-1390.

Rajan, P., McKay, R.D., 1998. Multiple routes to astrocytic differentiation in the CNS. J Neurosci. 18, 3620-9.

Reynolds, B.A., Weiss, S., 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 255, 1707-10.

Rivera, E.J., Goldin, A., Fulmer, N., Tavares, R., Wands, J.R., de la Monte, S.M., 2005. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. J Alzheimers Dis. 8, 247-68.

Rocha, N.P., Scalzo, P.L., Barbosa, I.G., de Sousa, M.S., Morato, I.B., Vieira, É.L.M., Christo, P.P., Reis, H.J., Teixeira, A.L., Circulating levels of adipokines in Parkinson's disease. Journal of the Neurological Sciences. 339, 64-68.

Rozengurt, E., 2014. Mechanistic target of rapamycin (mTOR): a point of convergence in the action of insulin/IGF-1 and G protein-coupled receptor agonists in pancreatic cancer cells. Front Physiol. 5, 357.

Salewski, R.P., Buttigieg, J., Mitchell, R.A., van der Kooy, D., Nagy, A., Fehlings, M.G., 2012. The generation of definitive neural stem cells from PiggyBac transposon-induced pluripotent stem cells can be enhanced by induction of the NOTCH signaling pathway. Stem Cells Dev. 22, 383-96.

Sanchez Alvarado, A., Yamanaka, S., 2014. Rethinking differentiation: stem cells, regeneration, and plasticity. Cell. 157, 110-9.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., Brivanlou, A.H., 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med. 10, 55-63.

Sauler, M., Bucala, R., Lee, P.J., 2015. Role of macrophage migration inhibitory factor in age-related lung disease. Am J Physiol Lung Cell Mol Physiol. 309, L1-10.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods. 9, 676-82.

Simon, A.R., Rai, U., Fanburg, B.L., Cochran, B.H., 1998. Activation of the JAK-STAT pathway by reactive oxygen species. Am J Physiol. 275**,** C1640-52.

Sinnett-Smith, J., Kisfalvi, K., Kui, R., Rozengurt, E., 2013. Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: dependence on glucose concentration and role of AMPK. Biochem Biophys Res Commun. 430**,** 352-7.

Song, W.J., Schreiber, W.E., Zhong, E., Liu, F.F., Kornfeld, B.D., Wondisford, F.E., Hussain, M.A., 2008. Exendin-4 stimulation of cyclin A2 in beta-cell proliferation. Diabetes. 57**,** 2371-81.

Stadelmann, C., Albert, M., Wegner, C., Brück, W., 2008. Cortical pathology in multiple sclerosis. Current Opinion in Neurology. 21**,** 229-234.

Steen, E., Terry, B.M., Rivera, E.J., Cannon, J.L., Neely, T.R., Tavares, R., Xu, X.J., Wands, J.R., de la Monte, S.M., 2005. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis. 7**,** 63-80.

Sun, D., 2014. The potential of endogenous neurogenesis for brain repair and regeneration following traumatic brain injury. Neural Regen Res. 9**,** 688-92.

Sun, J., Zhao, M., Jia, P., Wang, L., Wu, Y., Iverson, C., Zhou, Y., Bowton, E., Roden, D.M., Denny, J.C., Aldrich, M.C., Xu, H., Zhao, Z., 2015. Deciphering Signaling Pathway Networks to Understand the Molecular Mechanisms of Metformin Action. PLoS Comput Biol. 11**,** e1004202.

Talbot, K., Wang, H.Y., Kazi, H., Han, L.Y., Bakshi, K.P., Stucky, A., Fuino, R.L., Kawaguchi, K.R., Samoyedny, A.J., Wilson, R.S., Arvanitakis, Z., Schneider, J.A., Wolf, B.A., Bennett, D.A., Trojanowski, J.Q., Arnold, S.E., 2012. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. J Clin Invest. 122**,** 1316-38.

Toutouna, L., Nikolakopoulou, P., Poser, S.W., Masjkur, J., Arps-Forker, C., Troullinaki, M., Grossklaus, S., Bosak, V., Friedrich, U., Ziemssen, T., Bornstein, S.R., Chavakis, T., Androutsellis-Theotokis, A., 2016. Hes3 expression in the adult mouse brain is regulated during demyelination and remyelination. Brain Res. 1642**,** 124-30.

Trapp, B.D., Nave, K.-A., 2008. Multiple Sclerosis: An Immune or Neurodegenerative Disorder? Annual Review of Neuroscience. 31**,** 247-269.

Wilkin, T.J., 2001. The accelerator hypothesis: weight gain as the missing link between Type I and Type II diabetes. Diabetologia. 44**,** 914-22.

World Health Organization, 2017. Life expectancy. Vol., ed.^eds.

Xu, G., Stoffers, D.A., Habener, J.F., Bonner-Weir, S., 1999. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes. 48**,** 2270-6.

Yamanaka, K., Boillee, S., Roberts, E.A., Garcia, M.L., McAlonis-Downes, M., Mikse, O.R., Cleveland, D.W., Goldstein, L.S.B., 2008. Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice. Proceedings of the National Academy of Sciences. 105**,** 7594-7599.

Ziegler, A.N., Levison, S.W., Wood, T.L., 2015. Insulin and IGF receptor signalling in neural-stem-cell homeostasis. Nat Rev Endocrinol. 11**,** 161-170.

Erklärungen zur Eröffnung des Promotionsverfahrens

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- Herrn PD Dr. Androutsellis-Theotokis, Innate Repair Laboratory, Medizinische Klinik III, Universitätsklinikum Carl Gustav Carus, Technische Universität Dresden
- Herrn Prof. Dr. rer. nat. Henning Morawietz, Bereich Gefäßendothel und Mikrozirkulation, Universitätsklinikum Carl Gustav Carus, Technische Universität Dresden

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Polyxeni Nikolakopoulou

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☐ das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, die das Medizinproduktegesetz betreffen

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