Regulation of Glucose Uptake in Neonatal Rat Cardiomyocytes by Neuregulin1β

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Riehen (BS), Schweiz

Basel, 2018

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 16. Oktober 2018

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Abbreviations

Akt	Protein kinase B
ARVMs	Adult rat ventricular myocytes
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
GTP	Guanine triphosphate
BCAA	Branched chain amino acid
CMs	Cardiomyocytes
EGF	Epidermal growth factor
ErbB	Erythroblastic leukemia viral oncogene homolog
Erk1/2	Extracellular signal-regulated kinases 1/2
4E-BP1	eIF4E-binding protein 1
FAK	Focal adhesion kinase
GLUT	Glucose transporter
GSK3β	Glycogen synthase kinase 3β
IGF-I	Insulin-like growth factor 1
IGF-IR	IGF-I receptor
InsR	Insulin receptor
i.p.	Intraperitoneal
IRS-1/2	Insulin receptor substrate 1/2
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
Nrg1β	Neuregulin 1 beta
NRVMs	Neonatal rat ventricular myocytes
PDK1	Phosphoinositide-dependent kinase-1
p-HH3	Phospho-Histone H3-pSer^{10}
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PI3K	Phosphatidylinositol-3-kinase

PTB	Phosphotyrosine-binding domain
p70S6K1	Ribosomal protein S6 kinase beta-1
qRT-PCR	Quantitative real-time polymerase chain reaction
Rab	Ras-related in brain
Rictor	Rapamycin-insensitive companion of mTOR
Ser	Serine
SGLT	Sodium-glucose linked transporter
siRNA	Small interfering ribonucleic acid
STDV	Standard deviation
c-Src	Proto-oncogene tyrosine-protein kinase Src
STZ	Streptozotocin
Thr	Threonine
Tyr	Tyrosine
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

Abstract

Purpose:

Neuregulin (Nrg) signaling plays an indispensable role in cardiac development and homeostasis. Since Nrg1β was shown to have beneficial effects on the heart in animal models of cardiac disease, several clinical trials are investigating its therapeutic value. The present study examines metabolic effects and molecular mechanisms that are induced by Nrg1β in cardiomyocytes, which could help to understand the positive effects observed in disease models *in vivo* and in clinics.

Methods:

Isolated neonatal rat ventricular myocytes (NRVMs), isolated adult rat ventricular myocytes (ARVMs), neonatal rats and adult mice served as models for our investigations. By pharmacological inhibition or siRNA knockdown, the role of proteins of interest was analyzed. For signaling analysis, proteins were extracted and Western blotting was performed. Glucose uptake was measured by [^{3}H]-deoxy-D-glucose incorporation, glycolysis was investigated by Seahorse assay and protein synthesis was detected by [^{3}H]-phenylalanine incorporation. GLUT4 translocation was evaluated by expressing a c-Myc-GLUT4-mCherry construct that was transfected into NRVMs. Proliferation of NRVMs was analyzed by immunofluorescence. In addition, neonatal rats and adult streptozotocin (STZ) mice were treated with Nrg1β or insulin to assess activation of glucose uptake pathways in the heart *in vivo*.

Results:

Similar to insulin and IGF-I, Nrg1β caused an 80% increase in glucose uptake and elevated the level of glycolysis in NRVMs. Dose-response curves of combinations of insulin and Nrg1β showed no additive effects on glucose uptake. Nrg1β activated the PI3K/Akt, the c-Src/FAK and the MAPK/Erk1/2 pathways, whereas only the PI3K/Akt and c-Src/FAK pathways appeared implicated in the glucose uptake. Pharmacological inhibition demonstrated that Nrg1β- and insulin-induced glucose uptake require PI3Kα and Akt. Both Nrg1β and insulin increased phosphorylation of Akt and AS160, whereas phosphorylation of IRS-1 at Tyr^{612} and IRS-1/2 degradation was only induced by insulin. Interestingly, Nrg1β and insulin showed an

additive effect on Akt phosphorylation, whereas this was not the case for Akt's target AS160. Knockdown of GLUT4 and an increase in GLUT4 translocation indicated its involvement in Nrg1β- and insulin-induced glucose uptake in NRVMs. Since glucose uptake is known to contribute to protein synthesis, we measured protein synthesis after Nrg1β stimulation, which was 50% increased, matching with elevated p70S6K1 and 4E-BP1 phosphorylation. Knockdown experiments revealed that the ErbB2/ErbB4 homodimer is required for the Nrg1β-induced glucose uptake as well as protein synthesis. Given the fact that glucose uptake and protein synthesis are part of proliferative responses, we analyzed proliferation of NRVMs in presence of Nrg1β. Interestingly, our preliminary data showed that Nrg1β increases proliferation of NRVMs, however only in combination with IGF-I. Furthermore, in order to compare our *in vitro* findings with an *in vivo* model, we analyzed the effect of Nrg1β on the neonatal rat heart. Western blot analysis of Nrg1β-stimulated neonatal hearts revealed comparable signaling effects as observed *in vitro*. In contrast to NRVMs and neonatal rat hearts, ARVMs did not increase the phosphorylation of Akt or AS160 and showed no effect on glucose uptake after Nrg1β stimulation. Moreover, while Nrg1β injection of diabetic STZ mice revealed a systemic effect on blood glucose clearance likely mediated by the liver, no activation of the PI3K/Akt/AS160 pathway was detectable in the hearts of these mice.

Conclusions:

Our major finding is that Nrg1β induces glucose uptake in NRVMs by a similar mechanism as insulin. This mechanism requires the ErbB2/ErbB4 homodimer, PI3Kα, Akt and AS160. Consistently, Nrg1β increases glucose uptake by GLUT4 translocation to a similar extent as insulin. Compared to insulin, Nrg1β signaling has distinct effects on IRS phosphorylation, which is not followed by IRS degradation. Altogether, our findings on glucose uptake, protein synthesis and proliferation contribute to a better understanding of the molecular mechanisms of Nrg1β in CMs, which may be applied to improve treatments of heart disease and promote heart regeneration in humans.

1. Introduction

1.1. The heart and cardiomyocytes

The heart acts as a pump and moves blood through the whole body. During evolution, the vertebral lineage gave rise to a closed circulation system, which consists of a cardiac muscle and a huge network of blood vessels. The vertebral heart needs to maintain a constant blood pressure by continuous contraction. In contrast to skeletal muscle, the heart is self-excitable, meaning that its contraction does not depend on an electrical stimulus from the nervous system, however the autonomous nervous system can influence heart activity. In the right atrium, the sinoatrial node generates electrical impulses, which spread into the wall of the heart and induce contraction of the cardiomyocytes (CMs). Contraction starts with the opening of voltage-gated sodium channels, which triggers the release of calcium ions from the sarcoplasmic reticulum through ryanodine receptors. This induces a conformational change of the sarcomeres, resulting in a shortening of the CMs. Shortly after contraction, calcium pumps bring the calcium back into the sarcoplasmic reticulum, consuming adenosine triphosphate (ATP). Since the whole process of excitation-contraction coupling needs a lot of energy, the heart has to be constantly supplied with nutrients and oxygen (Zipes et al., 2005). To meet this high demand of energy, the heart has a broad range of possible energy substrates. Besides glucose, lactate, ketones and amino acids, the main energy source of the adult heart are fatty acids (Bing, 1965; Lopaschuk and Jaswal, 2010; Neely et al., 1972).

The mammalian heart consists of several cell types, mainly CMs, fibroblasts, smooth muscle cells, endothelial cells, Purkinje fibers and pacemaker cells (Xin et al., 2013). In the adult heart, the majority of CMs are post-mitotic cells, highly differentiated and have a low capacity to replicate. Fully differentiated CMs have a high number of thin invaginations of the sarcolemma, the so-called t-tubules, which facilitate ion exchange with the extracellular space (Fig. 1). The whole cytoplasm of CMs is filled with a network of sarcomeres, the indispensable unit for contraction. CMs are tightly linked with desmosomes and connected with gap junctions at the intercalated discs to be able to conduct the electrical current, which triggers contraction. Since performing rhythmic contractions requires a lot of energy, CMs have a high number of mitochondria (Walker and Spinale, 1999). Due to the fact that CMs are

highly differentiated cells, their ability to proliferate is very limited. In the human heart, it was observed that the turnover rate of CMs is only about 1% per year, which declines with higher age (Bergmann et al., 2009; Mollova et al., 2013). This low proliferation capacity of CMs is very likely a reason why the human heart undergoes scarring instead of replacement of cardiac tissue after injury. This is in contrast to other species like certain fishes, amphibians or reptiles, which are able to regenerate their heart after damage (Garbern et al., 2013; Yester and Kuhn, 2017). Excitingly, it was observed that even young mammals have the capacity to regenerate their heart (Porrello et al., 2011; Ye et al., 2018; Zhu et al., 2018). In the last years, many studies aimed to induce heart regeneration by activating CM proliferation (Leone et al., 2015). One promising candidate in the field of heart regeneration is Neuregulin (Nrg) (Polizzotti et al., 2015).

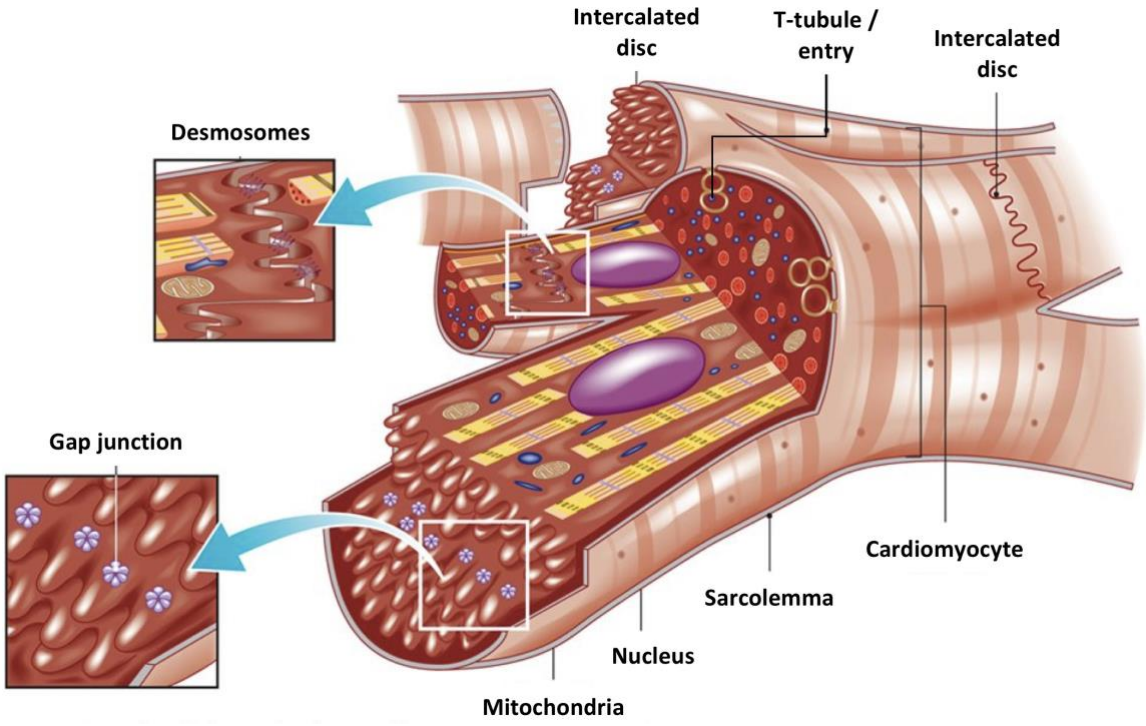

Figure 1. Cardiomyocyte model (adapted from V. Mayoral, P. Francisco, Á. Rodríguez. 2014. Patologia.)

1.2. Neuregulin and ErbB receptors

Nrg, which is also known as glial growth factor 1, Heregulin, Neu differentiation factor or acetylcholine receptor-inducing activity (ARIA), was first discovered in neural and cancer cells and belongs to the epidermal growth factor (EGF) family of growth factors (Falls et al., 1993; Holmes et al., 1992; Marchionni et al., 1993; Peles et al., 1992). So far, there are six neuregulin genes (NRG1-6) identified, of which NRG1 is the best characterized (Mei and Nave, 2014). NRG1 is widely expressed, namely in the brain, heart, breast, eye, skin, lung, gut, testis and skeletal muscle. NRG1 is a large gene (1'400 kb) on chromosome 8 in the human and mice genome and it encodes six types of proteins, which include more than 31 isoforms. The isoforms can be distinguished by their N-terminal region (I-VI), the EGF-like domain (α, β or γ), and the juxtamembrane extracellular region (a, b, or c) (Falls, 2003). All isoforms have a variable cytoplasmic tail, a transmembrane domain and some isoforms include an immunoglobulin-like (Ig) domain (Parodi and Kuhn, 2014). The formation of most isoforms starts with a membrane-bound precursor. Upon activation by oxidative stress (Kuramochi et al., 2004a), the extracellular domain is cleaved off by A disintegrin and metalloproteinases (ADAM 10, ADAM17, ADAM 19) and gets released into the extracellular space (Fleck et al., 2013; Horiuchi et al., 2005; Willem, 2016). Some Nrgs are cut intracellularly in the cytoplasmic tail and migrate to the nucleus for the promotion of gene expression (Bao et al., 2004). Nrgs mainly act paracrine/juxtacrine and bind to erythroblastic leukemia viral oncogene homolog (ErbB) receptor tyrosine kinases (Parodi and Kuhn, 2014). For our study, we used the extracellular domain or only the EGF domain of Nrg1β, the isoform which was demonstrated to be the most potent to bind and activate the ErbB receptors (Pinkas-Kramarski et al., 1996; Tzahar et al., 1994).

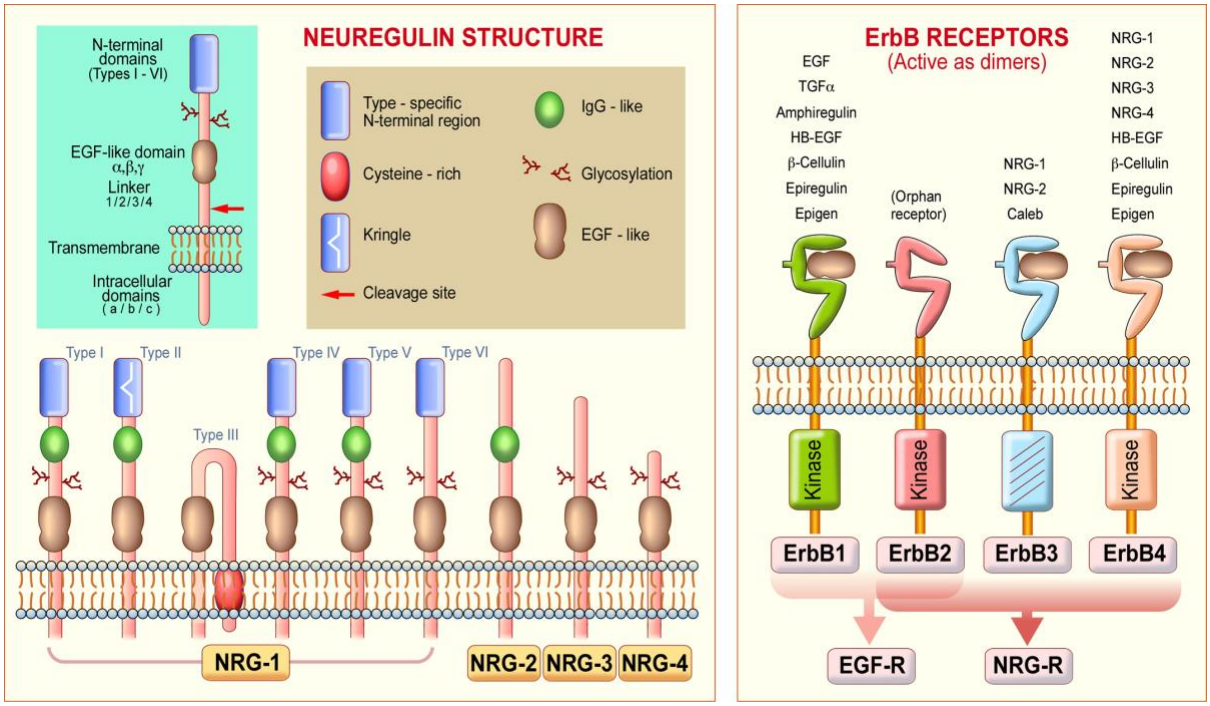

Figure 2. Neuregulin1-4 and ErbB1-4 receptors (Guma et al., 2010)

The ErbB receptor tyrosine kinases are closely related to the epidermal growth factor receptor (EGFR or ErbB1). There are three receptors involved in Nrg signaling, called ErbB2, ErbB3 and ErbB4, which are expressed in several organs including the heart (Parodi and Kuhn, 2014). At the N-terminus, the ErbB pro-receptor has a signal peptide that is necessary for correct localization to the plasma membrane. The mature receptor consists of an intracellular domain, a transmembrane domain and an extracellular domain (Fig. 2). The intracellular domain includes a juxtamembrane sub-domain, a phosphotyrosine kinase sub-domain and a non-catalytic C-terminal regulatory domain (Burgess et al., 2003). Each receptor has its own repertoire of ligands that can bind to its extracellular domain (Wilson et al., 2009). Upon ligand binding, the receptor undergoes a conformational change from a closed to an open conformation. In this open conformation, the receptor is able to dimerize and transphosphorylate tyrosine (Tyr) residues on the non-catalytic C-terminal regulatory domain. Except ErbB2, all receptors have a binding site for ligands. ErbB2 is constantly in the open conformation, but it needs to form a heterodimer with another ligand-bound ErbB receptor to become active (Cho et al., 2003). Besides receptor dimerization, the ErbB2 receptor can also be activated in a different way. Cleavage of the extracellular receptor domain by a metalloprotease can lead to a constitutively active receptor, which may play a

role in certain breast cancers where ErbB2 is overexpressed. (Codony-Servat et al., 1999). ErbB3 has no functional catalytic domain, meaning it is able to bind ligands but cannot create a downstream signal. Like ErbB2, ErbB3 needs to form a heterodimer to transmit the signal (Citri et al., 2003). Only ErbB4 can form a fully functional homodimer. Upon receptor activation, the C-terminal domain is phosphorylated at Tyr residues, which serve as docking platform for intracellular signaling proteins with Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. Depending on the combination of receptors, different signaling pathways are activated (Burgess et al., 2003; Fuller et al., 2008).

1.2.1. ErbB signaling

Binding of Nrg to the ErbB3 or ErbB4 receptors and subsequent dimerization with the ErbB2, ErbB3 or ErbB4 activates the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt), the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinases 1/2 (Erk1/2) and the proto-oncogene tyrosine-protein kinase Src (c-Src)/Focal adhesion kinase (FAK) pathway, which influence cellular processes like proliferation, migration, differentiation and survival (Fig. 3) (Pentassuglia and Sawyer, 2009).

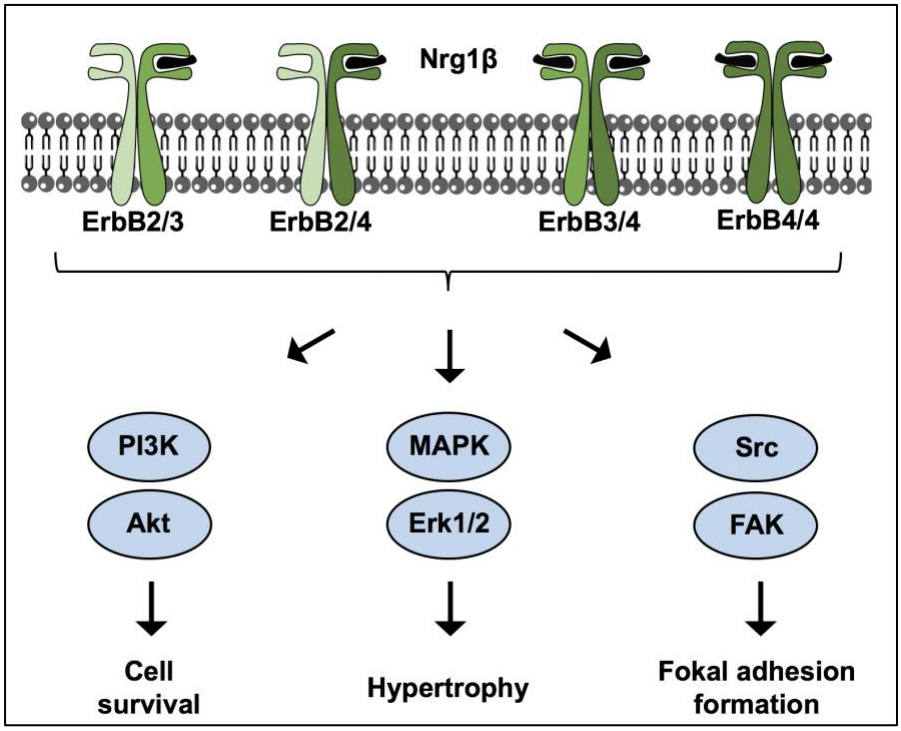

Figure 3. The ErbB receptors activate several signaling pathways

The PI3K/Akt pathway is a well-studied signaling pathway known to be involved in the regulation of many cellular processes like protein synthesis, apoptosis or glucose uptake (Hemmings and Restuccia, 2012). In case of insulin-induced glucose uptake, insulin binding to its receptor activates PI3K via insulin receptor substrate-1 (IRS-1) and/or IRS-2 and produces the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 is necessary for the activation of 3-phosphoinositide-dependent protein kinase-1 (PDK1), which finally phosphorylates Akt at threonine (Thr)^{308}. To fully activate Akt, the mammalian target of rapamycin complex 2 (mTORC2) needs to phosphorylate serine (Ser)^{473}. Akt then phosphorylates Tuberous Sclerosis Complex 2 (TSC2) and proline-rich Akt substrate of 40 kDa (PRAS40), which leads to mTORC1 activation. mTOR phosphorylates then eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase beta-1 (p70S6K1). 4E-BP1 and p70S6K1 are involved in the regulation of protein synthesis. Subsequent to phosphorylation by mTOR, 4E-BP1 is inhibited and releases eIF4E, a translation initiation factor. p70S6K1 has a key role in regulation of cell growth and proliferation and it is activated by mTOR phosphorylation. Prolonged activation of PI3K, mTORC1 and p70S6K1 induces a negative feedback by phosphorylating IRS-1, which may cause its degradation by the proteasome (Fig. 4) (Harrington et al., 2005; Laplante and Sabatini, 2009).

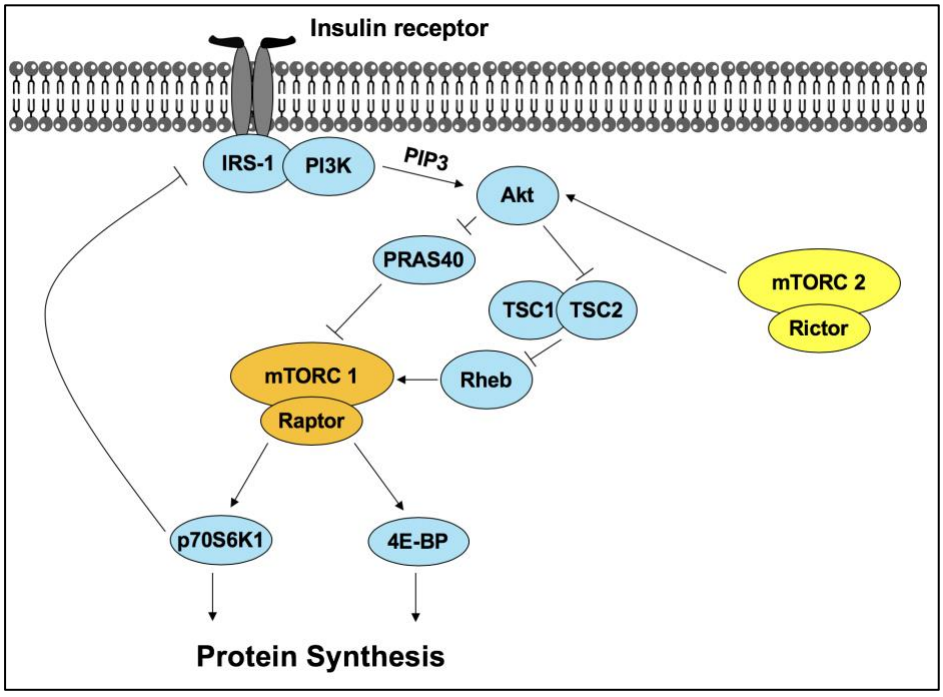

Figure 4. PI3K/Akt signaling pathway

Another pathway that is activated by Nrg is the MAPK/Erk1/2 pathway. The MAPK/Erk1/2 pathway is mainly induced by the stimulation of growth factors. Other intensively studied MAPK pathways are the p38 and the c-Jun N-terminal kinases (JNK) pathways, which are primarily activated by a variety of stimuli related to stress like cytokines or DNA damage (Pearson et al., 2001). All MAPK pathways share some common features. They are composed of three kinases that phosphorylate themselves in a sequential manner: a MAPK, a MAPK kinase (MAPKK) and a MAPK kinase kinase (MAPKKK). Triggered by an interaction with a protein of the Rat sarcoma (Ras)/Ras homologue (Rho) family, the MAPKKK phosphorylate and activate a MAPKK, which then itself phosphorylates the MAPK. The activated MAPK, for example Erk1/2, is a Ser/Thr kinase that phosphorylates different substrates like transcription factors or several protein kinases named MAPK-activated protein kinases (MK) that regulate many biological functions. The MAPK/Erk1/2 pathway has been demonstrated to be a key regulator of cell proliferation and thus, some anticancer drugs are targeting this pathway (Roux and Blenis, 2004).

The c-Src/FAK pathway is as well activated by Nrg stimulation (Kuramochi et al., 2006). c-Src and FAK are non-receptor protein tyrosine kinases involved in cytoskeleton regulation, survival and proteins synthesis (Thomas and Brugge, 1997). c-Src belongs to the Src family of protein tyrosine kinases, which can interact with a broad spectrum of cellular receptors and other targets. FAK is part of the focal adhesion complex that links the cytosol with the extracellular matrix via integrins (Graham et al., 2015). Nrg1β was demonstrated to trigger phosphorylation of c-Src at Tyr^{416} and Tyr^{215}, two known kinase-activating sites of c-Src, and FAK at Tyr^{861} in adult CMs (Kuramochi et al., 2006). Moreover, Kuramochi et al. concluded that c-Src/FAK signaling contributes to cytoskeletal remodeling and thereby enables isolated adult rat ventricular myocytes (ARVMs) to form cell-to-cell contacts.

Since many signaling molecules of these pathways have been shown to be implicated in different kind of cancer types, drugs against several targets have been developed. To analyze the role of these signaling molecules, a couple of pharmacologic inhibitors were applied in this thesis.

1.2.2. Neuregulin in the heart

Since more than 20 years, it is known that Nrg and its receptors (ErbB2-4) are indispensable for proper heart development and homeostasis (Odiete et al., 2012). Besides the important role of the Nrg/ErbB signaling in the heart, it is also fundamental for the nervous system regulating myelination, synaptic plasticity, neural circuitry and neurotransmission (Mei and Nave, 2014). Even more, it was shown that Nrg1, ErbB2 and ErbB4 are involved in early embryogenesis (Fig. 5). The knockout of each gene is embryonically lethal because of severe problems with heart formation, namely impaired ventricular trabeculation, and thus the animals die due to heart failure (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). Embryos that are lacking ErbB3 have defects in the endocardial cushion formation, which as well results in embryonic lethality (Erickson et al., 1997). Recently, Nrg1 was demonstrated to promote synthesis of myocardial extracellular matrix, which is necessary for proper formation of the trabeculae (Del Monte-Nieto et al., 2018). Interestingly, Rentschler et al. observed that Nrg1 contributes also to the development of the heart conduction system (Rentschler et al., 2002). In addition, Rentschler et al. showed that Nrg1 triggered differentiation of embryonic CMs into cells of the cardiac conduction system and fetal CMs into cardiac pacemaker-like cells.

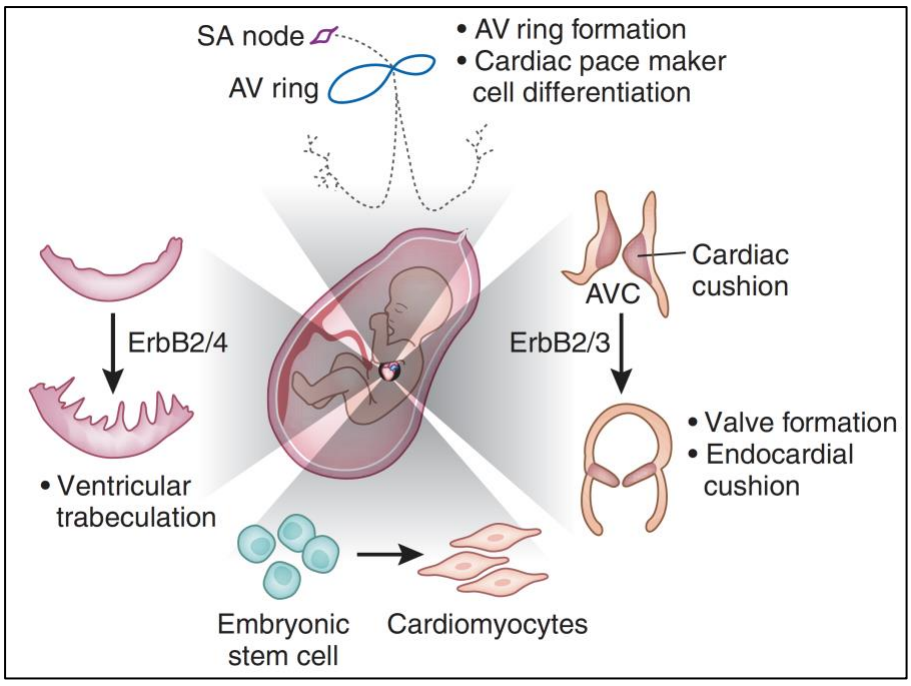

Figure 5. The roles of Nrg/ErbB in heart development (Odiete et al., 2014)

In contrast to earlier publications, which detected only ErbB1/EGFR, ErbB2 and ErbB4 in the adult heart, recently it was demonstrated that all four ErbB receptors are expressed (Camprecios et al., 2011). They showed that the expression of ErbB2 and ErbB4 decreases within the first week after birth, whereas ErbB3 expression increases and remains high at later time points. Conditional cardiomyocyte-specific disruption of ErbB2 or ErbB4 leads to dilated cardiomyopathy. Moreover, cancer treatment, where chemotherapy is combined with a recombinant monoclonal antibody against ErbB2 (Trastuzumab), may result in cardiac dysfunction (Crone, 2002; Garcia-Rivello et al., 2005; Ozcelik et al., 2002; Slamon et al., 2001). This demonstrates that ErbB/Nrg signaling is necessary to keep homeostasis of the adult heart. In addition, Nrg is important for the heart during stress situations. It was observed that Nrg expression is increased upon mechanical strain (Lemmens et al., 2006) and during pregnancy, a time when cardiac demand is elevated (Lemmens et al., 2011). Lemmens et al. showed that when pregnant mice were treated with Lapatinib, a dual ErbB1/ErbB2 tyrosine kinase inhibitor, cardiac function was reduced and about 25% of the pregnant animals died prematurely.

In the heart, Nrg is released by the microvascular endothelium and binds the ErbB receptors on CMs (Cote et al., 2005). It was observed that Nrg1 has an anti-apoptotic effect on neonatal and adult CMs cultured in serum-free medium (Zhao et al., 1998). In addition, Baliga et al. demonstrated that the recombinant human glial growth factor (rhGGF-2), a soluble Nrg1 peptide, induces hypertrophy in NRVMs (Baliga et al., 1999). Nrg1β protects CMs from β1-adrenergic receptor–induced cell death and has a negative inotropic effect in isolated papillary muscles (Kuramochi et al., 2004b; Lemmens et al., 2004). Furthermore, it was shown that Nrg1 has anti-fibrotic and anti-remodeling effects in a swine model of heart failure (Galindo et al., 2014). Moreover, several studies claim that Nrg1 enhances proliferation of CMs *in vitro* and *in vivo* (Bersell et al., 2009; D'Uva et al., 2015; Gemberling et al., 2015; Ma et al., 2016; Polizzotti et al., 2015; Zhao et al., 1998). Recently, our group demonstrated that Nrg1β is as potent as insulin to induce glucose uptake in neonatal CMs by a mechanism that implicates PI3K, Akt and Akt substrate of 160 kDa (AS160, also known as TBC1D4) (Pentassuglia et al., 2016). Previously, Canto et al. observed that Nrg1β increased glucose uptake as well in L6E6 skeletal muscle cells, but independently of Akt (Canto et al., 2004). These data show that the Nrg1β-induced signaling mechanism leading to increased glucose uptake differs between CMs and skeletal muscle cells.

1.3. Glucose metabolism

For many organisms, glucose is a very important source of energy. Glucose is catabolically processed during glycolysis to pyruvate or besides its intermediates, serves as substrate for several anabolic pathways. Rapidly after glucose uptake, glucose gets phosphorylated by hexokinase turning into glucose-6-phosphate, which is hydrophilic and therefore is retained in the cell. Glucose-6-phosphate can either be stored as glycogen during glycogen synthesis, enter the pentose phosphate pathway and the polyol pathway, or it gets degraded by glycolysis. During glycolysis, glucose-6-phosphate turns into fructose-6-phosphate, which can enter the hexosamine biosynthetic pathway that delivers substrates for glycosylation of proteins or it can be further degraded. When fructose-6-phosphate is further degraded by glycolysis, after several enzymatic steps, pyruvate is produced. Finally, pyruvate can be turned into acetyl-CoA and enter the tricarboxylic acid (TCA) cycle for ATP production in the mitochondria or it can be turned into lactate, which might be secreted by the cell (Shao and Tian, 2015).

1.3.1. The role of insulin and IGF-I in glucose metabolism

Insulin is a hormone that regulates blood glucose levels and influences anabolic effects like protein, fatty acid and carbohydrate synthesis. Its major effects on glucose metabolism are stimulation of glucose uptake and glycolysis in muscle and adipose tissue, activation of glycogen synthesis in several tissues and inhibition of glycogenolysis and gluconeogenesis in the liver (Dimitriadis et al., 2011). In comparison to insulin, the insulin-like growth factor (IGF-I) has similar effects on glucose handling but its mitogenic potential is much higher (Beguinot et al., 1985). IGF-I is structurally related with proinsulin and shares 48% of the amino acid sequence. At physiologic concentrations, Insulin binds the insulin receptor (InsR) and IGF-I binds the IGF-I receptor (IGF-IR). Interestingly, IGF-I seems to enhance insulin sensitivity, very likely by an indirect mechanism and at higher concentrations, IGF-I can also bind the InsR (Clemmons, 2006). In addition, the InsR and IGF-IR have a very similar structure and can form hybrid heterodimers, thereby activating both receptor substrates. The two receptors have a common substrate, namely IRS (Chang et al., 2004; Clemmons, 2006).

1.3.2. IRS proteins

There are four IRS isoforms in human, namely IRS-1, IRS-2, IRS-3 and IRS-4. IRS-1 and IRS-2 are involved in the regulation of glucose uptake and they are part of the classical insulin-induced signaling cascade. Both are activated by the InsR and the IGF-IR and serve as scaffolding proteins to attract downstream effector proteins. Upon activation of the receptors, the PI3K pathway is strongly induced. IRS proteins are mainly regulated by ubiquitin-mediated degradation and phosphorylation at Tyr, Ser and Thr residues. The available phosphorylation sites on IRS are very numerous and their effect on IRS activity is difficult to elucidate. A negative feedback loop via mTOR/S6K1 has been associated with increased phosphorylation on Ser^{307} (rat) or Ser^{312} (human) respectively, which leads to its inactivation (Copps and White, 2012; Greene et al., 2003; Harrington et al., 2005). On the other hand, Tyr^{612} and Tyr^{632} have been shown to be required for insulin-induced PI3K activation and glucose transporter 4 (GLUT4) translocation (Esposito et al., 2001). In the heart and other organs, IRS-1 and IRS-2 are thought to contribute to the development of insulin resistance in type 2 diabetes mellitus (T2DM) (Lavin et al., 2016). Showing the importance of IRS for the heart, myocardium-specific double knockout mice of IRS-1 and IRS-2 die due to heart failure within 6-8 weeks after birth (Qi et al., 2013).

1.3.3. Cardiac glucose metabolism

The healthy adult heart consumes primarily fatty acids and only to a minor part glucose. Once glucose enters a cell, it is immediately phosphorylated and eventually stored as glycogen. In contrast to the fetal and neonatal heart, the adult heart has only little glycogen storage (about 30% vs. 2%). The level of glycogen can be increased by hyperinsulinemia, fasting or elevated levels of fatty acids in the blood (Depre et al., 1999). Under stress conditions like ischemia or cardiac hypertrophy, the adult heart increases the consumption of glucose at the expense of fatty acids to sustain the high energy demand needed for continuous contraction (Szablewski, 2017). This is associated with a changed pattern of gene expression, resembling that of the fetal and neonatal heart (Razeghi et al., 2001). In contrast to the adult heart, the fetal and neonatal heart rely mainly on glycolysis as energy source, which correlates with higher proliferation capacity. Soon after birth, the heart undergoes a metabolic switch, increasing

oxidative phosphorylation and reducing glycolysis (Lopaschuk and Jaswal, 2010). This metabolic switch likely occurs because there is suddenly more oxygen available and in addition, many species feed their offspring with milk, which is rich in fat (Onay-Besikci, 2006).

1.3.4. Glucose transporters in the heart

Since glucose cannot cross the plasma membrane, the cells have glucose transporters that allow facilitative diffusion of hydrophilic sugar molecules. In the process of glucose uptake several glucose transporters are involved, namely the GLUT, SGLT (Sodium-glucose linked transporters) and SWEET proteins. The predominant transporters in the heart are GLUT1 and GLUT4, whereas GLUT3, GLUT8, GLUT10, GLUT12 and SGLT1 were detected as well (Aerni-Flessner et al., 2012; Szablewski, 2017).

GLUT1 is encoded by the SLC2A1 gene and the protein consists of 492 amino acid residues that form 12 transmembrane segments located in the plasma membrane. GLUT1 mainly mediates passive diffusion transport of glucose, but also mannose, galactose, glucosamine and reduced vitamin C are physiological substrates. GLUT1 can be inhibited by Cytochalasin B, which binds to the sugar-binding site (Carruthers and Helgerson, 1991). It was shown that GLUT1 is indispensable for proper development, since GLUT1 knockout mice are embryonically lethal (Heilig et al., 2003). In general, GLUT1 is ubiquitously expressed, whereas in the heart, GLUT1 is mainly expressed during development (Mueckler and Thorens, 2013; Shao and Tian, 2015).

GLUT4 is implicated in insulin-stimulated glucose uptake. GLUT4 is structurally very similar to GLUT1, however GLUT4 has a higher affinity for glucose (Km ≈ 4–7 mM vs. Km ≈ 20–26 mM) (Nishimura et al., 1993; Palfreyman et al., 1992). It is highly expressed in insulin-sensitive tissues like skeletal muscle, adipose tissue and the heart. GLUT4 dominates in adult CMs and is primarily found in intracellular vesicles. Upon increased levels of insulin or muscle contraction, GLUT4 vesicles translocate to the plasma membrane and facilitate glucose uptake. A defect of this translocation mechanism paralleled with increased insulin secretion is referred to insulin resistance, leading to T2DM (Mueckler and Thorens, 2013; Shao and Tian, 2015). GLUT4 can be inhibited by human immunodeficiency virus (HIV) protease inhibitors, for example Indinavir (Hresko and Hruz, 2011).

1.3.5. Glucose uptake by GLUT4

Upon insulin stimulation, the InsR is autophosphorylated and turns on its tyrosine kinase activity. The active InsR phosphorylates IRS proteins, which recruit PI3K through the p85 regulatory subunit that triggers activation of the catalytic subunit p110. PI3K induces the formation of PIP3 from phosphatidylinositol (4,5)-bisphosphate (PIP2) that serves as docking platform for Akt. Once Akt has bound it gets phosphorylated at Thr^{308} by PDK1. Active Akt leads to increased phosphorylation of AS160 at Thr^{642} amongst other sites, which inhibits AS160 activity. AS160 is a Rab (Ras-related in brain) guanine triphosphatase (GTPase)-activating protein that inhibits Rab-GTP by converting guanine triphosphate (GTP) to guanine diphosphate (GDP). When AS160 is phosphorylated by Akt, its inhibitory effect is blocked and Rab-GTP can trigger the translocation of GLUT4 storage vesicles (GSV) and fusion with the plasma membrane, enabling glucose uptake (Fig. 6) (Rowland et al., 2011).

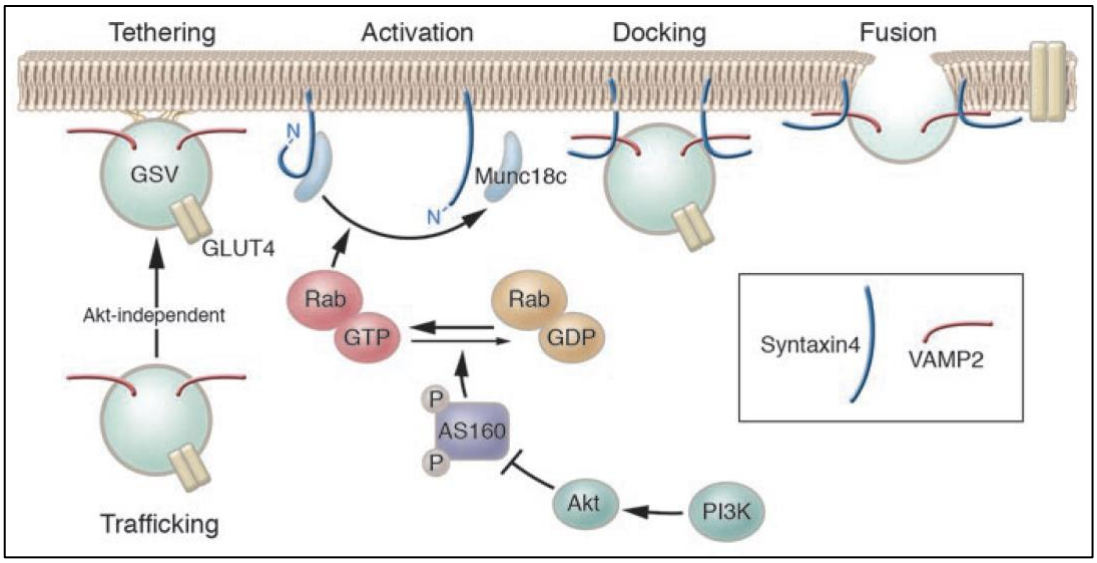

Figure 6. Process of GLUT4 translocation (James D. E., 2005)

1.4. Neuregulin and glucose metabolism

Nrg signaling was shown to play a role in metabolic regulation in skeletal muscle and the liver (Guma et al., 2010). Nrg induces glucose uptake in skeletal muscle cells by triggering translocation of glucose transporters, namely GLUT1, GLUT3 and GLUT4 (Suarez et al., 2001). In 2004, Canto et. al demonstrated that Nrg induces GLUT4 translocation in skeletal muscle

cells in an additive manner to insulin, proposing different regulatory mechanisms of these two stimuli (Canto et al., 2004). In addition, Nrg is also involved in contraction-induced glucose uptake in skeletal muscle. This was demonstrated by blocking the ErbB4 receptor after a caffeine-induced cytosolic calcium (Ca^{2+}) increase, which resulted in reduced glucose uptake. The proposed mechanism involves the interaction of protein kinase ζ (PKCζ) with calmodulin-dependent protein kinase II (CAMKII) triggering GLUT4 translocation (Canto et al., 2006). Furthermore, exposure of skeletal muscle cells to low Nrg concentrations over a long period of time stimulated mitochondrial biogenesis and increased GLUT4 levels (Canto et al., 2007). In contrast, chronic exposure of skeletal muscle cells to Nrg resulted in reduced GLUT4 expression and elevated levels of GLUT1 and GLUT3 (Suarez et al., 2001). Like in skeletal muscle, Nrg also influences glucose consumption of the liver. In diabetic rats and mice, Nrg1 recently was shown to improve the response to the glucose tolerance test (Ennequin et al., 2015; Lopez-Soldado et al., 2016).

1.5. Clinical relevance of Neuregulin1

Worldwide, the number of people suffering from cardio vascular diseases increased tremendously during the last century (Cooper et al., 2000; Sanchis-Gomar et al., 2016). Prolonged lifetime and changes in lifestyle are the main factors that contribute to this observation. Not only in the Western world, but also in the Third world, more and more people nourish from a high-caloric diet and do not practice enough sports anymore, which favors the development of heart disease. Especially in Western countries, this is a severe problem, which causes increasing health costs every year. At the same time, medicine has found ways to prevent the development of heart disease, which is reflected by a decrease in morbidity due to heart failure in the last 50 years. However, the World Health Organization published that cardiovascular diseases remains the leading cause of death worldwide (www.who.int).

A big issue is the limited capacity of the human heart to recover after an injury. In contrast to other species, the human heart cannot replace the damaged cardiac muscle tissue but undergoes scarring. Unfortunately, after the formation of a scar the heart shows reduced contractility, which results in impaired cardiac function (Weber et al., 2013). The prevention

of scarring and the improvement of cardiac function of the stressed heart is an ongoing field of research. People state, that under stress conditions like pathological hypertrophy or ischemia the diseased heart changes its energy substrate from fatty acids to an increased consumption of glucose, resembling the energy profile of a heart during development (Allard, 2004; Opie, 1990; Taegtmeyer et al., 2010).

There are two kinds of cardiac hypertrophies, physiological and pathophysiological hypertrophy. Physiological hypertrophy is an adaptation of the healthy heart due to repetitive exercise and pathological hypertrophy is triggered by heart disease that alter upstream stimuli and signaling mechanisms, which are harmful to the heart and may finally lead to heart failure (Nakamura and Sadoshima, 2018). It was observed that the metabolic profile of both types of cardiac hypertrophies are distinct (Strom et al., 2005). Whereas physiologic cardiac hypertrophy is related to increase β-oxidation of fatty acids, during pathologic cardiac hypertrophy glucose metabolism is increased. As previously mentioned, the healthy adult heart relies mainly on fatty acids and only to a minor part on glucose (Bing, 1965). Interestingly, NRG1 was shown to be upregulated after endurance exercise that leads to physiologic cardiac hypertrophy (Waring et al., 2014). Previously, Nrg1β has been demonstrated to induce hypertrophy in neonatal CMs (Baliga et al., 1999). Moreover, reduced levels of ErbB2 and ErbB4 were associated with the transition from compensatory hypertrophy to heart failure (Rohrbach et al., 1999). Very likely, Nrg1β plays a role in both types of hypertrophic responses of the heart, however further studies are required.

Also during an ischemic period, the heart adapts its energy substrate to increased glucose and reduced fatty acids consumption due to the lack of oxygen necessary for oxidative phosphorylation (Stanley et al., 2005). After an ischemic insult, ErbB3 was demonstrated to contribute to the recovery of the heart (Morano et al., 2017) and it was observed that the myocardial endothelium releases Nrg1 during ischemia-reperfusion (Kuramochi et al., 2004a). In addition, it was shown that administration of Nrg1β to swine with heart failure after myocardial infarction has beneficial effects on cardiac function (Galindo et al., 2014).

Changes in the metabolic profile of the heart were as well observed in T2DM. Well known features of T2DM are hyperglycemia, hyperinsulinemia, increased levels of fatty acids, elevated levels of inflammatory cytokines and changes in molecular pathways in CMs triggered by high-fat diet, inactivity and increased body weight (Bugger and Abel, 2014; Fonseca, 2009; Kota et al., 2011). Patients with T2DM may develop diabetic cardiomyopathy

(DCM), also known as lipotoxic cardiomyopathy, which is characterized by diastolic dysfunction due to reduced glucose metabolism and increased fatty acids consumption that leads to intramyocardial lipid accumulation (Miki et al., 2013; Szczepaniak et al., 2007). In the diabetic heart, the CMs are deprived of glucose and limited to fatty acids as energy source. These factors lead to impaired cardiac contractility, myocytes dysfunction and cell death. Recently, it was shown that intraperitoneal (i.p.) injections of Nrg1β enhances systemic glucose uptake in mice (Ennequin et al., 2015) and Nrg1β was observed to increase glucose uptake in skeletal muscle (Canto et al., 2004). Moreover, Nrg1β reduced CMs stiffening caused by insulin-dependent titin modifications in T2DM (Hopf et al., 2018). Interestingly, in a rat streptozotocin (STZ) model for diabetic cardiomyopathy, reduced phosphorylation of ErbB2 and ErbB4 was detected, indicating impaired Nrg signaling (Gui et al., 2012).

In the last years, several clinical studies were performed, showing that Nrg1β is a promising drug candidate for the treatment of chronic heart failure (Gao et al., 2010; Jabbour et al., 2011b; Lenihan et al., 2016). However, the molecular mechanisms behind these positive effects of Nrg1β are not fully understood.

2. Aims of the thesis

Nrg1β is known to be very important for heart development and it was shown to have beneficial effects on the stressed heart *in vivo* and in clinical trials. However, concrete molecular mechanisms in CMs that are related to these beneficial effects induced by Nrg1β are scarcely known so far. Therefore, we were interested in mechanisms that are induced by Nrg1β in NRVMs, specifically glucose uptake, which may lead to a better performance of the heart under stress conditions. Our findings might contribute to improve the treatment of heart disease, using Nrg1β as drug to ameliorate heart function. The specific aims of our studies were to:

1. *examine the effect of Nrg1*β *on glucose uptake, glycolysis and protein synthesis in NRVMs.* We assess glucose uptake after Nrg1β stimulation by [^{3}H]-deoxy-D-glucose incorporation, estimate the rate of glycolysis with the glycolytic stress test from Seahorse and measure protein synthesis by [^{3}H]-phenylalanine incorporation. Moreover, we analyze the signaling pathways and proteins that are involved in Nrg1β stimulation in NRVMs, focusing on glucose uptake-related signaling and the mTOR pathway and compare the effects of Nrg1β stimulation with insulin and IGF-I.

2. *elucidate the molecular mechanism whereby Nrg1*β *triggers glucose uptake in NRVMs*. We analyze the contribution of GLUT1 and GLUT4 with an siRNA approach and detect GLUT4 translocation after Nrg1β and insulin stimulation by immunofluorescence.

3. *investigate the effect of Nrg1*β *in vivo, using neonatal rats and STZ mice as models.* We treat the animals with Nrg1β and insulin and analyze glucose uptake-related signaling effects in the heart.

4. *clarify if Nrg1*β *influences glucose uptake as well in isolated ARVMs.* We assess glucose uptake by [^{3}H]-deoxy-D-glucose incorporation and analyze signaling effects of Nrg1β and insulin.

3. Results

3.1. Neuregulin-1β promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes

3.1.1. Published article

Am J Physiol Endocrinol Metab 310: E782–E794, 2016.
First published March 15, 2016; doi:10.1152/ajpendo.00259.2015.

Neuregulin-1β promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes

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Submitted 4 June 2015; accepted in final form 2 March 2016

Pentassuglia L, Heim P, Lebboukh S, Morandi C, Xu L, Brink M. Neuregulin-1β promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes. *Am J Physiol Endocrinol Metab* 310: E782–E794, 2016. First published March 15, 2016; doi:10.1152/ajpendo.00259.2015.—Nrg1β is critically involved in cardiac development and also maintains function of the adult heart. Studies conducted in animal models showed that it improves cardiac performance under a range of pathological conditions, which led to its introduction in clinical trials to treat heart failure. Recent work also implicated Nrg1β in the regenerative potential of neonatal and adult hearts. The molecular mechanisms whereby Nrg1β acts in cardiac cells are still poorly understood. In the present study, we analyzed the effects of Nrg1β on glucose uptake in neonatal rat ventricular myocytes and investigated to what extent mTOR/Akt signaling pathways are implicated. We show that Nrg1β enhances glucose uptake in cardiomyocytes as efficiently as IGF-I and insulin. Nrg1β causes phosphorylation of ErbB2 and ErbB4 and rapidly induces the phosphorylation of FAK (Tyr^{861}), Akt (Thr^{308} and Ser^{473}), and its effector AS160 (Thr^{642}). Knockdown of ErbB2 or ErbB4 reduces Akt phosphorylation and blocks the glucose uptake. The Akt inhibitor VIII and the PI3K inhibitors LY-294002 and Byl-719 abolish Nrg1β-induced phosphorylation and glucose uptake. Finally, specific mTORC2 inactivation after knockdown of rictor blocks the Nrg1β-induced increases in Akt-p-Ser^{473} but does not modify AS160-p-Thr^{642} or the glucose uptake responses to Nrg1β. In conclusion, our study demonstrates that Nrg1β enhances glucose uptake in cardiomyocytes via ErbB2/ErbB4 heterodimers, PI3Kα, and Akt. Furthermore, although Nrg1β activates mTORC2, the resulting Akt-Ser^{473} phosphorylation is not essential for glucose uptake induction. These new insights into pathways whereby Nrg1β regulates glucose uptake in cardiomyocytes may contribute to the understanding of its regenerative capacity and protective function in heart failure.

phosphatidylinositol 3-kinase; metabolism; tyrosine kinase; ErbB; signaling; protein synthesis

NEUREGULIN-1β (Nrg1β) and its receptors ErbB2 and ErbB4 are essential for cardiac development and also play a critical role in the healthy and diseased adult heart (45, 49, 50). Cardiomyocyte-specific ablation of ErbB2 (12, 47) or ErbB4 (21) leads to dilated cardiomyopathy with diminished contractility under basal or pressure-overload conditions. Experimental studies in which Nrg1β was administered in various rodent models of cardiac disease confirmed its beneficial action, which together with mechanistic insights obtained in cultured cardiomyocytes (17, 50, 73) led to its use in clinical trials to treat heart failure (19, 43, 58). The first two trials indeed showed a transient improvement of cardiac function (20, 30), whereas the results of several other trials, either with a recombinant human Nrg1β isoform or with the naturally occurring neuronal isoform glial growth factor 2 (GGF2), are to be expected in the near future. Recent work also implicated Nrg1β in the regenerative potential of neonatal and adult hearts (3, 25, 53). Nevertheless, the molecular mechanisms whereby Nrg1β exerts these effects in cardiac cells are still poorly understood.

Upon stimulation of cardiomyocytes by Nrg1β, the ErbB receptors act via the Src/focal adhesion kinase (FAK), the extracellular-regulated kinase (Erk)1 and 2, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways, which have been linked to distinct functions (50). Cardiac developmental and postnatal growth as well as physiological or pathological adaptations of the adult heart are regulated by the serine/threonine kinase mammalian target of rapamycin (mTOR). mTOR modulates cellular processes such as protein synthesis and energy metabolism (10, 37) and has distinct functions depending on whether it is part of mTOR complex (mTORC)1 or mTORC2. In the developing and adult heart mTORC1 activity is associated with protein synthesis and physiological hypertrophy, and mTORC2 may modulate glucose uptake, as demonstrated previously in skeletal muscle (35, 40, 57). Increased glucose uptake is critical for the survival of cardiomyocytes during the acute phase of ischemic injury, when lipid metabolism will become not only insufficient for the energy demands of the heart but will also lead to a significant increase in oxidative stress (3, 46, 72). Nrg1β is cardioprotective during ischemia (38), but its effects on glucose uptake and the involvement of mTOR have not been investigated.

In other contexts, ErbB receptor activity has been related to mTOR signaling. In breast cancer, pathological ErbB2 overexpression is associated with constitutive activation of Akt/mTOR and predicts tumor progression (52, 67, 79), and mTOR inhibitors improve the outcome of ErbB2-positive breast cancer (72). Whereas mTOR inhibition appears to be of therapeutic value in cancer, cardiomyocyte mTORC1 deficiency leads to cardiac dysfunction in mice (62, 77). The observation that recombinant human GGF2 causes phosphorylation of the mTORC1 target 70-kDa ribosomal S6 kinase (p70S6K) in cardiomyocytes (2) and that Nrg1β causes phosphorylation of Akt on the mTORC2 target site Ser^{473} (59, 76) led us to investigate whether and how mTORC1 and mTORC2 mediate one or more of the Nrg/ErbB-related cardioprotective activities.

Our study demonstrates in a model of rat neonatal cardiomyocytes that Nrg/ErbB signaling enhances glucose uptake and protein synthesis. The glucose uptake is mediated by PI3Kα/Akt/AS160. Nrg1β-induced mTORC1 activation plays a small role in the protein synthesis, whereas mTORC2 appears to not be implicated in the glucose uptake.

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MATERIALS AND METHODS

Growth factors and inhibitors. Nrg1β was from R & D Systems, IGF-I was from Genentech, insulin, PP242, and wortmannin were from Sigma, Lapatinib and Dasatinib were from LC Laboratories, and PP2, PP3, LY-294002, Akt inhibitor VIII, U-0126, SB-203580, rapamycin, and PF573228 were from Calbiochem. Byl-719, TGX-221, Cal101, and AS605240 were kind gifts from Matthias P. Wymann, University of Basel.

Antibodies. Antibodies against mTOR-p-Ser^{2448}, mTOR-p-Ser^{2481}, mTOR, Erk1/2-p-Thr^{202}/Tyr^{204}, Akt-p-Thr^{308}, Akt-p-Ser^{473}, Akt, p70S6K1-p-Thr^{389}, p70S6K1, ULK1-p-Ser^{757}, eukaryotic initiation factor 4E-binding protein-1 (4E-BP1)-p-Ser^{65}, 4E-BP1, phosphorylated Akt substrate, AS160, and AS160-p-Thr^{642} were from Cell Signaling Technology. Antibodies against GAPDH, FAK, c-Src, ErbB2-p-Tyr^{1248}, ErbB2, and ErbB4 as well as normal goat IgG were from Santa Cruz Biotechnology. Antibodies against FAK-p-Tyr^{861} and ErbB4-p-Tyr^{1248} were from Abcam. Antibodies against FAK-p-Tyr^{397} were from BD Biosciences. Antibodies against Src-p-Tyr^{215} were from ECM Biosciences, and antibodies to ULK1 were from Sigma.

Primary neonatal cardiomyocyte isolation and transfection. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old rats and transfected with nontarget ErbB2 and ErbB4 siRNA (Dharmacon) at 1 μg/3 × 10^6 cells using cardiomyocyte AMAXA nucleofactor (Lonza), as published previously (26). Two days later, the cells were treated with inhibitors and growth factors after an overnight incubation in serum-free albumin, carnitine, creatine and taurine-enriched medium (ACCT). ACCT medium consisted of 2 g/l albumin, 2 mM L-carnitine, 5 mM creatine, and 5 mM taurine (all from Sigma) in DMEM (Gibco).

Glucose uptake. NRVMs were treated with inhibitors for 30 min, followed by 30 min in the presence of growth factors and another 30 min in the presence of deoxy-D-glucose, 2-[1,2-^{3}H(N)] (Perkin-Elmer) and D-(+)-glucose (1 μCi/ml and 100 μM, respectively) in Krebs-Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 10 mM HEPES, pH = 7.4, and 0.1% BSA). The glucose uptake was stopped by three washes with ice-cold PBS and lysis in NaOH. Part of the lysate was mixed with scintillation liquid, and ^{3}H was measured with a β-counter. A micro BCA protein assay (Thermo Scientific) was performed with the remaining lysate to normalize the counts/min.

Protein synthesis. To analyze the pathways by which Nrg1β stimulates protein synthesis, NRVMs were incubated for 30 min with inhibitors, as indicated in RESULTS, and then stimulated in the presence of 1 μCi/ml [^{3}H]phenylalanine (Amersham Biosciences) for 24 h. Cells were then washed with ice-cold PBS, precipitated with 10% ice-cold trichloroacetic acid for 30 min, washed with glacial EtOH 95%, dried, and lysed in NaOH for 45 min. Part of the lysate was mixed with scintillation liquid for measurement of ^{3}H with a β-counter. A DNA assay with Hoechst (Invitrogen) was performed with the remaining lysate and used to normalize the counts/min.

Protein extraction and Western blot analysis. Total protein was extracted with RIPA buffer [50 mM Tris·HCl, pH = 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 0.1% SDS, 5 mM EDTA, and 0.5% phosphatase inhibitor cocktail 2 and 3 (Sigma)], and 1% protease inhibitor cocktail (Sigma) was separated by SDS-PAGE and transferred to a PVDF membrane (Amersham-GE Healthcare). After incubation with antibodies, the signal was revealed with Super-Signal West Pico Chemiluminescent Substrate (Thermo Scientific), CL-XPosure Film (Thermo Scientific), or the ChemiDoc MP System (Bio-Rad). Blots were quantified with Image Lab (Bio-Rad) and ImageJ (National Institutes of Health).

Immunoprecipitation. All procedures for immunoprecipitation were done at 4°C. Protein lysates (300 μg) were cleared with 25 μl of protein A-sepharose (Amersham-GE Healthcare) and incubated overnight with 2 μg of antibody to AS160 or normal goat IgG. A 50% slurry of protein A-sepharose (40 μl) was added for 4 h, and the beads were then washed five times with RIPA buffer and collected by centrifugation for 3 min at 3,000 rpm. The beads were resuspended in loading buffer and heated at 95°C. Supernatants were loaded on an 8% SDS-PAGE, and proteins transferred to PVDF, and phosphorylated Akt substrate and AS160 were detected as described above.

Isolation of adult mouse ventricular myocytes. Hearts were dissected from C57BL/6 mice, briefly washed in ice-cold Ca^{2+}-free perfusion buffer (135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.33 mM NaH_2PO_4, 10 mM glucose, 10 mM 2,3-butanedione-monoxime, and 5 mM taurine), and cannulated through the aorta for retrograde perfusion. After 5 min of acclimatization at 37°C, hearts were perfused for 7 min with digestion solution, consisting of 5,000 U collagenase (Worthington) and 5.24 U protease (Sigma) in Ca^{2+}-free perfusion buffer. The dissociated myocytes were passed through a 100-μm cell strainer and incubated with increasing concentrations of Ca^{2+} (0, 0.06, 0.24, 0.6, and 1.2 mM) that were obtained by mixing appropriate amounts of transfer *buffer A* (perfusion buffer with 5 mg/ml of BSA) with transfer *buffer B* (137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, 1.2 mM $CaCl_2 \cdot H_2O$, and 5 mM glucose). Cells were seeded on laminin-coated dishes and kept in ACCT medium overnight before treatment.

Statistics. All results are expressed as means ± SE. One-way ANOVA analysis was followed by Sidak's post hoc testing using Prism 6 (GraphPad).

RESULTS

Nrg1β induces phosphorylation of the mTORC1 targets 4E-BP1, S6K, and ULK and the mTORC2 target Akt-p-Ser^{473}. First, we analyzed the temporal pattern of activation of kinases known to be part of the mTOR and Akt signaling pathways using NRVMs. Figure 1*A* shows that at 5 min, Nrg1β treatment caused phosphorylation of both ErbB2 and ErbB4 at Tyr^{1248}. At the same time, the phosphorylation of FAK and Akt was already strongly increased. The phosphorylated amounts of mTOR and the mTORC1 targets 4E-BP1, p70-S6K1, and ULK were increased later at 15 and 30 min. Lapatinib, a well known inhibitor of ErbB1 and ErbB2, blocked the phosphorylation of ErbB2 and ErbB4 as well as that of all downstream effectors (Fig. 1*A*). Moreover, a dose response experiment at 30 min confirmed specificity of the Akt response and established 10 ng/ml as optimal Nrg1β concentration for further experiments (Fig. 1*B*). Nrg1β also activated mTOR signaling in cardiomyocytes isolated from adult mouse hearts, in which increases in mTOR-pS2448 were similar to those obtained with IGF-I, and an upward bandshift was observed for 4E-BP1, indicating increased phosphorylation (Fig. 1*C*). Thus, we conclude that Nrg1β has immediate and specific stimulatory effects on mTOR-mediated signaling cascades in cardiomyocytes.

In Fig. 1*D*, the effects of Nrg1β are compared with those of IGF-I to evaluate the potency of either growth factor to activate mTOR and its effectors over a longer period of time. At 30 min, IGF-I and Nrg1β similarly increased mTOR-p-Ser^{2448}, p70-S6K1-p-Thr^{389}, Akt-p-Ser^{473}, and 4E-BP1 phosphorylation. For Nrg1β, the p70-S6K1-p-Thr^{389} and Akt-p-Ser^{473} signals decreased more rapidly than for IGF-I. Thus, at 3 h the Nrg1β-treated samples already displayed a much lesser increase compared with controls than the IGF-I-treated samples, for which the increase remained very pronounced for at least 6 h. On the other hand, the phosphorylation of 4E-BP1 persisted for up to 24 h for both growth factors. PP242, an mTOR inhibitor that blocks mTORC1 as well as mTORC2, abolished all of the Nrg1β-induced increases in phosphorylation (Fig.

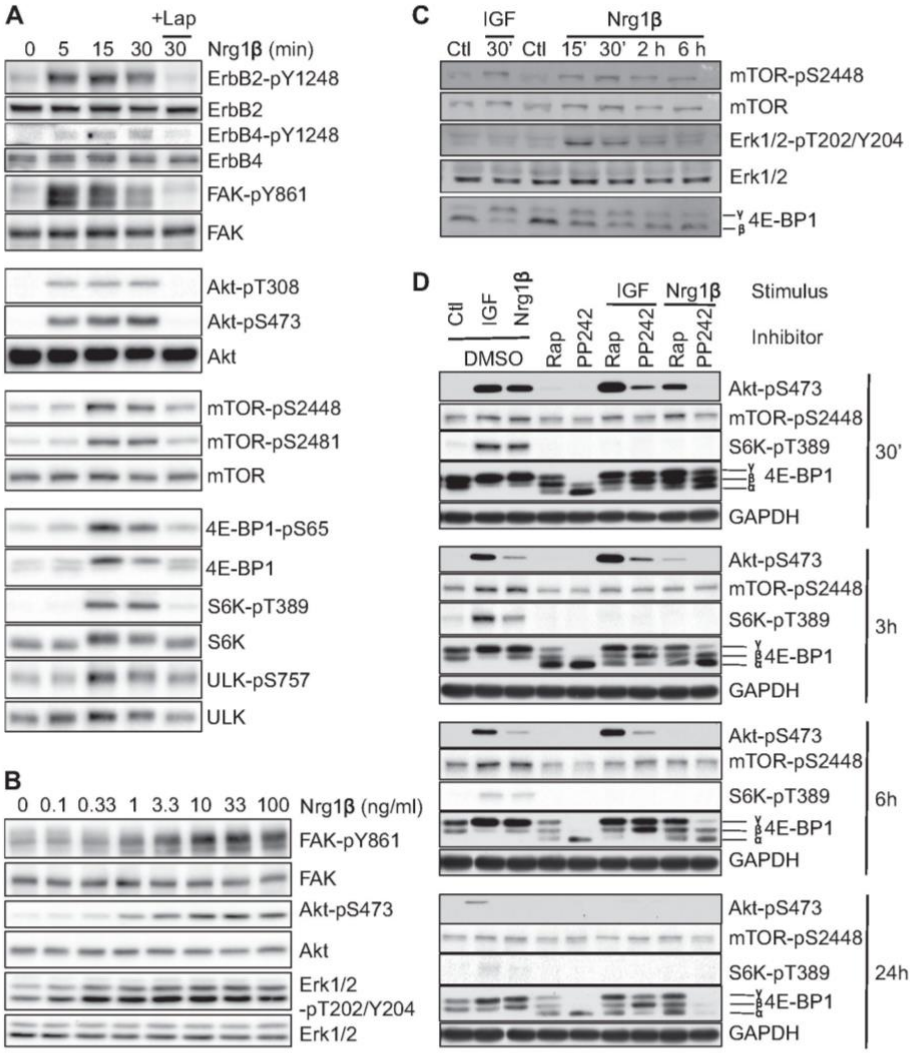

Fig. 1. Neuregulin-1β (Nrg1β) activates mammalian target of rapamycin (mTOR) complex (mTORC)1 and mTORC2 in ventricular myocytes. *A*: after overnight incubation in serum-free albumin, carnitine, creatine, and taurine-enriched medium, neonatal rat ventricular myocytes (NRVMs) were treated with Nrg1β (10 ng/ml) for 5, 15, and 30 min. Cells were pretreated with Lapatinib (Lap; 10 μM) or vehicle (DMSO) for 30 min. *B*: dose response for Nrg1β in NRVMs at 30 min. *C*: adult mouse ventricular myocytes were treated with Nrg1β (10 ng/ml) or IGF-I (20 ng/ml) and lysed for analysis by Western blotting at the time points indicated. *D*: NRVMs were treated with Nrg1β (10 ng/ml) or IGF-I (20 ng/ml) for ≤24 h in the absence or presence of rapamycin (Rap; 20 ng/ml) or PP242 (20 μM), and lysates were analyzed as in *A*. *D*: total proteins (20 μg) were analyzed by Western blotting to test for total and phosphorylated proteins as indicated. FAK, focal adhesion kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; Ctl, control; S6K, p70S6K1.

1*D*). As expected, rapamycin decreased phosphorylation of p70-S6K1 and 4E-BP1 in line with its function as mTORC1 inhibitor in short-term experiments. Interestingly, rapamycin increased IGF-I-induced phosphorylation of the mTORC2 target site Ser^{473} in Akt, consistent with earlier studies (44, 68, 69, 74), whereas it did not modify the Nrg1β-induced phosphorylation of Akt at Ser^{473}. We conclude that Nrg1β/ErbB activates mTORC1-4E-BP1 in a similar manner as IGF-I, whereas it activates p70-S6K1 and mTORC2-Akt more transiently than IGF-I.

Nrg1β stimulates protein synthesis. Given the well-established function of mTORC1, we tested whether Nrg1β stimulates protein synthesis in cardiomyocytes via mTORC1. Incubation with Nrg1β for 24 h increased phenylalanine incorporation in a Lapatinib-sensitive manner (Fig. 2*A*), and rapamycin and PP242 both diminished this effect to a similar

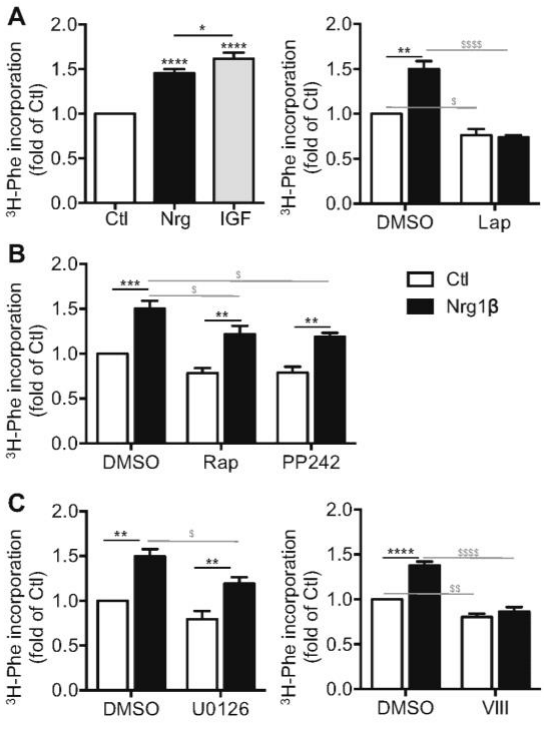

Fig. 2. Nrg1β enhances protein synthesis in part via mTORC1, Erk1/2, and Akt. After preincubation with vehicle (DMSO), Lap (10 μM), Rap (20 ng/ml), PP242 (20 μM), U-0126 (10 μM), or Akt inhibitor VIII (VIII; 20 μM) for 30 min, NRVMs were treated with Nrg1β (10 ng/ml) in the presence of [^{3}H]phenylalanine (1 μCi) for 24 h. The [^{3}H]phenylalanine incorporated into the cells was normalized for DNA content; $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, Nrg-treated vs. Ctl; \$$P < 0.05$, \$\$$P < 0.01$, and \$\$\$\$$P < 0.0001$, inhibitor- vs. corresponding DMSO-treated control.

extent (Fig. 2*B*), which suggests that mTORC1 contributes to the Nrg1β-induced protein synthesis. However, since a strong increase persisted after PP242 or Rap inhibition, mTOR-independent alternate pathways are also important in the Nrg1β-induced phenylalanine uptake. Consistent with previous studies, the Erk1/2 inhibitor U-0126 partially inhibited phenylalanine incorporation (Fig. 2*C*). Moreover, the Akt inhibitor VIII abolished the protein synthesis response, in line with the established role of Akt as upstream activator of mTORC1. We conclude that Akt and Erk1/2, as well as mTORC1 via sustained phosphorylation of 4E-BP1 (Fig. 1*D*), are implicated in the enhanced global protein synthesis after Nrg1β treatment of neonatal cardiomyocytes.

Nrg1β enhances glucose uptake. Given our observation that the effect of PP242 on protein synthesis was only modest, we next aimed at identifying other functions of mTOR in cardiomyocytes. Very little is known about the cardiac function of mTORC2, and therefore, we decided to focus on the function of the Nrg1β-induced phosphorylation of Akt at Ser^{473}, which in Fig. 1*D* was shown to be rapamycin resistant and PP242 sensitive and, therefore, most likely mTORC2 dependent. Akt is implicated in the translocation of glucose transporters to the plasma membrane and thereby contributes to insulin-induced glucose uptake after feeding (34). As mTORC2 has been reported to regulate glucose metabolism in some tissues (35, 40), we hypothesized that mTORC2-mediated phosphorylation of Akt contributes to Nrg1β-induced glucose uptake in our cardiomyocyte model. We tested this hypothesis while using IGF-I and insulin as a reference. The three stimuli induced similar increases in glucose uptake, and combinations of Nrg1β with either IGF-I or insulin did not yield any further increase (Fig. 3*A*), indicating that Nrg1β depends at least in part on the same signaling molecules as IGF-I and insulin. Lapatinib abolished the effect of Nrg1β on glucose uptake (Fig. 3*B*), implicating ErbB2 and/or ErbB4 in the response. Figure 3*C* shows that rapamycin did not have any effect at all, which excludes a role for mTORC1. On the other hand, PP242 preincubation reduced the Nrg1β-induced glucose uptake compared with that of DMSO-preincubated cells (Fig. 3*C*). In fact, PP242 reduced the Nrg1β-induced glucose uptake from 1.77- to 1.56-fold compared with the corresponding unstimulated DMSO- and PP242-preincubated controls, respectively, and therefore, the inhibition was 27% of the total increase. In additional independent experiments, PP242 reduced Nrg1β-, IGF-I-, and insulin-induced glucose uptake from 1.88-, 1.64-, and 1.90- to 1.52-, 1.56-, and 1.52-fold, respectively, vs. the corresponding unstimulated controls. This represents an inhibition of 41, 13, and 42%, respectively, for the three growth factors. Together, our data suggest that common pathways are used by the three growth factors to link receptor activation to glucose uptake and that mTORC2 may mediate part of the insulin- and Nrg1β-induced responses. Additional nonpharmacological approaches are required to prove this further (see below).

The Nrg1β-induced glucose uptake is mediated by Akt and AS160. To further characterize the PP242-sensitive signaling branch of the Nrg1β-induced glucose uptake, we assessed which kinases, in addition to those already shown in Fig. 1, were inhibited by PP242 (Fig. 4). At 30 min, Nrg1β enhanced Akt phosphorylation at Thr^{308} and Ser^{473}, although the effect was somewhat weaker than that observed for IGF-I. PP242 abolished both increases completely for Nrg1β and partially for IGF-I (Fig. 4*A*). Together with the generally accepted view that Akt-p-Ser^{473} is a direct target of mTORC2 and our finding that rapamycin does not reduce Akt-p-Ser^{473} (Fig. 1*D*), this complete inhibition by PP242 supports that mTORC2 is implicated in this Nrg1β-induced Akt phosphorylation in cardiomyocytes. In Fig. 1*A*, we showed that Nrg1β increases Akt-p-Ser^{473} already at 5 min, before mTOR phosphorylation is detectable, and therefore, we tested whether Nrg1β has a rapid, mTOR-independent phase of Akt activation. Figure 4*B* shows that PP242 inhibited the Akt-Ser^{473} phosphorylation at 5 min after Nrg1β stimulation, which indicates that mTORC2 was active and required for this early phosphorylation of Akt. Notably, Akt-p-Thr^{308} was somewhat reduced but not abolished in the PP242-pretreated cells, consistent with the notion that PDK1-mediated Akt-Thr^{308} phosphorylation is possible in the absence of mTORC2-mediated Akt-Ser^{473} phosphorylation. These reduced Akt-p-Thr^{308} amounts may explain the partially reduced glucose uptake response to Nrg1β after PP242 pretreatment. Figure 4*C* shows that the Akt inhibitor VIII abolished the Nrg1β-induced glu-

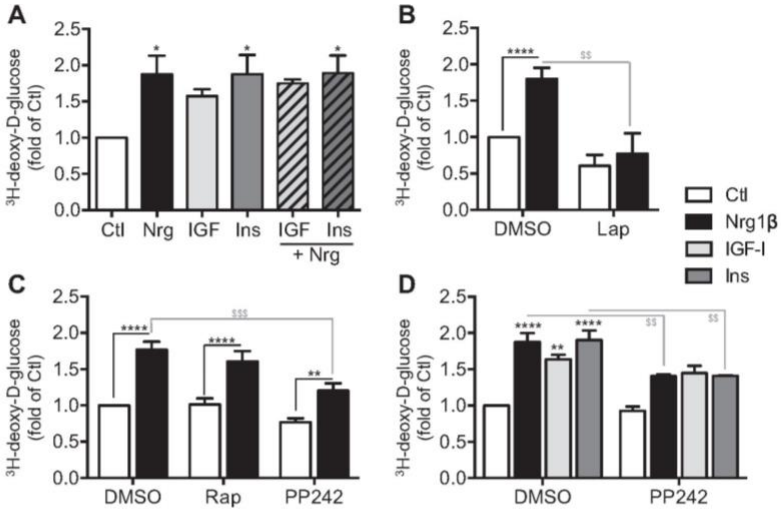

Fig. 3. Nrg1β increases glucose uptake, which is partially inhibited by PP242. *A*: NRVMs were treated for 30 min with Nrg1β (10 ng/ml), IGF-I (20 ng/ml), or insulin (Ins; 20 ng/ml) either alone or in combination as indicated. This was followed by 30 min of incubation with 1 μCi/ml [^{3}H]deoxy-D-glucose. Data were normalized for the total protein/well; $n = 3$. *B–D*: NRVMs were treated with Nrg1β (10 ng/ml) after 30 min of preincubation with vehicle (DMSO), Lap (10 μM), Rap (20 ng/ml), or PP242 (20 μM), and glucose uptake was assessed as in *A*; $n = 6$ (*B*), 7 (*C*), and 3 (*D*). $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ (vs. Ctl); $\$\$\$P < 0.001$ inhibited vs. corresponding DMSO; $\$\$P < 0.01$, inhibitor- vs. corresponding DMSO-treated control.

cose uptake, which indeed supports that Akt is a main actor in this effect.

IGF/insulin-induced Akt activation is known to cause the translocation of glucose transporters to the sarcolemma via its downstream mediator AS160 (56), but whether or not Nrg1β acts via this mechanism in cardiomyocytes is not known. Immunoprecipitation experiments demonstrated that Nrg1β raised the phosphorylation of Akt substrate AS160 in a manner similar to IGF-I (Fig. 4*D*). Moreover, an antibody to AS160-p-Thr^{642} confirmed its phosphorylation after Nrg1β stimulation (Fig. 4*E*), which is consistent with the known function of this specific phosphorylation site being indicative of enhanced glucose transporter type 4 (GLUT4) translocation to the sarcolemma. Together, these data support that Nrg1β induces glucose uptake in NRVMs via Akt and AS160 and suggest that mTORC2-mediated Akt-Ser^{473} phosphorylation may contribute to this response.

Nrg1β-induced mTORC2-mediated Akt phosphorylation at Ser^{473} is not required for the glucose uptake response. To further investigate the role of mTORC2, we knocked down its specific and essential component rictor using siRNA technologies. Figure 4*F* shows that reduced rictor protein levels were associated with lower Akt-p-Ser^{473}, confirming that mTORC2 activity was efficiently reduced. However, this was not associated with lower AS160-p-Thr^{642} (Fig. 4*F*), and consistently, glucose uptake responses to Nrg1β were not impaired (Fig. 4*G*). Together with our observations that PP242 only modestly and Akt inhibitor VIII completely inhibited the glucose uptake, these data indicate that mTORC2-mediated Akt phosphorylation is not essential for this response to Nrg1β. Thus, whereas our data demonstrate that Nrg1β activates mTORC2 and stimulates glucose uptake, the mTORC2-mediated Akt-p-Ser^{473} phosphorylation appears not to modulate this glucose uptake.

Upstream of Akt, PI3K is implicated in the Nrg1β-induced glucose uptake. We next aimed at identifying other upstream signaling molecules that mediate the Nrg1β-induced Akt activation and glucose uptake. The class 1 PI3K inhibitor LY-294002 (LY) abolished the Nrg1β-induced glucose uptake (Fig. 5*A*), whereas effects of the p38 inhibitor SB-203580 (SB) and the Erk1/2 inhibitor U-0126 did not reach significance (Fig. 5*B*). Consistently, LY blocked whereas SB only had small effects on Akt phosphorylation (Fig. 5*E*). Others have demonstrated that SB decreases glucose uptake independently of p38 MAPK inhibition (1). Therefore, PI3K is most likely the main actor upstream of Akt in the Nrg1β-induced glucose uptake. LY has been reported to also inhibit other kinases, including mTORC1, at concentrations similar to those that inhibit PI3K (13). Additional experiments with the isoform-specific PI3K inhibitor Byl-719 revealed that Nrg1β activates glucose uptake via PI3Kα (Fig. 5*C*), and this was associated with lower Akt (Thr^{308} and Ser^{473}) and Akt substrate (160 kDa) phosphorylation (Fig. 5*D*), which suggests that PI3Kα is activating both PDK1 and mTORC2. Consistently, inhibition of PI3Kβ, -δ, and -γ with TGX-221, Cal101, and AS605240, respectively, had no effect (data not shown).

Effect of Src family kinase inhibitors on the Nrg1β-induced glucose uptake. How does Nrg1β-induced ErbB2/ErbB4 phosphorylation lead to PI3K activation? Given our observation that the Nrg1β-induced Akt phosphorylation was paralleled over time by increased FAK-p-Tyr^{861} (Fig. 1*A*), we tested whether Src family kinases are implicated, because FAK is one of their direct targets (7, 61). PP2, a compound that inhibits Src family protein kinases such as c-Src, reduced the Nrg1β-induced glucose uptake (Fig. 6*A*) as well as phosphorylation of FAK at Tyr^{861} and Akt at Thr^{308} and Ser^{473} (Figs. 5*E* and 6*E*), whereas it had no effect on p70-S6K1-p-Thr^{389} (Fig. 5*E*). None of the effects was observed with PP3, a negative control for PP2. Recent studies reported that PP2 may inhibit other kinases with similar affinities and that it is less Src selective than the Src family kinase inhibitor dasatinib (Das) (4). Therefore, we also tested Das and found that it efficiently reduced FAK-p- Tyr^{861}, Akt at both tested

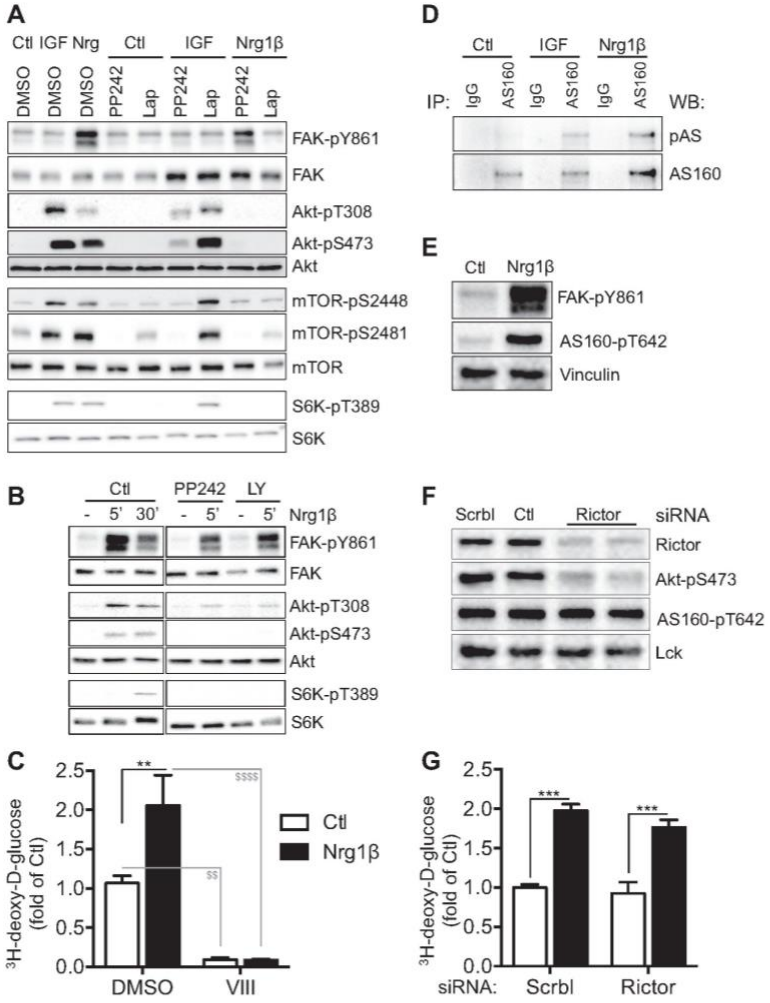

Fig. 4. The Nrg1β-induced glucose uptake is mediated by ErbB2, phosphatidylinositol 3-kinase (PI3K), and Akt. *A*: NRVMs were treated for 30 min with Nrg1β, IGF-I, or vehicle (Ctl) after 30 min of pretreatment with DMSO, PP242 (20 μM), or Lap (10 μM). Western blot (WB) analysis was performed to assess the phosphorylation status of the kinases indicated. *B*: as in *A*, LY-294002 (LY; 10 μM) was used during the preincubation, and Nrg1β treatment was done for 5 and 30 min. Images shown are cut from the same Western blot. *C*: after 30 min of pretreatment with DMSO or Akt inhibitor VIII (20 μM), NRVMs were treated for 30 min with Nrg1β or vehicle (Ctl), followed by 30 min of 1 μCi/ml [^{3}H]deoxy-D-glucose; $n = 4$. *D*: NRVMs were treated with IGF-I or Nrg1β for 30 min, and total protein extracts were immunoprecipitated (IP) for Akt substrate of 160 kDa (AS160). WBs of the precipitated proteins were analyzed with antibodies to phosphorylated Akt substrate (pAS) and AS160. *E*: after Nrg1β stimulation, total protein extracts were analyzed with a phosphospecific antibody to AS160-p-Thr^{642}. *F* and *G*: cardiomyocytes were transfected with small-interfering RNA (siRNA) to rictor immediately after their isolation using AMAXA. Stimulation with Nrg1β and protein extraction (*F*) or glucose uptake assays (*G*) followed 2 days after the transfection. $^{**}P < 0.01$ and $^{***}P < 0.001$, Nrg1β vs. Ctl; $\$\$P < 0.01$ and $\$\$\$P < 0.0001$, inhibitor vs. corresponding DMSO. Lck, lymphocyte-specific protein tyrosine kinase; Scrbl, scrambled.

sites (Figs. 5*E* and 6*E*), and the 160-kDa Akt substrate (not shown). A dose response experiment demonstrated that strong inhibition was obtained already at 100 ng/ml and was complete at 1 μg/ml (data not shown). Notably, Das potently reduced the Nrg1β- but not the IGF-I-induced glucose uptake (Fig. 6*B*), indicating that Das sensitivity is a unique feature of Nrg1β-induced ErbB signaling. Similarly, only Nrg1β led to increased phosphorylation of FAK at Tyr^{861} (Fig. 6*C*). In an attempt to further identify the implicated kinase, we analyzed over time the phosphorylation of Src at Tyr^{215} and Tyr^{416}, the latter site being indicative of c-Src activation. Src-p-Tyr^{215} increased at 30 min, which is much later than FAK-p- Tyr^{861}, Akt, and Akt substrate phosphorylation, excluding a role of this site in the rapid activation of Akt (Fig. 6*D*). Src-p-Tyr^{416} was already high under basal conditions and did not increase any further after Nrg1β stimulation. Das but not PP2 inhibited phosphorylation of this site (Fig. 6*E*), whereas both inhibitors negatively affected Nrg1β-induced Akt activation and glucose uptake (Fig. 6, *A* and *B*), which altogether excludes a causative role of c-Src. In conclusion, our findings indicate that one or more members of the Src kinase family (but not c-Src) are involved in the phosphorylation of FAK at Tyr^{861} and in the glucose uptake response to Nrg1β, although a causal relationship between FAK-p861 and glucose uptake and the precise mechanism involved remain to be established.

Nrg1β/ErbB-induced glucose uptake does not depend on integrin/FAK397. Src family kinases have been implicated in integrin signaling, and recent data suggest that integrin activation may promote glucose uptake (29). Ligand-induced integrin activation involves, as a first step, autophosphorylation of FAK at Tyr^{397}, which induces a conformational change and exposes a binding motif for SH2-domain-

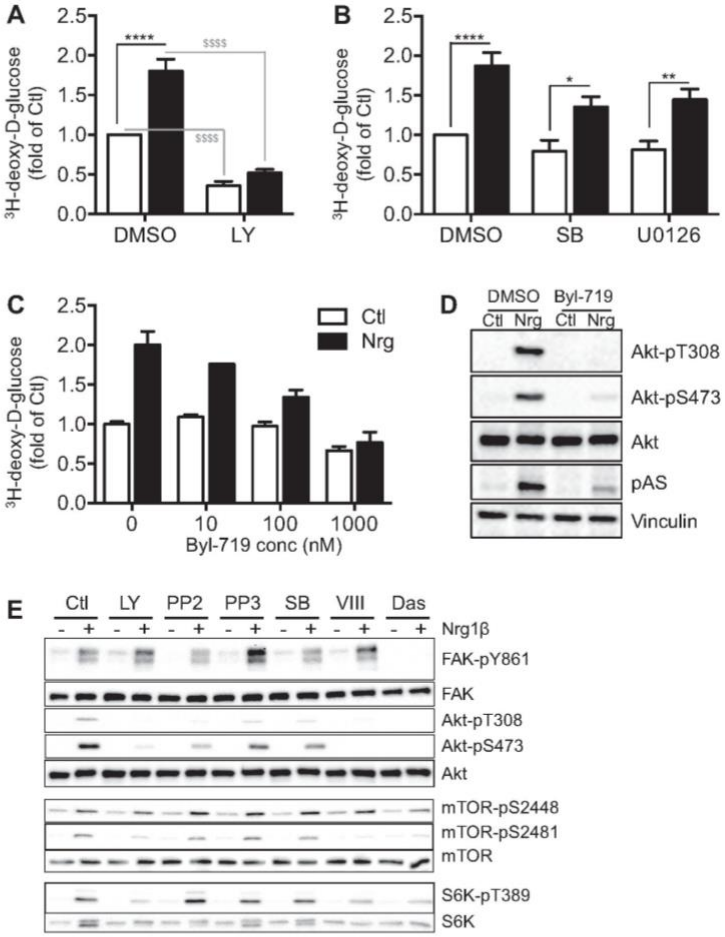

Fig. 5. Upstream of Akt, PI3Kα is implicated in the Nrg1β-induced glucose uptake. *A–D*: NRVMs were pretreated with the PI3K inhibitor LY (10 μM), the p38 inhibitor SB-203580 (SB; 10 μM), and the PI3Kα inhibitor Byl-719 (1 μM; *D*) for 30 min. NRVMs were then stimulated with vehicle (Ctl) or Nrg1β (10 ng/ml) for 30 min, and glucose uptake was assessed as in Figs. 3 and 4. *E*: inhibitors were used and cells stimulated as in *A–D* and Fig. 6. Protein extracts were analyzed by Western blotting as in Fig. 1. $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$ (vs. Ctl); $\$\$\$\$P < 0.0001$, inhibitor vs. DMSO.

containing Src-family kinases. After binding, these kinases further phosphorylate FAK at multiple sites and thereby activate it (60). To investigate whether the Nrg1β-induced glucose uptake requires signaling via integrin/FAK-p-Tyr^{397}, we used the potent FAK inhibitor PF-573228 (PF). Figure 7*A* shows that Nrg1β increased FAK-p-Tyr^{861} but did not change FAK-p-Tyr^{397} compared with untreated controls. Consistent with an earlier report (33), PF had no effect on the Nrg1β-stimulated FAK-p-Tyr^{861}, but it effectively reduced FAK-p-Tyr^{397}, Src-p-Tyr^{416}, and phosphorylation of the integrin-FAK target paxillin. Nevertheless, PF neither inhibited Nrg1β-induced mTOR and Akt signaling, as phosphorylation of mTOR and its targets Akt (Ser^{473}) and p70S6K remained high (Fig. 7*A*), nor did it affect the glucose uptake response (Fig. 7*B*), from which we conclude that these effects are integrin/FAK-p-Tyr^{397}/c-Src-independent. The data also indicate the presence of high basal integrin/FAK/Src activity in our model.

Specific downregulation of ErbB2 and ErbB4 with siRNA. Lapatinib blocked the glucose uptake and all Nrg1β-induced signaling, but as it reduced phosphorylation of both ErbB2 and ErbB4 it did not distinguish between the two receptors (Fig. 1). To define the specific role of ErbB2 and ErbB4 in the Nrg1β-induced pathways that enhance glucose uptake, we used pools of siRNA specific for ErbB2 and ErbB4. Both targeted proteins were effectively downregulated, whereas GAPDH and vinculin were not altered (Fig. 8). Knockdown of ErbB4 resulted in reduced Nrg1β-stimulated levels of FAK-p-Tyr^{861}, Akt-p-Ser^{473}, Akt-p-Thr^{308}, mTOR-p-Ser^{2448}, ULK-p-Ser^{757}, and p70-S6K1-p-Thr^{389}. ErbB2 knockdown, on the other hand, only reduced Akt-p-Ser^{473} and Akt-p-Thr^{308} (Fig. 8). Both the ErbB2 and ErbB4 siRNA knockdown abolished the Nrg1β-induced glucose uptake, showing that both ErbB2 and ErbB4 are needed for this response. The signaling data suggest that ErbB4 is implicated in the Nrg1β-induced activation of mTORC1 as well as mTORC2. ErbB2 is implicated in mTORC2 but does not seem to be required for mTORC1 activation by Nrg1β. However, as our knockdown approach was more efficient for ErbB4 than for ErbB2, coimmunoprecipitation and com-

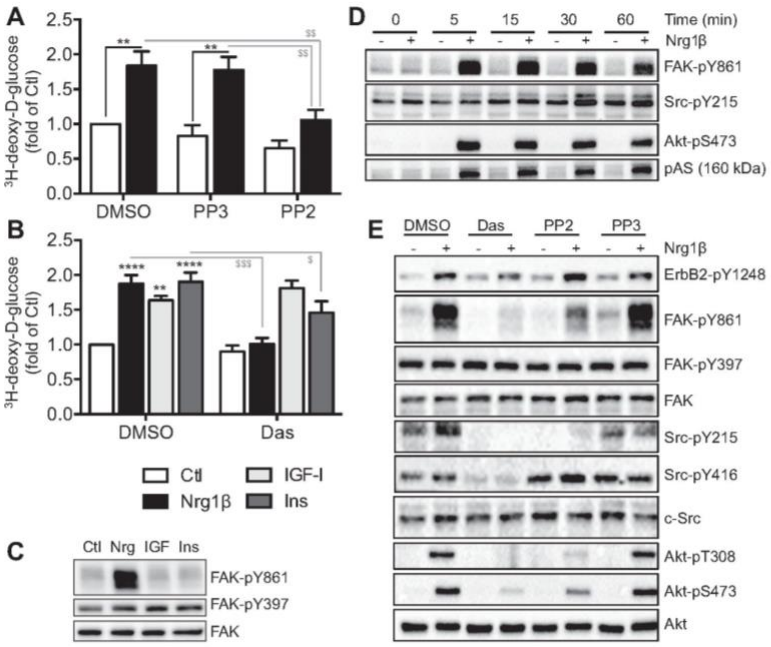

Fig. 6. Effect of Src family kinase inhibitors on Nrg1β-induced signaling and glucose uptake. *A* and *B*: NRVMs were pretreated with the inhibitor PP2 (5 μM) or dasatinib (Das; 1 μM) for 30 min. PP3 (5 μM) was used as negative control for PP2. NRVMs were then stimulated with vehicle (Ctl) or Nrg1β (10 ng/ml) or IGF-I (20 ng/ml) for 30 min, and glucose uptake was assessed as in Figs. 3–5. *C–E*: protein extracts were analyzed by Western blotting as in Fig. 1. $^{**}P < 0.01$ and $^{****}P < 0.0001$ vs. Ctl; $\$P < 0.05$, $\$\$P < 0.01$, and $\$\$\$P < 0.001$, inhibitor vs. corresponding DMSO.

plete knockout approaches are required to further investigate receptor involvement. Our data are in line with the notion that ErbB4 is the main receptor for Nrg1β, whereas ErbB2 has no extracellular binding pocket for Nrg1β but transduces specific signals when it is part of a heterodimer complex with one of the other ErbB isoforms.

DISCUSSION

Under stress conditions, cardiac microvascular endothelial cells release Nrg1β, which in a paracrine manner activates the receptor dimers ErbB2/ErbB4 and ErbB4/ErbB4, both of which are expressed in cardiomyocytes (11, 78). Upon stimulation, the ErbB receptors may act via the Erk1/2, PI3K/Akt, and Src/FAK pathways, each of which has been linked to distinct protective functions. For example, Nrg1β diminishes doxorubicin-induced sarcomere disarray in cardiomyocytes via Erk (48, 51, 59), whereas PI3K/Akt activation is responsible for the protection against basal or anthracycline-induced apoptosis (16, 22, 78) in part by reducing oxidative stress and improving mitochondrial function, calcium handling, and contractility (22, 23, 66). Independently of PI3K/Akt and Erk1/2, Nrg1β influences focal adhesion formation via Src/FAK (36).

Consistent with previous studies (2, 11, 78), we show here that Nrg1β increases global protein synthesis in neonatal cardiomyocytes. Whereas early studies implicated ErbB2 and Erk1/2 in the protein synthesis response to GGF2, here we extend the mechanistic insights by demonstrating how Nrg1β activates mTORC1 over time and by providing data that suggest its involvement in protein synthesis. However, it should be mentioned here that a large part of the Nrg1β-induced protein synthesis was not inhibitable by the mTOR inhibitor PP242, indicating pathway redundancy. Nevertheless, we show that Nrg1β increases mTOR-p-Ser^{2448} (a site that indicates mTORC1 activity) and the phosphorylated levels of two direct mTORC1 targets that regulate protein synthesis, namely p70-S6K and 4E-BP1. The Nrg1β-induced increase in 4E-BP1 phosphorylation lasted as long as that induced by IGF-I, which may explain the similar potency of the two factors to enhance protein synthesis. In contrast to IGF-I, Nrg1β only transiently affects p70-S6K phosphorylation, and rapamycin does not lead to hyperphosphorylation of Akt. These findings suggest that Nrg1β activates mTORC1 and protein synthesis without inducing the negative feedback loop that is perceived as one of the causes of insulin resistance.

Our study also demonstrates that Nrg1β increases glucose uptake. Nrg1β was reported to enhance glucose uptake in cardiomyocytes only in one earlier study, in which it was shown that the endothelium generates both Nrg1α and Nrg1β, but that only Nrg1β causes ErbB2 tyrosine phosphorylation with functional consequences such as increased glucose uptake (11). Whereas that study did not analyze the underlying pathways, our study now shows that Nrg1β increases glucose uptake via ErbB2/ErbB4 heterodimers and enhanced signaling via PI3Kα, Akt, and AS160. Given the well-established function of Akt/AS160, our data suggest that the increase in glucose uptake after Nrg1β stimulation is a consequence of GLUT4 translocation to the sarcolemma (56). Thus, whereas others have linked Akt activation by Nrg1β to pro-survival pathways (16, 22, 23, 66, 78), here we provide evidence that Nrg1β also triggers glucose uptake via this kinase.

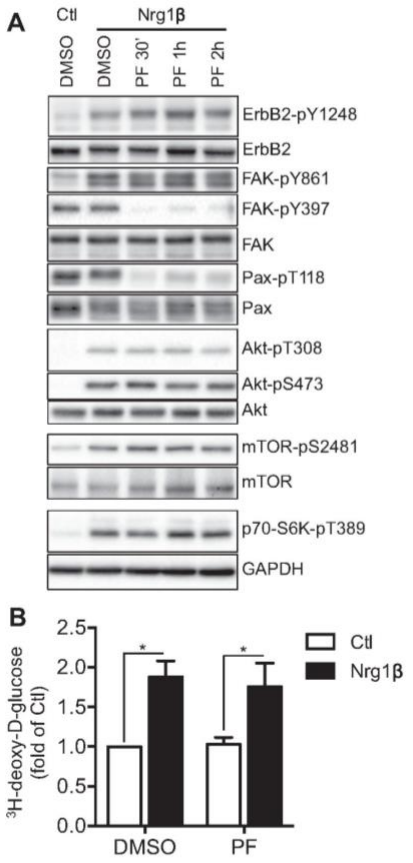

Fig. 7. FAK-p-Tyr^{397} is not implicated in the glucose uptake response to Nrg1β. *A*: NRVMs were pretreated with FAK inhibitor PF-573228 (PF; 1 μM), followed by Nrg1β treatment for 30 min. *A*: WB analysis was performed for FAK and mTOR signaling. PF preincubation was done for 30 min, 1 h, and 2 h; the DMSO control matched the 30-min time point. *B*: glucose uptake was performed as in Fig. 3 to assess the influence of integrin/FAK-p-Tyr^{397} inhibition on the Nrg1β-stimulated glucose uptake; $n = 4$. *$P < 0.05$ vs. Ctl.

This novel ErbB2/ErbB4-specific mechanism of glucose uptake may be particularly important under conditions of acute stress such as ischemic events, when the heart has to rapidly respond to maintain performance and survival of contractile cells. Whereas fatty acid and glucose oxidation are tightly regulated in the healthy heart to optimally provide it with the high amounts of energy needed for contraction, substrate use for ATP production changes under hypoxic conditions associated with, e.g., ischemia or hypertrophy. A shift from predominant fatty acid oxidation to increased carbohydrate use and glycolysis ensures continued ATP production under conditions of oxygen deficiency (31, 39). Ischemia has previously been associated with the translocation of glucose transporters to the sarcolemma (5, 46, 75). At the early stages of ischemia, the glucose allows a better adaptation and survival (5). In support of this concept, cardiac deletion of GLUT4 leads to a lower tolerance to ischemic events associated with a higher rate of ATP depletion (65). Indeed, ischemic insults acutely caused the rapid release of Nrg1β from microendothelial cells and phosphorylation of ErbB4 (15, 36), and Nrg1β ablation in endothelial cells aggravated the harmful consequences of ischemia (38), whereas intravenous injections of the EGF-like domain of Nrg1β or GGF2 improved ventricular function in rat and swine models of myocardial infarction (18, 27, 41). Our present findings suggest that an increase in glucose uptake is one of the protective mechanisms induced by Nrg1β in these conditions.

The ability of Nrg1β to stimulate glucose uptake has been reported previously for a skeletal muscle cell line (8, 64), and consistent with those in vitro studies, acute infusion of GGF2 or Nrg1β was recently shown to lower blood glucose in swine (18), rats (6), and a mouse model of type 2 diabetes (14). Whereas in L6E9 myotubes PKCζ was implicated in the glucose uptake response, here we show that in cardiomyocytes Akt activation is implicated, because Nrg1β increases AS160 phosphorylation, and the Akt inhibitor VIII abolishes this as well as the glucose uptake response. Another new finding of our study is that Nrg1β activates mTORC2 because it phosphorylated the mTORC2-specific site Ser^{473} of Akt in a PP242-sensitive manner. However, consistent with our recently published in vivo data in adult mice (63), Akt-p-Ser^{473} phosphorylation appears to not be needed for phosphorylation of substrates involved in glucose uptake. This conclusion is supported by our observation that rictor knockdown efficiently reduces Akt-p-Ser^{473} without having any effects on AS160 phosphorylation and glucose uptake. Thus, whereas a role has previously been attributed to mTORC2 in skeletal muscle (35, 40, 57), fat, and liver (24), our own data demonstrate that mTORC2 is not implicated in glucose uptake of the heart. In this setting, it should be mentioned that PI3K was recently reported to directly phosphorylate Akt at Ser^{473} and induce glucose transporter translocation (70), and thus mTORC2-dependent Ser^{473} phosphorylation of Akt appears to be dispensable in this pathway.

To the best of our knowledge, our study is the first to identify Nrg1β as an activator of PI3K/Akt-mediated glucose uptake in cardiac cells. We show unique features of this Nrg1β-induced pathway, such as its Das sensitivity, which was not observed in IGF-I-stimulated cells. Consistently, Nrg1β, but not IGF-I or insulin, induced phosphorylation of FAK at Tyr^{861}. LY did not block FAK phosphorylation, whereas Das and PP2 blocked phosphorylation of FAK and Akt as well as the glucose uptake response, suggesting that Src family kinases and FAK may be implicated either upstream of or in parallel to PI3K, consistent with previous studies in the heart (9) and noncardiac cells (42). Whether or not Src-dependent FAK-Tyr^{861} phosphorylation is implicated in transmitting the signal from ErbB to PI3K remains to be proven. Others have demonstrated that ErbB activation may also directly stimulate PI3K in cardiomyocytes (70).

Our observation that Nrg1β rapidly increases FAK-p- Tyr^{861} is consistent with a previous study on adult cardiomyocytes (36). In contrast to that study, phosphorylation of c-Src at Tyr^{416} was high in our unstimulated NRVMs, and Nrg1β did not increase this further. Das decreased c-Src-p-Tyr^{416}, suggesting that it was already active under basal conditions in our

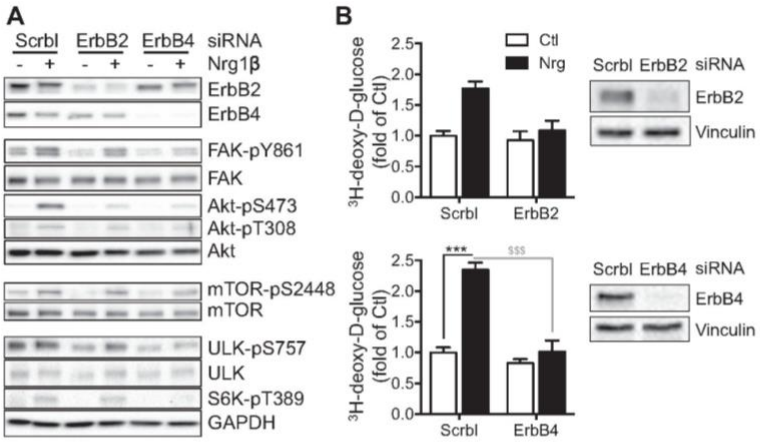

Fig. 8. The Nrg1β-stimulated glucose uptake is mediated by the ErbB2/ErbB4 heterodimer. *A*: ErbB2 and ErbB4 receptors were down-regulated with pools of specific siRNAs. WB analysis was performed to assess the efficiency of the ErbB2 and ErbB4 knockdown as well as the effects on mTORC1 and mTORC2 targets. *B*: glucose uptake was measured as in Fig. 3 at 2 days after the siRNA transfection. ***$P < 0.001$, Nrg-treated vs. control; $$$$P < 0.001$, inhibitor- vs. corresponding DMSO-treated control.

model. On the other hand and consistent with Kuramochi et al. (36), Nrg1β increased the signal detected with an antibody to Src-p-Tyr^{215}. PP2 and Das, but not PP3, blocked this increase as well as the glucose uptake response. However, our observation that this phosphorylation happens later than the Akt phosphorylation excludes its role in the specific activation by ErbB2/ErbB4. We conclude that the presence of a PP2/Das-sensitive kinase, but most likely not c-Src, is important for the phosphorylation of FAK at Tyr^{861} and the glucose uptake response to Nrg1β, although the causal relationship between FAK-p-Tyr^{861} and Akt remains to be proven.

Interestingly, we found that phosphorylation of FAK at Tyr^{861} by Nrg1β does not depend on FAK phosphorylation at Tyr^{397}, a site that autophosphorylates upon integrin stimulation and leads to additional phosphorylation events after Src family kinase recruitment to SH2 domains. Our conclusion is supported by the observation that PF reduces integrin-related FAK-p-Tyr^{397} and paxillin-p-Tyr^{118} but not the Nrg1β-induced phosphorylation of FAK at Tyr^{861}. Differential inhibition by PF of these two phosphorylation events has also been reported for lung and breast cancer cells (28, 33). Thus, Nrg1β induces FAK phosphorylation independently of the classical integrin pathway.

A limitation of our own as well as other studies with different cell types and hormonal stimuli (32, 54, 71) is that pharmacological inhibition was used to implicate Src family kinases in glucose uptake responses. Since PP2 and Das, besides inhibiting multiple Src family kinases, have recently been described to have off-target effects, further studies are needed to determine which kinases inhibited by PP2 and Das are responsible for FAK-Tyr^{861} phosphorylation and the glucose uptake response to Nrg1β. It also remains possible that although the time course and PP2/Das sensitivity of FAK-Tyr^{861} phosphorylation parallels that of Akt and AS160 phosphorylation, FAK is not causally implicated in the glucose uptake. Moreover, it is also possible that ErbB directly activates PI3K (55).

Taken together, we show that Nrg1β enhances glucose uptake via ErbB2/ErbB4, PI3Kα, Akt, and AS160 (Fig. 9) and that these effects are sensitive to PP2 and Das. These novel insights provide a basis for future experimental and clinical studies in which this pathway may be exploited to increase glucose uptake, especially in states of irresponsiveness to insulin.

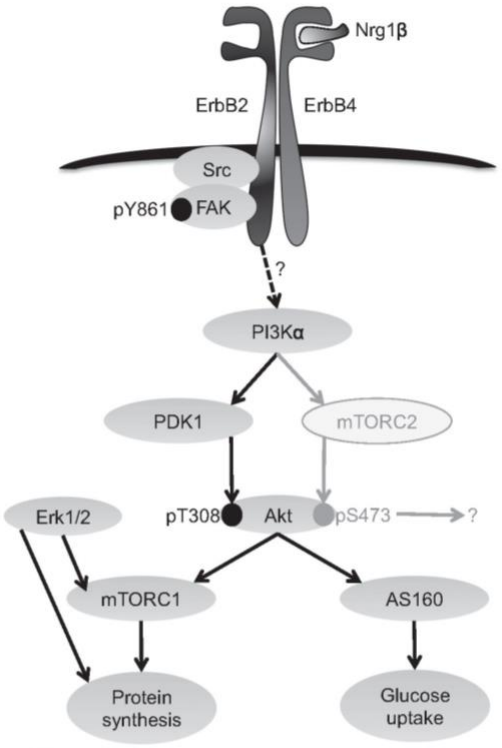

Fig. 9. Working model for the pathways that link Nrg1β to glucose uptake in cardiomyocytes.

ACKNOWLEDGMENTS

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GRANTS

This work was supported by Swiss National Science Foundation Grant No. 31-135559/1, the "Stiftung für kardiovaskuläre Forschung Basel," and a career grant from the University of Basel to L. Pentassuglia.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.P., P.H., and M.B. conception and design of research; L.P., P.H., S.L., C.M., and L.X. performed experiments; L.P., P.H., C.M., and M.B. analyzed data; L.P., P.H., and M.B. interpreted results of experiments; L.P., P.H., and M.B. prepared figures; L.P. drafted manuscript; L.P., P.H., S.L., C.M., L.X., and M.B. edited and revised manuscript; L.P., P.H., S.L., C.M., L.X., and M.B. approved final version of manuscript.

REFERENCES

1. **Antonescu CN, Huang C, Niu W, Liu Z, Eyers PA, Heidenreich KA, Bilan PJ, Klip A.** Reduction of insulin-stimulated glucose uptake in L6 myotubes by the protein kinase inhibitor SB203580 is independent of p38MAPK activity. *Endocrinology* 146: 3773–3781, 2005.
2. **Baliga RR, Pimental DR, Zhao YY, Simmons WW, Marchionni MA, Sawyer DB, Kelly RA.** NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, $p70^{S6K}$, and MEK-MAPK-RSK. *Am J Physiol Heart Circ Physiol* 277: H2026–H2037, 1999.
3. **Bersell K, Arab S, Haring B, Kuhn B.** Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 138: 257–270, 2009.
4. **Brandvold KR, Steffey ME, Fox CC, Soellner MB.** Development of a highly selective c-Src kinase inhibitor. *ACS Chem Biol* 7: 1393–1398, 2012.
5. **Broderick TL, Quinney HA, Barker CC, Lopaschuk GD.** Beneficial effect of carnitine on mechanical recovery of rat hearts reperfused after a transient period of global ischemia is accompanied by a stimulation of glucose oxidation. *Circulation* 87: 972–981, 1993.
6. **Caillaud K, Boisseau N, Ennequin G, Chavanelle V, Etienne M, Li X, Denis P, Dardevet D, Lacampagne A, Sirvent P.** Neuregulin 1 improves glucose tolerance in adult and old rats. *Diabetes Metab*. In press.
7. **Calalb MB, Zhang X, Polte TR, Hanks SK.** Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem Biophys Res Commun* 228: 662–668, 1996.
8. **Canto C, Suarez E, Lizcano JM, Grino E, Shepherd PR, Fryer LG, Carling D, Bertran J, Palacin M, Zorzano A, Guma A.** Neuregulin signaling on glucose transport in muscle cells. *J Biol Chem* 279: 12260–12268, 2004.
9. **Clemente CF, Xavier-Neto J, Dalla Costa AP, Consonni SR, Antunes JE, Rocco SA, Pereira MB, Judice CC, Strauss B, Joazeiro PP, Matos-Souza JR, Franchini KG.** Focal adhesion kinase governs cardiac concentric hypertrophic growth by activating the AKT and mTOR pathways. *J Mol Cell Cardiol* 52: 493–501, 2012.
10. **Cornu M, Albert V, Hall MN.** mTOR in aging, metabolism, and cancer. *Curr Opin Genet Dev* 23: 53–62, 2013.
11. **Cote GM, Miller TA, Lebrasseur NK, Kuramochi Y, Sawyer DB.** Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. *Exp Cell Res* 311: 135–146, 2005.
12. **Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J Jr, Chien KR, Lee KF.** ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med* 8: 459–465, 2002.
13. **Davies SP, Reddy H, Caivano M, Cohen P.** Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351: 95–105, 2000.
14. **Ennequin G, Boisseau N, Caillaud K, Chavanelle V, Etienne M, Li X, Sirvent P.** Neuregulin 1 Improves Glucose Tolerance in db/db Mice. *PLoS One* 10: e0130568, 2015.
15. **Fang SJ, Wu XS, Han ZH, Zhang XX, Wang CM, Li XY, Lu LQ, Zhang JL.** Neuregulin-1 preconditioning protects the heart against ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism. *Chin Med J (Engl)* 123: 3597–3604, 2010.
16. **Fukazawa R, Miller TA, Kuramochi Y, Frantz S, Kim YD, Marchionni MA, Kelly RA, Sawyer DB.** Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of PI3-kinase/Akt. *J Mol Cell Cardiol* 35: 1473–1479, 2003.
17. **Fuller SJ, Sivarajah K, Sugden PH.** ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. *J Mol Cell Cardiol* 44: 831–854, 2008.
18. **Galindo CL, Kasasbeh E, Murphy A, Ryzhov S, Lenihan S, Ahmad FA, Williams P, Nunnally A, Adcock J, Song Y, Harrell FE, Tran TL, Parry TJ, Iaci J, Ganguly A, Feoktistov I, Stephenson MK, Caggiano AO, Sawyer DB, Cleator JH.** Anti-remodeling and anti-fibrotic effects of the neuregulin-1β glial growth factor 2 in a large animal model of heart failure. *J Am Heart Assoc* 3: e000773, 2014.
19. **Galindo CL, Ryzhov S, Sawyer DB.** Neuregulin as a heart failure therapy and mediator of reverse remodeling. *Curr Heart Fail Rep* 11: 40–49, 2014.
20. **Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, Li T, Liu X, Xu Y, Li X, Zhou M.** A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. *J Am Coll Cardiol* 55: 1907–1914, 2010.
21. **Garcia-Rivello H, Taranda J, Said M, Cabeza-Meckert P, Vila-Petroff M, Scaglione J, Ghio S, Chen J, Lai C, Laguens RP, Lloyd KC, Hertig CM.** Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. *Am J Physiol Heart Circ Physiol* 289: H1153–H1160, 2005.
22. **Gordon LI, Burke MA, Singh AT, Prachand S, Lieberman ED, Sun L, Naik TJ, Prasad SV, Ardehali H.** Blockade of the erbB2 receptor induces cardiomyocyte death through mitochondrial and reactive oxygen species-dependent pathways. *J Biol Chem* 284: 2080–2087, 2009.
23. **Grazette LP, Boecker W, Matsui T, Semigran M, Force TL, Hajjar RJ, Rosenzweig A.** Inhibition of ErbB2 causes mitochondrial dysfunction in cardiomyocytes: implications for herceptin-induced cardiomyopathy. *J Am Coll Cardiol* 44: 2231–2238, 2004.
24. **Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN.** Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab* 15: 725–738, 2012.
25. **Hao J, Galindo CL, Tran TL, Sawyer DB.** Neuregulin-1beta induces embryonic stem cell cardiomyogenesis via ErbB3/ErbB2 receptors. *Biochem J* 458: 335–341, 2014.
26. **Hauselmann SP, Rose-Schlüter BI, Lorenz V, Plaisance I, Brink M, Pfister O, Kuster GM.** β1-Integrin is up-regulated via Rac1-dependent reactive oxygen species as part of the hypertrophic cardiomyocyte response. *Free Radic Biol Med* 51: 609–618, 2011.
27. **Hill MF, Patel AV, Murphy A, Smith HM, Galindo CL, Pentassuglia L, Peng X, Lenneman CG, Odiete O, Friedman DB, Kronenberg MW, Zheng S, Zhao Z, Song Y, Harrell FE Jr, Srinivas M, Ganguly A, Iaci J, Parry TJ, Caggiano AO, Sawyer DB.** Intravenous glial growth factor 2 (GGF2) isoform of neuregulin-1β improves left ventricular function, gene and protein expression in rats after myocardial infarction. *PLoS One* 8: e55741, 2013.
28. **Hiscox S, Barnfather P, Hayes E, Bramble P, Christensen J, Nicholson RI, Barrett-Lee P.** Inhibition of focal adhesion kinase suppresses the adverse phenotype of endocrine-resistant breast cancer cells and improves endocrine response in endocrine-sensitive cells. *Breast Cancer Res Treat* 125: 659–669, 2011.
29. **Hsieh IS, Yang RS, Fu WM.** Osteopontin upregulates the expression of glucose transporters in osteosarcoma cells. *PLoS One* 9: e109550, 2014.
30. **Jabbour A, Hayward CS, Keogh AM, Kotlyar E, McCrohon JA, England JF, Amor R, Liu X, Li XY, Zhou MD, Graham RM, Macdonald PS.** Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *Eur J Heart Fail* 13: 83–92, 2011.
31. **Jaswal JS, Keung W, Wang W, Ussher JR, Lopaschuk GD.** Targeting fatty acid and carbohydrate oxidation—a novel therapeutic intervention in the ischemic and failing heart. *Biochim Biophys Acta* 1813: 1333–1350, 2011.
32. **Kanda Y, Watanabe Y.** Thrombin-induced glucose transport via Src-p38 MAPK pathway in vascular smooth muscle cells. *Br J Pharmacol* 146: 60–67, 2005.

33. **Kline ER, Shupe J, Gilbert-Ross M, Zhou W, Marcus AI.** LKB1 represses focal adhesion kinase (FAK) signaling via a FAK-LKB1 complex to regulate FAK site maturation and directional persistence. *J Biol Chem* 288: 17663–17674, 2013.
34. **Kohn AD, Summers SA, Birnbaum MJ, Roth RA.** Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271: 31372–31378, 1996.
35. **Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, Lawrence JC Jr..** Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. *Mol Cell Biol* 28: 61–70, 2008.
36. **Kuramochi Y, Guo X, Sawyer DB.** Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. *J Mol Cell Cardiol* 41: 228–235, 2006.
37. **Laplante M, Sabatini DM.** mTOR signaling in growth control and disease. *Cell* 149: 274–293, 2012.
38. **Lemmens K, Doggen K, De Keulenaer GW.** Activation of the neuregulin/ErbB system during physiological ventricular remodeling in pregnancy. *Am J Physiol Heart Circ Physiol* 300: H931–H942, 2011.
39. **Lionetti V, Stanley WC, Recchia FA.** Modulating fatty acid oxidation in heart failure. *Cardiovasc Res* 90: 202–209, 2011.
40. **Liu H, Liu R, Xiong Y, Li X, Wang X, Ma Y, Guo H, Hao L, Yao P, Liu L, Wang D, Yang X.** Leucine facilitates the insulin-stimulated glucose uptake and insulin signaling in skeletal muscle cells: involving mTORC1 and mTORC2. *Amino Acids* 46: 1971–1979, 2014.
41. **Liu X, Gu X, Li Z, Li X, Li H, Chang J, Chen P, Jin J, Xi B, Chen D, Lai D, Graham RM, Zhou M.** Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. *J Am Coll Cardiol* 48: 1438–1447, 2006.
42. **Liu X, Marengere LE, Koch CA, Pawson T.** The v-Src SH3 domain binds phosphatidylinositol 3′-kinase. *Mol Cell Biol* 13: 5225–5232, 1993.
43. **Mendes-Ferreira P, De Keulenaer GW, Leite-Moreira AF, Brás-Silva C.** Therapeutic potential of neuregulin-1 in cardiovascular disease. *Drug Discov Today* 18: 836–842, 2013.
44. **Mieulet V, Lamb RF.** Tuberous sclerosis complex: linking cancer to metabolism. *Trends Mol Med* 16: 329–335, 2010.
45. **Odiete O, Hill MF, Sawyer DB.** Neuregulin in cardiovascular development and disease. *Circ Res* 111: 1376–1385, 2012.
46. **Opie LH.** Effects of regional ischemia on metabolism of glucose and fatty acids. Relative rates of aerobic and anaerobic energy production during myocardial infarction and comparison with effects of anoxia. *Circ Res* 38: I52–I74, 1976.
47. **Ozcelik C, Erdmann B, Pilz B, Wettschureck N, Britsch S, Hubner N, Chien KR, Birchmeier C, Garratt AN.** Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci USA* 99: 8880–8885, 2002.
48. **Pentassuglia L, Graf M, Lane H, Kuramochi Y, Cote G, Timolati F, Sawyer DB, Zuppinger C, Suter TM.** Inhibition of ErbB2 by receptor tyrosine kinase inhibitors causes myofibrillar structural damage without cell death in adult rat cardiomyocytes. *Exp Cell Res* 315: 1302–1312, 2009.
49. **Pentassuglia L, Sawyer DB.** ErbB/integrin signaling interactions in regulation of myocardial cell-cell and cell-matrix interactions. *Biochim Biophys Acta* 1833: 909–916, 2013.
50. **Pentassuglia L, Sawyer DB.** The role of Neuregulin-1beta/ErbB signaling in the heart. *Exp Cell Res* 315: 627–637, 2009.
51. **Pentassuglia L, Timolati F, Seifriz F, Abudukadier K, Suter TM, Zuppinger C.** Inhibition of ErbB2/neuregulin signaling augments paclitaxel-induced cardiotoxicity in adult ventricular myocytes. *Exp Cell Res* 313: 1588–1601, 2007.
52. **Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L, Gibbons J, Frost P, Dreisbach V, Blenis J, Gaciong Z, Fisher P, Sawyers C, Hedrick-Ellenson L, Parsons R.** An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/− mice. *Proc Natl Acad Sci USA* 98: 10320–10325, 2001.
53. **Polizzotti BD, Ganapathy B, Walsh S, Choudhury S, Ammanamanchi N, Bennett DG, dos Remedios CG, Haubner BJ, Penninger JM, Kühn B.** Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. *Sci Transl Med* 7: 281ra45, 2015.
54. **Rosenzweig T, Aga-Mizrachi S, Bak A, Sampson SR.** Src tyrosine kinase regulates insulin-induced activation of protein kinase C (PKC) delta in skeletal muscle. *Cell Signal* 16: 1299–1308, 2004.
55. **Roskoski R Jr.** The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacol Res* 79: 34–74, 2014.
56. **Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE.** Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278: 14599–14602, 2003.
57. **Sato M, Dehvari N, Oberg AI, Dallner OS, Sandström AL, Olsen JM, Csikasz RI, Summers RJ, Hutchinson DS, Bengtsson T.** Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. *Diabetes* 63: 4115–4129, 2014.
58. **Sawyer DB, Caggiano A.** Neuregulin-1beta for the treatment of systolic heart failure. *J Mol Cell Cardiol* 51: 501–505, 2011.
59. **Sawyer DB, Zuppinger C, Miller TA, Eppenberger HM, Suter TM.** Modulation of anthracycline-induced myofibrillar disarray in rat ventricular myocytes by neuregulin-1beta and anti-erbB2: potential mechanism for trastuzumab-induced cardiotoxicity. *Circulation* 105: 1551–1554, 2002.
60. **Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT.** Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14: 1680–1688, 1994.
61. **Schlaepfer DD, Hunter T.** Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* 16: 5623–5633, 1996.
62. **Shende P, Plaisance I, Morandi C, Pellieux C, Berthonneche C, Zorzato F, Krishnan J, Lerch R, Hall MN, Ruegg MA, Pedrazzini T, Brink M.** Cardiac raptor ablation impairs adaptive hypertrophy, alters metabolic gene expression, and causes heart failure in mice. *Circulation* 123: 1073–1082, 2011.
63. **Shende P, Xu L, Morandi C, Pentassuglia L, Heim P, Lebboukh S, Berthonneche C, Pedrazzini T, Kaufmann BA, Hall MN, Ruegg MA, Brink M.** Cardiac mTOR complex 2 preserves ventricular function in pressure-overload hypertrophy. *Cardiovasc Res* 109: 103–114, 2016.
64. **Suarez E, Bach D, Cadefau J, Palacin M, Zorzano A, Guma A.** A novel role of neuregulin in skeletal muscle. Neuregulin stimulates glucose uptake, glucose transporter translocation, and transporter expression in muscle cells. *J Biol Chem* 276: 18257–18264, 2001.
65. **Tian R, Abel ED.** Responses of GLUT4-deficient hearts to ischemia underscore the importance of glycolysis. *Circulation* 103: 2961–2966, 2001.
66. **Timolati F, Ott D, Pentassuglia L, Giraud MN, Perriard JC, Suter TM, Zuppinger C.** Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction coupling and reduces oxidative stress in adult rat cardiomyocytes. *J Mol Cell Cardiol* 41: 845–854, 2006.
67. **Tomioka H, Mukohara T, Kataoka Y, Ekyalongo RC, Funakoshi Y, Imai Y, Kiyota N, Fujiwara Y, Minami H.** Inhibition of the mTOR/S6K signal is necessary to enhance fluorouracil-induced apoptosis in gastric cancer cells with HER2 amplification. *Int J Oncol* 41: 551–558, 2012.
68. **Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A.** Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. *Endocrinology* 146: 1328–1337, 2005.
69. **Tremblay F, Marette A.** Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J Biol Chem* 276: 38052–38060, 2001.
70. **Tsuchiya A, Kanno T, Nishizaki T.** PI3 kinase directly phosphorylates Akt1/2 at Ser473/474 in the insulin signal transduction pathway. *J Endocrinol* 220: 49–59, 2014.
71. **Valle-Casuso JC, Gonzalez-Sanchez A, Medina JM, Tabernero A.** HIF-1 and c-Src mediate increased glucose uptake induced by endothelin-1 and connexin43 in astrocytes. *PLoS One* 7: e32448, 2012.
72. **Vicier C, Dieci MV, Arnedos M, Delaloge S, Viens P, Andre F.** Clinical development of mTOR inhibitors in breast cancer. *Breast Cancer Res* 16: 203, 2014.
73. **Wadugu B, Kuhn B.** The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. *Am J Physiol Heart Circ Physiol* 302: H2139–H2147, 2012.
74. **Wang X, Yue P, Kim YA, Fu H, Khuri FR, Sun SY.** Enhancing mammalian target of rapamycin (mTOR)-targeted cancer therapy by

preventing mTOR/raptor inhibition-initiated, mTOR/rictor-independent Akt activation. *Cancer Res* 68: 7409–7418, 2008.

75. **Young LH, Renfu Y, Russell R, Hu X, Caplan M, Ren J, Shulman GI, Sinusas AJ.** Low-flow ischemia leads to translocation of canine heart GLUT-4 and GLUT-1 glucose transporters to the sarcolemma in vivo. *Circulation* 95: 415–422, 1997.
76. **Zagozdzon R, Bougeret C, Fu Y, Avraham HK.** Overexpression of the Csk homologous kinase facilitates phosphorylation of Akt/PKB in MCF-7 cells. *Int J Oncol* 21: 1347–1352, 2002.
77. **Zhang D, Contu R, Latronico MV, Zhang JL, Rizzi R, Catalucci D, Miyamoto S, Huang K, Ceci M, Gu Y, Dalton ND, Peterson KL, Guan KL, Brown JH, Chen J, Sonenberg N, Condorelli G.** MTORC1 regulates cardiac function and myocyte survival through 4E-BP1 inhibition in mice. *J Clin Invest* 120: 2805–2816, 2010.
78. **Zhao YY, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, Kelly RA.** Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J Biol Chem* 273: 10261–10269, 1998.
79. **Zhou X, Tan M, Stone Hawthorne V, Klos KS, Lan KH, Yang Y, Yang W, Smith TL, Shi D, Yu D.** Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. *Clin Cancer Res* 10: 6779–6788, 2004.

3.1.2. Supplementary data (unpublished results)

The data presented here were not included in our publication, although the experiments were part of my PhD project. Some results did not reach significance due to limited repetitions of the experiments, whereas other experiments had a negative outcome. Nevertheless, as my preliminary data may lead to interesting future studies, I would like here to present some of them. The aims of the experiments were:

(1) to examine the role of c-Src and FAK in Nrg1β-induced glucose uptake by siRNA knockdown.

(2) to investigate the contribution of ErbB2 and ErbB4 to protein synthesis using siRNA knockdown.

c-Src and FAK may contribute to Nrg1β-induced glucose uptake

c-Src and FAK are involved in cytoskeleton regulation, survival and proteins synthesis (Thomas and Brugge, 1997). c-Src can interact with a broad spectrum of cellular receptors and other targets like FAK, which is part of the focal adhesion complex that links the cytosol with the extracellular matrix via integrins (Graham et al., 2015). Previously, FAK was demonstrated to interact and increase PI3K activity (Chen and Guan, 1994). Furthermore, Nrg1β in known to trigger phosphorylation of c-Src at Tyr^{416} and Tyr^{215} and FAK at Tyr^{861} in adult CMs (Kuramochi et al., 2006). Therefore, we investigated a possible link between c-Src/FAK and Nrg1β - induced glucose uptake signaling.

We found that Nrg1β-stimulated glucose uptake was blocked after pharmaceutical inhibition of c-Src with Dasatinib or PP2 (Pentassuglia et al., 2016). Since these inhibitors may have some off-target effects, we also used a non-pharmacological approach to further prove the involvement of FAK and c-Src. Knockdown of c-Src and FAK in NRVMs with specific siRNAs partially reduced basal glucose uptake, but the Nrg1β-stimulated glucose uptake was not affected (Fig. 1). The knockdown was not complete, which can be seen on the Western blots of c-Src and FAK. Therefore, while not providing any proof, these results also do not exclude a contribution of c-Src and FAK to the Nrg1β-induced glucose uptake. Further studies are required to elucidate the role of c-Src/FAK in Nrg1β-related glucose uptake signaling in NRVMs.

Figure 1

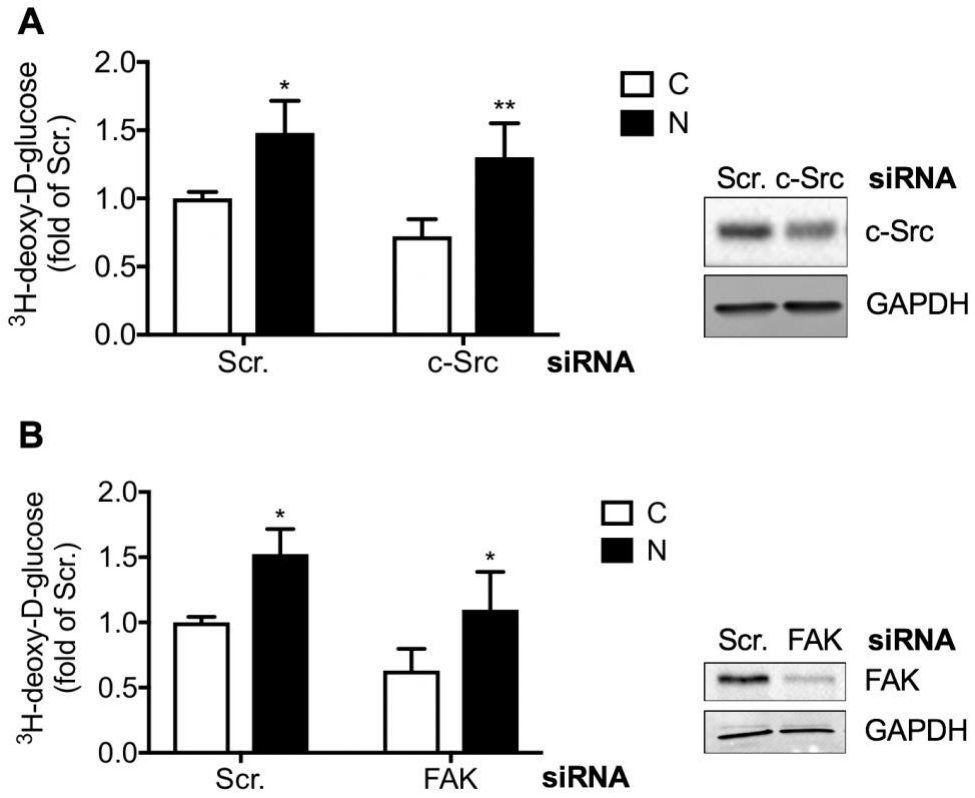

Figure 1. Glucose uptake after siRNA knockdown of c-Src and FAK

A: NRVMs were transfected with siRNAs specific for c-Src. 48 h after transfection, NRVMs were stimulated for 30 min with Nrg1β (10 ng/ml) or control vehicle. Glucose uptake was assessed by 30 min incubation with 1 µCi/ml [^{3}H]-deoxy-D-glucose. Counts were normalized to total protein/well. siRNA-transfected NRVMs were treated for 30 min with Nrg1β (10 ng/ml). Western blot analysis was performed to detect c-Src (representative experiment, mean +/- standard deviation, STDV). **B:** NRVMs were transfected with siRNAs specific for FAK and treated in the same way as above (representative experiment, mean +/- STDV). *P < 0.05, **P < 0.01 Nrg1β vs. vehicle. Control vehicle (C), Nrg1β (N), scrambled (Scr.).

ErbB2 and ErbB4 are required for Nrg1β-induced protein synthesis

Protein synthesis plays an important role in physiological as well as pathological cardiac hypertrophy (Nakamura and Sadoshima, 2018). Moreover, glucose uptake was demonstrated to be required for protein synthesis in CMs by regulating branched chain amino acid (BCAA) degradation (Shao et al., 2018). Nrg1β has been shown to increase protein synthesis in CMs (Baliga et al., 1999), but the responsible receptor has not been identified. Therefore, as first aim, we analyzed protein synthesis in NRVMs and tried to identify the responsible receptors necessary for Nrg1β-induced protein synthesis. We observed a complete inhibition of Nrg1β-induced protein synthesis after treatment with the ErbB1/2 inhibitor Lapatinib (Pentassuglia et al., 2016). Since Western blot analysis showed that Lapatinib not only blocked phosphorylation of ErbB2 but also ErbB4, protein synthesis was measured after ErbB2 and ErbB4 knockdown by siRNA. Reduced protein levels of either receptor lowered the incorporation of [^{3}H]-phenylalanine (Fig. 2A). The high efficiency of the knockdown was revealed by Western blot analysis (Fig. 2B). Due to variability between the three experiments, the effect of Nrg1β on protein synthesis did not reach significance (P=0.053) in the scrambled controls. However, in previous experiments with scrambled siRNA, Nrg1β significantly increased protein synthesis (Pentassuglia et al., 2016). Therefore, similar to what we found for the glucose uptake, both ErbB2 and ErbB4 seem to be involved in the Nrg1β-induced protein synthesis. In conclusion, these data show the involvement of the ErbB2/4 heterodimer in Nrg1β-induced protein synthesis in NRVMs.

As second aim, we wanted to test if Nrg1β-induced glucose uptake contributes to protein synthesis in NRVMs. Therefore, we blocked glucose uptake pharmacologically with a GLUT4 inhibitor (Indinavir) or with siRNAs against GLUT1 or GLUT4. Indinavir reduced basal glucose uptake and protein synthesis but had no effect on the insulin- and Nrg1β-induced response (n = 3/1, data not shown). These data demonstrate that glucose uptake contributes to overall protein synthesis in NRVMs. Furthermore, it might indicate that next to GLUT4 one or more additional glucose transporters are involved in insulin- and Nrg1β-stimulated glucose uptake in NRVMs. Moreover, our preliminary data of the siRNA approach did not reveal any effect on protein synthesis. Hence, further experiments are required to elucidate the contribution of Nrg1β-induced glucose uptake to protein synthesis. In this context, one could analyze the

role of BCAA regulation, which links glucose uptake with protein synthesis as proposed by Shao et al. (Shao et al., 2018).

Figure 2

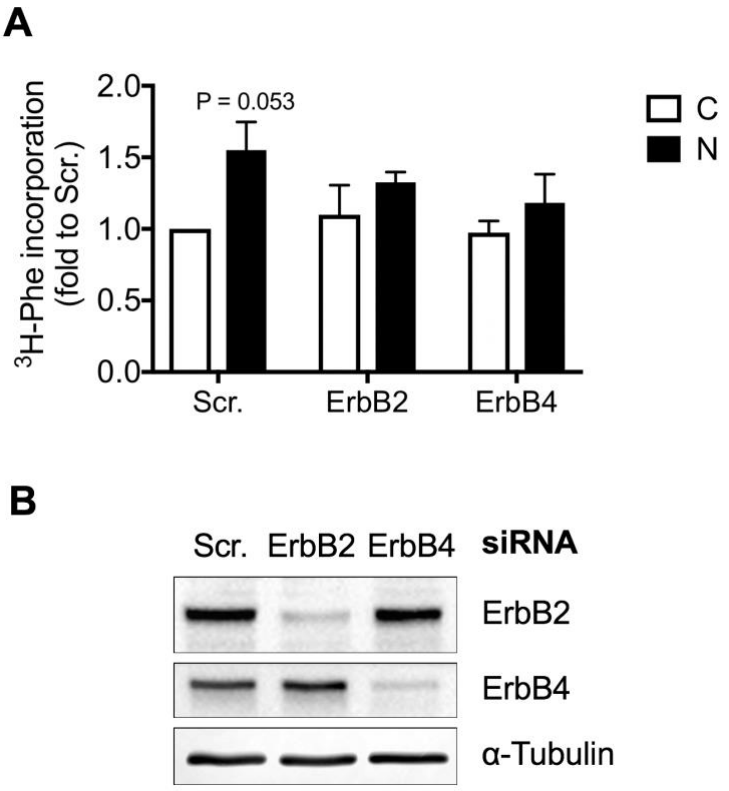

Figure 2. Protein synthesis after siRNA knockdown of ErbB2 and ErbB4

A: NRVMs were transfected with siRNA specific for ErbB2 and ErbB4. 48 h after transfection, NRVMs were stimulated for 24 h with Nrg1β (10 ng/ml) or control vehicle in presence of 2 µCi/ml [^{3}H]-phenylalanine for protein synthesis measurement. Counts were normalized to total DNA/well (n=3, mean +/- STDV). **B:** siRNA-transfected NRVM protein extracts were analyzed by Western blot analysis to detect ErbB2 and ErbB4 (representative experiment). Control vehicle (C), Nrg1β (N), scrambled (Scr.).

3.2. Manuscript: Neuregulin-1β Stimulates Glucose Uptake in Neonatal Rat Cardiomyocytes by Regulating GLUT4 Translocation

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Short Title: Neuregulin-1β causes GLUT4 translocation

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Keywords
Neuregulin, ErbB, cardiomyocytes, glucose uptake, GLUT4, metabolism

Abstract

Background/Aims: During stress conditions such as pressure overload and acute ischemia, the myocardial endothelium releases neuregulin (Nrg), which acts as cardioprotective factor and supports recovery of the heart. Recently, we demonstrated that Nrg1β enhances glucose uptake in neonatal rat ventricular myocytes (NRVMs) via PI3Kα and Akt. The present study aimed to elucidate the mechanism whereby Nrg1β activates glucose uptake in more detail in comparison to the well-studied insulin-induced glucose uptake. ***Methods:*** Isolated NRVMs and adult rat cardiomyocytes (CMs) were treated with Nrg1β or insulin for comparison. Prior to stimulation, proteins of interest were knocked down using siRNAs. Glucose uptake was measured by ^{3}H-deoxy-D-glucose incorporation, glycolysis by Seahorse and proteins by Western blot. To detect GLUT4 translocation, a c-Myc-GLUT4-mCherry construct was transfected using AMAXA Nucleofector and detected by immunofluorescence. Neonatal rats were treated with Nrg1β or insulin to assess activation of glucose uptake pathways in the intact heart. ***Results:*** Combinations of Nrg1β with increasing doses of insulin did not yield any additive effect on glucose uptake, indicating that the mechanisms of both stimuli are very similar. In c-Myc-GLUT4-mCherry transfected NRVMs, we demonstrate that Nrg1β increases sarcolemmal GLUT4 by 16-fold, similar to insulin. In contrast to insulin, IRS-1 is not phosphorylated at Tyr^{612} in presence of Nrg1β, indicating that IRS-1 is not implicated in signal transmission. Treatment of neonatal rats with Nrg1β showed a signaling response comparable with our *in vitro* findings, including increased phosphorylation of ErbB4 at Tyr^{1284}, Akt at Thr^{308} and Erk1/2 at Thr^{202}/Tyr^{204}. In contrast, in adult rat CMs Nrg1β only increased Erk1/2 phosphorylation without having any effect on AS160 and glucose uptake, indicating that Nrg1β signaling and function in NRVMs differs from that in adult rat CMs. ***Conclusions:*** Our results show that similar to insulin, Nrg1β induces glucose uptake in NRVMs by activating the PI3K pathway and GLUT4 translocation. Unlike insulin, the Nrg-induced effect is not mediated by IRS proteins and is observed only in NRVMs and not in adult rat CMs.

Introduction

Since more than 20 years it is known that neuregulin1 (Nrg1) and its receptors called ErbB2, ErbB3 and ErbB4, are indispensable for proper cardiac development and homeostasis (Erickson et al., 1997; Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). More recently, Nrg1 also gathered a lot of attention in the field of cardiac regeneration. In zebrafish and mouse models, Nrg1 was demonstrated to contribute to regeneration of the heart by inducing cardiomyocyte proliferation (Harvey et al., 2015; Yester and Kuhn, 2017). In addition, clinical trials are investigating the therapeutic value of Nrg1 and published data show its positive effects on cardiac function in patients with heart disease (Gao et al., 2010; Jabbour et al., 2011b; Lenihan et al., 2016). However, the cellular and molecular mechanisms responsible for these beneficial effects are poorly understood.

Nrg1/ErbB induces the PI3K, MAPK/Erk1/2 and Src/FAK pathways, which regulate several cellular functions including cytoskeletal organization, cell growth and survival, and angiogenesis (Pentassuglia and Sawyer, 2009). Only ErbB3 and ErbB4 can bind ligands, whereas ErbB2 is an orphan receptor (Fuller et al., 2008). Nrg1/ErbB signaling activation requires the formation of ErbB homo- or heterodimers. Recently, we demonstrated that Nrg1β enhances glucose uptake in NRVMs by a mechanism that implicates the ErbB2/ErbB4 heterodimer, PI3Kα and Akt (Pentassuglia et al., 2016). Similar to skeletal muscle and fat cells, glucose uptake by cardiac cells is thought to be mediated by glucose transporters (GLUT). The fetal heart mainly expresses GLUT1, whereas in the adult heart GLUT4 dominates (Riehle and Abel, 2016; Shao and Tian, 2015). This pattern of GLUT expression matches the well-studied energy substrate profile of the fetal vs. the adult heart, which primarily consume glucose vs. fatty acids, respectively (Lopaschuk and Jaswal, 2010). Under conditions of stress such as ischemia or pressure overload, the adult heart increases glucose uptake and reduces fatty acid consumption to sustain the high energy demand needed for continuous contraction (Riehle and Abel, 2016; Shao and Tian, 2015; Szablewski, 2017). Under such stress conditions, the myocardial endothelium also releases Nrg1 as cardioprotective factor (Hedhli et al., 2011; Kuramochi et al., 2004a). Our previously published finding that Nrg1 stimulates glucose uptake in NRVMs points to glucose uptake as one of the basic mechanisms underlying its cardioprotective properties. Given the important therapeutic potential of Nrg1, our present

study aimed to better understand the mechanisms whereby Nrg1 enhances glucose uptake in NRVMs, and to extend the findings to adult CM and *in vivo* models. The classical insulin-stimulated glucose uptake involves a cascade of enzymatic reactions comprising phosphorylation of the InsR, IRS, PI3K, PIP3, PDK1, Akt, AS160 and Rab, which finally triggers GLUT4 vesicles to translocate and fuse with the plasma membrane (Jaldin-Fincati et al., 2017; Leto and Saltiel, 2012). Our present study analyzes the mechanism of Nrg1β-stimulated glucose uptake in comparison to that induced by insulin. Moreover, we compare neonatal with adult rat CMs and evaluate the responses to Nrg1β using a neonatal *in vivo* model.

Materials and Methods

Isolation and transfection of primary cardiomyocytes

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-days-old rats. The hearts were predigested in 0.05% trypsin–EDTA (Gibco) over-night, followed by serial digestions in 0.09% collagenase type II (Worthington). To reduce the number of cardiac fibroblasts, cells were pre-plated twice in T75 culture flasks (Sarstedt). NRVMs were seeded on plastic culture dishes (BD Falcon) or transfected with siRNA (Dharmacon: scrambled, ErbB3, ErbB4, GLUT1 and GLUT4) or a plasmid (Addgene: 64049) according to Amaxa's Neonatal Rat Cardiomyocyte Nucleofector kit (VPE-1002) and kept at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml; Gibco), streptomycin (100 µg/ml; Gibco), and 5-bromo-2-deoxyuridine (BrdU; 100 µmol/L; Sigma). After 30 h, the medium was changed to ACCT medium, serum-free DMEM supplemented with 2 g/l albumin, 2 mM L-carnitine, 5 mM creatine and 5 mM taurine (all from sigma) and experiments were performed on the following day. Adult rat CMs were isolated as previously described by Asrih et al. (Asrih et al., 2011) and plated onto laminin-coated culture dishes in ACCT medium.

Neonatal heart dissection and protein isolation

1- to 2-days-old rats were injected i.p. with Nrg1β (PeproTech, 50 µg/kg body weight), insulin (1 U/kg body weight) or saline. After 30 min, the animals were sacrificed by decapitation, the heart was isolated and put immediately into ice-cooled phosphate-buffered saline. After washing, the atria were removed and the ventricles frozen in liquid nitrogen. The tissues were grinded with a Polytron homogenizer in RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 0.1% SDS, 5 mM EDTA, and 0.5% phosphatase inhibitor cocktail 2 and 3, 1% protease

inhibitor cocktail, 10 mM Na pyrophosphate, 10 mM glycerophosphate from Sigma) and proteins were analyzed by Western blot.

Protein extraction and Western blot analysis

Proteins were extracted with RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 0.1% SDS, 5 mM EDTA, and 0.5% phosphatase inhibitor cocktail 2 and 3, 1% protease inhibitor cocktail from Sigma), separated by SDS-PAGE and transferred to a PVDF membrane (Amersham-GE Healthcare). After antibody incubation, the signal was revealed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the ChemiDoc MP System (Bio-Rad). Blots were quantified with Image Lab (Bio-Rad) and ImageJ (National Institutes of Health).

Glucose uptake assay

NRVMs were treated with inhibitors for 30 min, followed by 30 min of stimulation by growth factors and another 30 min in the presence of deoxy-D-glucose, 2-[1,2-^{3}H(N)] (Perkin-Elmer) and D-glucose (1 µCi/ml and 100 µM, respectively) in Krebs-Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 24 mM NaHCO3, 10 mM HEPES, pH 7.4, and 0.1% BSA). Afterwards, the cells were washed with ice-cold PBS and lysed in 0.2 M NaOH. One part of the lysate was mixed with scintillation liquid (Perkin Elmer) to measure ^{3}H in a β-counter. With the other part, a micro BCA protein assay (Thermo Scientific) was performed for normalization of the counts/min. Glucose uptake in adult rat CM was measured as previously described by Montessuit et al. (Montessuit et al., 2008).

Seahorse assay

Freshly isolated NRVMs were seeded in a laminin (10 µg/ml) coated 24-well Seahorse plate (60'000 cells/well). After overnight incubation in ACCT medium, the procedure was done according to the manufacturer's protocol. The adapted glycolytic stress test Seahorse XF24 protocol consisted of 11 min calibration/equilibration, injection of the stimuli/drugs, followed by 4 min of mixing, 2 min waiting and 2 min measuring. The extracellular acidification rate (ECAR) was measured 4 times at base line and 3 times after each injection. The rate of glycolysis was determined by subtracting the basal ECAR value before glucose injection from the maximal ECAR value after glucose injection.

Immunofluorescence

Transfected NRVMs were seeded on 13 mm glass cover slips coated with laminin. The cells were washed with ice-cold PBS and fixed for 1 h at 4 °C with PBS + 4% formaldehyde (Polyscience). After

blocking with PBS + 3% bovine serum albumin for 1 h at RT, the first antibodies were incubated over night at 4°C. The samples were washed with PBS and incubated for 1 h at RT with a fluorescent secondary antibody and DAPI (1 µg/ml). The samples were washed again and mounted with mounting medium (3 mM polyvinyl alcohol 4-88, 33% glycerol, 133.3 mM Tris-HCl pH 8.5 and 2.5% diazabicyclooctane) onto a glass slide. The pictures were taken with an Olympus BX63 microscope and quantification was done blinded using ImageJ.

Gene expression analysis

RNA was extracted form NRVMs with Tri Reagent (Sigma) and treated with DNAse I (Ambion). cDNA was prepared using the high capacity DNA reverse transcription kit (Applied Biosystems). The product was amplified on a 7500 Fast Real-Time PCR system (Applied Biosystems), with GoTaq qPCR Master Mix (Promega) and 300 nM for forward and reverse primers in a total volume of 20 µl. Following primers were used: rat GLUT1 (5'-ATC AAC GCC CCC CAG AA-3' / 5'-AAT CAT GCC CCC GAC AGA-3'), rat GLUT4 (5'-CCC CCG ATA CCT CTA CAT-3' / 5'-GCA TCA GAC ACA TCA GCC CAG-3') and rat GAPDH (5'-GAT GGT GAA GGT CGG TGT GAA-3' / 5'-TTG AAC TTG CCG TGG GTA GAG-3'). The mRNA level was based on the critical threshold (Ct) value.

Reagents

Neuregulin1β was from R&D Systems (377-HB) for *in vitro* or PeproTech (100-03) for *in vivo* applications, Insulin and Oligomycin was from Sigma, MG132 was from Calbiochem and IGF-I was from Genentech. The antibodies against Akt-pSer^{473}, Akt-pThr^{308}, Akt2, AMPK-pThr^{172}, AMPK, AS160-pThr^{642}, AS160, ErbB3-pTyr^{1289}, ErbB3, Erk1/2-pThr^{202}/Tyr^{204}, Erk1/2, InsR/IGF-IR-pTyr^{1146}/Tyr^{1131}, IRS-1-pS$^{636/639}$, P70S6K1-pThr^{389} and P70S6K1 were from Cell Signaling Technology. The antibodies against ErbB2-pTyr^{1248}, ErbB4, GAPDH, IRS-1, c-Myc and Vinculin were from Santa Cruz Biotechnology. The antibody against ErbB4-pTyr^{1284} was from Abcam. The antibody against IRS-1-pTyr^{612} was from MyBioSource. The antibody against IRS-1-pS^{307} and IRS-2 were from Upstate. The antibody against α-Tubulin was from Sigma.

Statistical analysis

All results are expressed as means +/- STDEV. One-way or two-way ANOVA analysis was followed by Sidak's or Dunnetts's post hoc test using Prism 7 (GraphPad).

Results

The ErbB2/ErbB4 heterodimer mediates Nrg1β-induced glucose uptake

We recently reported that Nrg1β enhances glucose uptake in NRVMs via ErbB2/ErbB4 (Pentassuglia et al., 2016). For a long time, ErbB3 was considered to be expressed only in the embryonic heart, while being downregulated after birth (Zhao et al., 1998). However, using qRT-PCR and ErbB3-specific antibodies, Camprecios et al. demonstrated that ErbB3 may also be expressed in neonatal and adult CMs (Camprecios et al., 2011). We therefore tested whether ErbB3 is implicated in the Nrg1β-induced glucose uptake in our NRVM model. Knockdown of ErbB3 with siRNA had no effect on Nrg1β-induced glucose uptake, whereas reduced ErbB4 levels fully blocked it (Fig. 1A). The efficient knockdown of ErbB3 and ErbB4 was confirmed by Western blot analysis (Fig. 1B). Like for ErbB4, ErbB2 knockdown also blunted the effect of Nrg1β as previously published by our group (Pentassuglia et al., 2016). These data demonstrate that ErbB3 is not responsible for the glucose uptake response and confirms that ErbB2/ErbB4 heterodimers are implicated.

Nrg1β and insulin have no additive effect on glucose uptake

We previously demonstrated that Nrg1β induces glucose uptake in NRVMs with similar potency as insulin (Pentassuglia et al., 2016). We performed a combined dose-response of Nrg1β and insulin to investigate whether the two stimuli have an additive effect. In earlier dose-response experiments we established that 10 ng/mL of Nrg1β leads to maximal ErbB signaling activation (Pentassuglia et al., 2016). Fig. 2A shows that receptor-saturating concentrations of Nrg1β cause a 2-fold increase in glucose uptake. The addition of increasing doses of insulin to Nrg1β did not cause any further elevation in glucose uptake. Western blot analysis revealed that the two factors had no additive effect on AS160 phosphorylation, matching the glucose uptake result, despite their strong additive effect on Akt phosphorylation (Fig. 2B). Since insulin is known to increase glycolysis in the heart (Lawson and Uyeda, 1987), we next investigated the effect of Nrg1β on glycolysis in comparison to insulin. Indeed, Seahorse analysis indicated that elevated glucose uptake was associated with increases in glycolysis for both factors (Fig. 2C and D).

Nrg1β and insulin have distinct effects on IRS-1 phosphorylation

It is well-known that IRS mediates insulin- and IGF-induced signaling and in breast cancer cells, ErbB1 and ErbB3 were shown to interact with IRS-1 (Knowlden et al., 2011; Knowlden et al., 2008), and ErbB2 may also interact with IRS (Jones et al., 2006). To evaluate if IRS-1 is implicated in the PI3K activation observed in our model, we stimulated NRVMs with insulin or Nrg1β for 1, 15, 60 and 120 min and analyzed the protein extracts by Western blotting. Fig. 3A shows that both stimuli induce phosphorylation of IRS-1 at several sites. Insulin causes a rapid transient phosphorylation of Tyr^{612} with a maximum reached already after 1 min, whereas Nrg1β has no effect on that site (Fig. 3A). At later time points, both insulin and Nrg1β phosphorylate IRS-1 at $Ser^{636/639}$ and Ser^{307}. Notably, the effect of Nrg1β on these sites is stronger than that of insulin. In addition, for both hormones a band shift of total IRS-1 is visible on the Western blots, which is very likely due to its increased phosphorylation status. Since IRS-2 is known to play a major role in mediating peripheral glucose uptake (Kaburagi et al., 1999), we also analyzed total IRS-2. Similar to IRS-1, the IRS-2 band was shifted upwards for both insulin and Nrg1β. Thus, while both stimuli induce band shifts of IRS-1/2 and cause IRS-1 phosphorylation at $Ser^{636/639}$ and Ser^{307}, only insulin induces IRS-1 phosphorylation at Tyr^{612}. Increased phosphorylation of these Ser sites correlates with proteasomal degradation and disruption of the InsR-IRS-1 interaction, and are believed to serve as negative feedback loop after insulin stimulation (Copps and White, 2012; Rui et al., 2001). In our cells, insulin lowered total IRS-1 and IRS-2 at 24 h, and these effects were blocked by the proteasomal inhibitor MG132 (Fig. 3B). On the other hand, despite causing strong phosphorylation at the same serine sites, Nrg1β did not cause any degradation of IRS. We tested whether increased IRS-1 Ser phosphorylation changes the response to insulin. After 24 h of pre-incubation with insulin or Nrg1β, cells were re-stimulated with insulin for 30 min and glucose uptake was evaluated 30 min later. Supplementary Fig. 1 shows that both insulin- and Nrg-pre-incubation enhanced the insulin-stimulated glucose uptake, indicating that the different phosphorylation state and protein levels of IRS1/2 did not modify the response to insulin.

Nrg1β triggers GLUT4 translocation

Phosphorylation of AS160 is implicated in GLUT4 shuttling to the cell membrane (Jaldin-Fincati et al., 2017; Leto and Saltiel, 2012). Nrg1β increased AS160-$pThr^{642}$ and we therefore investigated whether Nrg1β induces GLUT4 translocation. To this end, NRVMs were

transfected with a plasmid expressing GLUT4-mCherry with a c-Myc tag located on the extracellular side (Lim et al., 2015). This allowed us to detect intracellular GLUT4 vesicles and identify translocated GLUT4 at the sarcolemma of non-permeabilized cells with an antibody specific for the c-Myc tag. Immunofluorescence with specific antibodies to sarcomeric actinin confirmed that the majority of GLUT4-positive cells are indeed NRVMs (Fig. 4A). Fig. 4B shows representative pictures of c-Myc-positive cells treated with insulin and Nrg1β, respectively. Quantification revealed that both stimuli significantly induced GLUT4 translocation to the sarcolemma (Fig. 4C).

To further investigate the role of the glucose transporters, we knocked down GLUT1 and GLUT4 (Fig. 4D and E). Knockdown of either GLUT1 or GLUT4 significantly reduced basal and insulin-stimulated as well as Nrg1β-stimulated glucose uptake. Taken together, the microscopy data indicates that GLUT4 translocation to the sarcolemma is involved in the Nrg1β-stimulated glucose uptake, while the knockdown data indicate that both GLUT1 and GLUT4 contributes to both basal and stimulated glucose uptake.

Nrg1β causes phosphorylation of cardiac Akt, AS160 and Erk1/2 in vivo

In our NRVM model, Nrg1β stimulated Akt, AS160 and Erk1/2 phosphorylation. To check whether the same signaling pathways are activated *in vivo*, we injected 1-2 days old neonatal rats with insulin and Nrg1β. Analysis of whole heart protein extracts revealed similar signaling events compared to our *in vitro* model. Fig. 5A shows increased ErbB4 phosphorylation after Nrg1β stimulation. In addition, Akt-pThr^{308} and AS160-pThr^{642} were higher in the presence of insulin and Nrg1β, with insulin showing stronger effects than Nrg1β (Fig. 5B). Consistent with its increased phosphorylation, the band of Akt2 was shifted upwards for both stimuli. Finally, and again similar to our *in vitro* findings, Nrg1β induced phosphorylation of Erk1/2 at Thr^{202}/Tyr^{204}.

Nrg1β does not increase glucose uptake in cardiomyocytes from adult rats

Nrg1β is known to be cardioprotective in adult rodent models (Liu et al., 2006). We therefore proceeded to investigate its effects on glucose uptake in adult rat CMs. Immediately after their isolation, the CMs were stimulated with insulin or Nrg1β and glucose uptake was measured (Fig. 6A). Insulin induced a significant increase in glucose uptake, whereas Nrg1β had no effect. A dose-response with Nrg1β showed that glucose uptake was not increased

even at the highest concentration used, which was 1 µg/ml (Fig. 6B). Signaling analysis matched the results of the glucose uptake assay as Nrg1β induced only very small increases in Akt and AS160 phosphorylation, whereas insulin had strong effects on both targets (Fig. 6C). Like in our NRVMs, Erk1/2 phosphorylation was elevated in presence of Nrg1β, confirming appropriate responsiveness of primary adult CM cultures to the peptide.

Discussion

The fetal heart primarily uses glucose as energy substrate. During the first week after birth substrate preference switches from glucose to fatty acids. Under stress conditions, the adult heart again increases glucose consumption at the expense of fatty acids to sustain the high energy demand needed for continuous contraction (Lopaschuk and Jaswal, 2010). This is associated with a changed pattern of gene expression, resembling that of the fetal and neonatal heart. Ischemic and hemodynamic stress conditions also trigger the myocardial endothelium to release Nrg1, favoring recovery and preserving function of the heart (Hedhli et al., 2011). It is currently not known whether these cardioprotective actions are explained by regulatory effects of Nrg1 on metabolism. Recently, we demonstrated that Nrg1β enhances glucose uptake in NRVMs via PI3Kα and Akt (Pentassuglia et al., 2016). In the present study, we investigated the implicated mechanism in comparison to the well-studied insulin-induced glucose uptake.

Our findings show that Nrg1β induces glucose uptake and increases glycolysis to the same extent as insulin and that the two factors have no additive effect in NRVMs. This is in contrast to skeletal muscle cells, in which an additive effect was observed (Canto et al., 2004). Previously, we concluded that the ErbB2/ErbB4 heterodimer is responsible for the Nrg1β-stimulated glucose uptake (Pentassuglia et al., 2016). Using siRNA knockdown, we here additionally show that ErbB3 is not involved, which strengthens our own earlier finding for NRVMs, but differs from that reported in L6E9 skeletal muscle cells (Canto et al., 2004).

Similar to other tyrosine kinase receptors including InsR/IGF-IR, the ErbB receptors can activate the PI3K pathway. Best-known for this activity is ErbB3 (Carraway et al., 1995; Hellyer et al., 1995), but in the present study we excluded its role in our cardiomyocyte model. Downstream of the InsR/IGF-IR, IRS-1/2 proteins mediate insulin-induced PI3K activation

(Peng and He, 2018). By analyzing several phosphorylation sites of IRS-1 over time, we showed that only insulin rapidly induces Tyr^{612} phosphorylation, a site that indicates increased activity of IRS-1 (Esposito et al., 2001). On the other hand, at later time points Nrg1β increased phosphorylation of IRS-1 on $Ser^{636/639}$ and Ser^{307}. While insulin also phosphorylated these sites, the Nrg-induced phosphorylation was much stronger. Since Nrg1β enhances mTORC1 activity in NRVMs (Pentassuglia et al., 2016), the elevated IRS-1 serine phosphorylation may be secondary to mTOR/S6K1 activation, consistent with the timepoint at which the effect occurs. IRS-1 phosphorylation at $Ser^{636/639}$ may also depend on Erk1/2 (Bouzakri et al., 2003), a MAPK strongly phosphorylated by Nrg1β in our model. Phosphorylation of these serine sites are thought to cause a negative feedback loop that leads to decreased activity and eventual proteasomal degradation of IRS-1 (Copps and White, 2012). Notably, total IRS-1 and IRS-2 protein levels were decreased after 24 h of insulin, but not after Nrg1β stimulation. The insulin-induced decrease was blocked by the proteasomal inhibitor MG132. This shows that insulin triggers proteasomal degradation of IRS-1/2 consistent with previous publications (Greene et al., 2003), whereas Nrg1β does not cause any IRS degradation, indicating that phosphorylation of the Ser sites by itself is not enough to trigger degradation. Whether ubiquitination and proteasomal degradation of IRS requires phosphorylation of the activation site and/or other sites remains to be investigated. Notably, the different phosphorylated and total protein levels of IRS did not modify the responsiveness to insulin in our model. Moreover, the lack of a negative feedback loop after Nrg stimulation suggests that IRS-1 and -2 are not implicated in mediating Nrg1β signaling. Instead, the ErbB2/4 heterodimer may directly activate PI3K, as proposed by Jones *et al* (Jones et al., 2006).

Downstream of PI3K and Akt and similar to insulin, Nrg1β causes AS160 phosphorylation, suggesting that GLUT4 translocation to the sarcolemma is mediating the increase in glucose uptake for both factors. Indeed, our microscopy data revealed that Nrg1β is as potent as insulin to induce GLUT4 translocation in NRVMs. Similarly, GLUT4 translocation in response to Nrg1 was also observed in L6L9 skeletal muscle cells (Canto et al., 2004). Moreover, our knockdown experiments provided support that GLUT4 contributes to Nrg1β-induced glucose uptake. The lack of a strong inhibition could be due to remaining GLUT4 protein or by compensatory uptake via one or more of the other GLUT transporters such as GLUT1, GLUT3, GLUT8, GLUT10, GLUT12 and SGLT1, that recently have been detected in the heart (Aerni-Flessner et al., 2012; Szablewski, 2017). GLUT1 knockdown reduced basal glucose uptake

more than GLUT4, consistent with GLUT1 being mainly responsible for basal glucose uptake (Kraegen et al., 1993).

Collectively, our data demonstrate that Nrg1β induces glucose uptake in NRVMs *in vitro* by activating the ErbB2/ErbB4 heterodimer, which triggers phosphorylation of Akt and AS160, leading to GLUT4 translocation (Fig. 7). Similarly, Nrg1β also successfully induced ErbB signaling in the hearts of neonatal rats, indicated by increases in ErbB4-pTyr^{1284}, Akt-pThr^{308}, AS160-pThr^{642} and Erk1/2-pThr^{202}/Tyr^{204}. Two out of four Nrg1β-treated hearts show only a small increase in ErbB4 phosphorylation, indicating that these animals responded less well to Nrg1β. Consistently, these hearts also have lower phosphorylated Akt, AS160 and Erk1/2. Still, the Nrg1β-induced increase was significant for Akt, whereas for AS160 the effect almost reached statistical significance (P=0.052). The data suggest that cardiac glucose uptake may be stimulated by Nrg1β *in vivo* like *in vitro*.

On the other hand, clear differences existed between neonatal and adult CMs. Nrg1β did not stimulate glucose uptake in adult CMs and consistently, Akt and AS160 phosphorylation was very low compared to NRVMs. At the same time, Nrg1β significantly increased Erk1/2 phosphorylation, indicating activation of the MAPK pathway and confirming responsiveness of the cells to Nrg1β. The difference between NRVMs and adult CMs could be due to different expression levels of the ErbB isoforms (Camprecios et al., 2011). In contrast to our data, Cote et al. showed that Nrg1β increased glucose uptake in adult rat CMs (Cote et al., 2005). This discrepancy could be due to the fact that they used the EGF domain of Nrg1β, whereas we used the full extracellular domain. Notably, in their dose response experiment no saturation of the glucose response was reached at very high concentrations of the peptide.

Our data revealed clear differences in the responsiveness to Nrg1β between CMs isolated from healthy neonatal and adult rats. Possibly, the activation of the Akt pathway and glucose uptake along with Erk1/2 activation in neonatal cells is related to the capacity of young mice to regenerate their heart (Polizzotti et al., 2015). On the other hand, it was observed that Nrg1β signaling is important in the adult heart under stress conditions. Nrg1 expression is increased upon mechanical strain as well as during pregnancy, situations when cardiac demand is increased (Lemmens et al., 2011; Lemmens et al., 2006). While depressed endothelial Nrg synthesis impairs recovery of cardiac contractile function after an ischemic insult (Hedhli et al., 2011), treatment with recombinant human Nrg1 peptide improved cardiac function in a rat myocardial ischemia model (Liu et al., 2006). Moreover, ErbB3 gene

expression is upregulated after ischemia/reperfusion injury and contributes to the recovery of the adult heart (Morano et al., 2017). Treating the ischemic heart with additional Nrg1β could lead to increased glucose consumption by the CMs, which would supply the heart with the energy needed to continue contracting when oxygen levels are too low to perform oxidative phosphorylation.

Taken together, we could show for the first time that Nrg1β induces GLUT4 translocation in NRVMs by a similar mechanism as insulin. It remains to be investigated whether Nrg1β activates the same mechanism in the adult heart under stress situations.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (Grant No. 310030-169996/1 and 310030-135559/1).

Disclosure Statement

The authors declare to have no conflict of interests.

References

1 Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G: Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 1995;378:390-394.

2 Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C: Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 1995;378:394-398.

3 Meyer D, Birchmeier C: Multiple essential functions of neuregulin in development. Nature 1995;378:386-390.

4 Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW: ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. Development 1997;124:4999-5011.

5 Harvey RP, Wystub-Lis K, Del Monte-Nieto G, Graham RM, Tzahor E: Cardiac Regeneration Therapies - Targeting Neuregulin 1 Signalling. Heart Lung Circ 2015

6 Yester JW, Kuhn B: Mechanisms of Cardiomyocyte Proliferation and Differentiation in Development and Regeneration. Curr Cardiol Rep 2017;19:13.

7 Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, Li T, Liu X, Xu Y, Li X, Zhou M: A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. J Am Coll Cardiol 2010;55:1907-1914.

8 Jabbour A, Hayward CS, Keogh AM, Kotlyar E, McCrohon JA, England JF, Amor R, Liu X, Li XY, Zhou MD, Graham RM, Macdonald PS: Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. Eur J Heart Fail 2011;13:83-92.

9 Lenihan DJ, Anderson SA, Lenneman CG, Brittain E, Muldowney JAS, Mendes L, Zhao PZ, Iaci J, Frohwein S, Zolty R, Eisen A, Sawyer DB, Caggiano AO: A Phase I, Single Ascending Dose Study of Cimaglermin Alfa (Neuregulin 1β3) in Patients With Systolic Dysfunction and Heart Failure. JACC: Basic to Translational Science 2016;1:576-586.

10 Pentassuglia L, Sawyer DB: The role of Neuregulin-1beta/ErbB signaling in the heart. Exp Cell Res 2009;315:627-637.

11 Fuller SJ, Sivarajah K, Sugden PH: ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. J Mol Cell Cardiol 2008;44:831-854.

12 Pentassuglia L, Heim P, Lebboukh S, Morandi C, Xu L, Brink M: Neuregulin-1beta promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes. Am J Physiol Endocrinol Metab 2016;310:E782-794.

13 Shao D, Tian R: Glucose Transporters in Cardiac Metabolism and Hypertrophy. Compr Physiol 2015;6:331-351.

14 Riehle C, Abel ED: Insulin Signaling and Heart Failure. Circ Res 2016;118:1151-1169.

15 Lopaschuk GD, Jaswal JS: Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. J Cardiovasc Pharmacol 2010;56:130-140.

16 Szablewski L: Glucose transporters in healthy heart and in cardiac disease. Int J Cardiol 2017;230:70-75.

17 Hedhli N, Huang Q, Kalinowski A, Palmeri M, Hu X, Russell RR, Russell KS: Endothelium-derived neuregulin protects the heart against ischemic injury. Circulation 2011;123:2254-2262.

18 Kuramochi Y, Cote GM, Guo X, Lebrasseur NK, Cui L, Liao R, Sawyer DB: Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. J Biol Chem 2004;279:51141-51147.

19 Jaldin-Fincati JR, Pavarotti M, Frendo-Cumbo S, Bilan PJ, Klip A: Update on GLUT4 Vesicle Traffic: A Cornerstone of Insulin Action. Trends Endocrinol Metab 2017

20 Leto D, Saltiel AR: Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol 2012;13:383-396.

21 Asrih M, Pellieux C, Papageorgiou I, Lerch R, Montessuit C: Role of ERK1/2 activation in microtubule stabilization and glucose transport in cardiomyocytes. Am J Physiol Endocrinol Metab 2011;301:E836-843.

22 Montessuit C, Papageorgiou I, Lerch R: Nuclear receptor agonists improve insulin responsiveness in cultured cardiomyocytes through enhanced signaling and preserved cytoskeletal architecture. Endocrinology 2008;149:1064-1074.

23 Zhao YY, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, Kelly RA: Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. J Biol Chem 1998;273:10261-10269.

24 Camprecios G, Lorita J, Pardina E, Peinado-Onsurbe J, Soley M, Ramirez I: Expression, localization, and regulation of the neuregulin receptor ErbB3 in mouse heart. J Cell Physiol 2011;226:450-455.

25 Lawson JW, Uyeda K: Effects of insulin and work on fructose 2,6-bisphosphate content and phosphofructokinase activity in perfused rat hearts. J Biol Chem 1987;262:3165-3173.

26 Knowlden JM, Gee JM, Barrow D, Robertson JF, Ellis IO, Nicholson RI, Hutcheson IR: erbB3 recruitment of insulin receptor substrate 1 modulates insulin-like growth factor

receptor signalling in oestrogen receptor-positive breast cancer cell lines. Breast Cancer Res 2011;13:R93.

27 Knowlden JM, Jones HE, Barrow D, Gee JM, Nicholson RI, Hutcheson IR: Insulin receptor substrate-1 involvement in epidermal growth factor receptor and insulin-like growth factor receptor signalling: implication for Gefitinib ('Iressa') response and resistance. Breast Cancer Res Treat 2008;111:79-91.

28 Jones RB, Gordus A, Krall JA, MacBeath G: A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature 2006;439:168-174.

29 Kaburagi Y, Yamauchi T, Yamamoto-Honda R, Ueki K, Tobe K, Akanuma Y, Yazaki Y, Kadowaki T: The mechanism of insulin-induced signal transduction mediated by the insulin receptor substrate family. Endocr J 1999;46 Suppl:S25-34.

30 Rui L, Fisher TL, Thomas J, White MF: Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J Biol Chem 2001;276:40362-40367.

31 Copps KD, White MF: Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 2012;55:2565-2582.

32 Lim CY, Bi XZ, Wu DH, Kim JB, Gunning PW, Hong WJ, Han WP: Tropomodulin3 is a novel Akt2 effector regulating insulin-stimulated GLUT4 exocytosis through cortical actin remodeling. Nat Commun 2015;6

33 Liu X, Gu X, Li Z, Li X, Li H, Chang J, Chen P, Jin J, Xi B, Chen D, Lai D, Graham RM, Zhou M: Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. J Am Coll Cardiol 2006;48:1438-1447.

34 Canto C, Suarez E, Lizcano JM, Grino E, Shepherd PR, Fryer LG, Carling D, Bertran J, Palacin M, Zorzano A, Guma A: Neuregulin signaling on glucose transport in muscle cells. J Biol Chem 2004;279:12260-12268.

35 Hellyer NJ, Kim HH, Greaves CH, Sierke SL, Koland JG: Cloning of the rat ErbB3 cDNA and characterization of the recombinant protein. Gene 1995;165:279-284.

36 Carraway KL, 3rd, Soltoff SP, Diamonti AJ, Cantley LC: Heregulin stimulates mitogenesis and phosphatidylinositol 3-kinase in mouse fibroblasts transfected with erbB2/neu and erbB3. J Biol Chem 1995;270:7111-7116.

37 Peng J, He L: IRS posttranslational modifications in regulating insulin signaling. J Mol Endocrinol 2018;60:R1-R8.

38 Esposito DL, Li Y, Cama A, Quon MJ: Tyr(612) and Tyr(632) in human insulin receptor substrate-1 are important for full activation of insulin-stimulated phosphatidylinositol 3-kinase activity and translocation of GLUT4 in adipose cells. Endocrinology 2001;142:2833-2840.

39 Bouzakri K, Roques M, Gual P, Espinosa S, Guebre-Egziabher F, Riou JP, Laville M, Le Marchand-Brustel Y, Tanti JF, Vidal H: Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 2003;52:1319-1325.

40 Greene MW, Sakaue H, Wang L, Alessi DR, Roth RA: Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by Serine 312 phosphorylation. J Biol Chem 2003;278:8199-8211.

41 Aerni-Flessner L, Abi-Jaoude M, Koenig A, Payne M, Hruz PW: GLUT4, GLUT1, and GLUT8 are the dominant GLUT transcripts expressed in the murine left ventricle. Cardiovasc Diabetol 2012;11:63.

42 Kraegen EW, Sowden JA, Halstead MB, Clark PW, Rodnick KJ, Chisholm DJ, James DE: Glucose transporters and in vivo glucose uptake in skeletal and cardiac muscle: fasting, insulin stimulation and immunoisolation studies of GLUT1 and GLUT4. Biochem J 1993;295 (Pt 1):287-293.

43 Cote GM, Miller TA, Lebrasseur NK, Kuramochi Y, Sawyer DB: Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. Exp Cell Res 2005;311:135-146.

44 Polizzotti BD, Ganapathy B, Walsh S, Choudhury S, Ammanamanchi N, Bennett DG, Dos Remedios CG, Haubner BJ, Penninger JM, Kuhn B: Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. Science translational medicine 2015;7:281ra245.

45 Lemmens K, Doggen K, De Keulenaer GW: Activation of the neuregulin/ErbB system during physiological ventricular remodeling in pregnancy. Am J Physiol Heart Circ Physiol 2011;300:H931-942.

46 Lemmens K, Segers VF, Demolder M, De Keulenaer GW: Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk. J Biol Chem 2006;281:19469-19477.

47 Morano M, Angotti C, Tullio F, Gambarotta G, Penna C, Pagliaro P, Geuna S: Myocardial ischemia/reperfusion upregulates the transcription of the Neuregulin1 receptor ErbB3, but only postconditioning preserves protein translation: Role in oxidative stress. Int J Cardiol 2017;233:73-79.

Figure 1

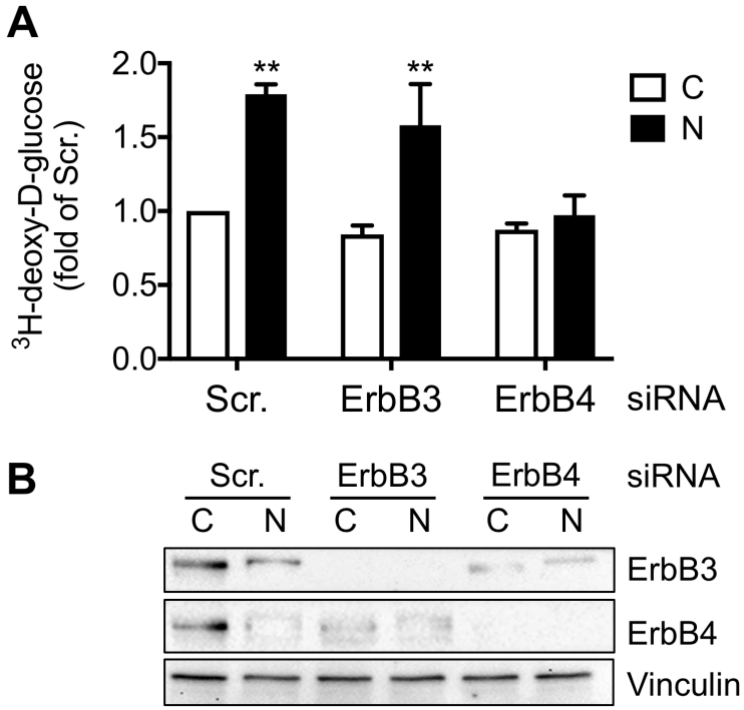


Figure 1. Nrg1β induces glucose uptake independently of ErbB3

A: NRVMs were transfected with siRNA specific for ErbB3 and ErbB4. 48 h after transfection, NRVMs were stimulated for 30 min with Nrg1β (10 ng/ml) or control vehicle. Glucose uptake was assessed by 30 min incubation with 1 µCi/ml [^{3}H]-deoxy-D-glucose. Counts were normalized to total protein/well (n = 3). **B:** siRNA-transfected NRVMs were treated for 30 min with Nrg1β (10 ng/ml). Western blot analysis was performed to detect ErbB3 and ErbB4. **P < 0.01 Nrg1β vs. vehicle. Control vehicle (C), Nrg1β (N), scrambled (Scr.).

Figure 2

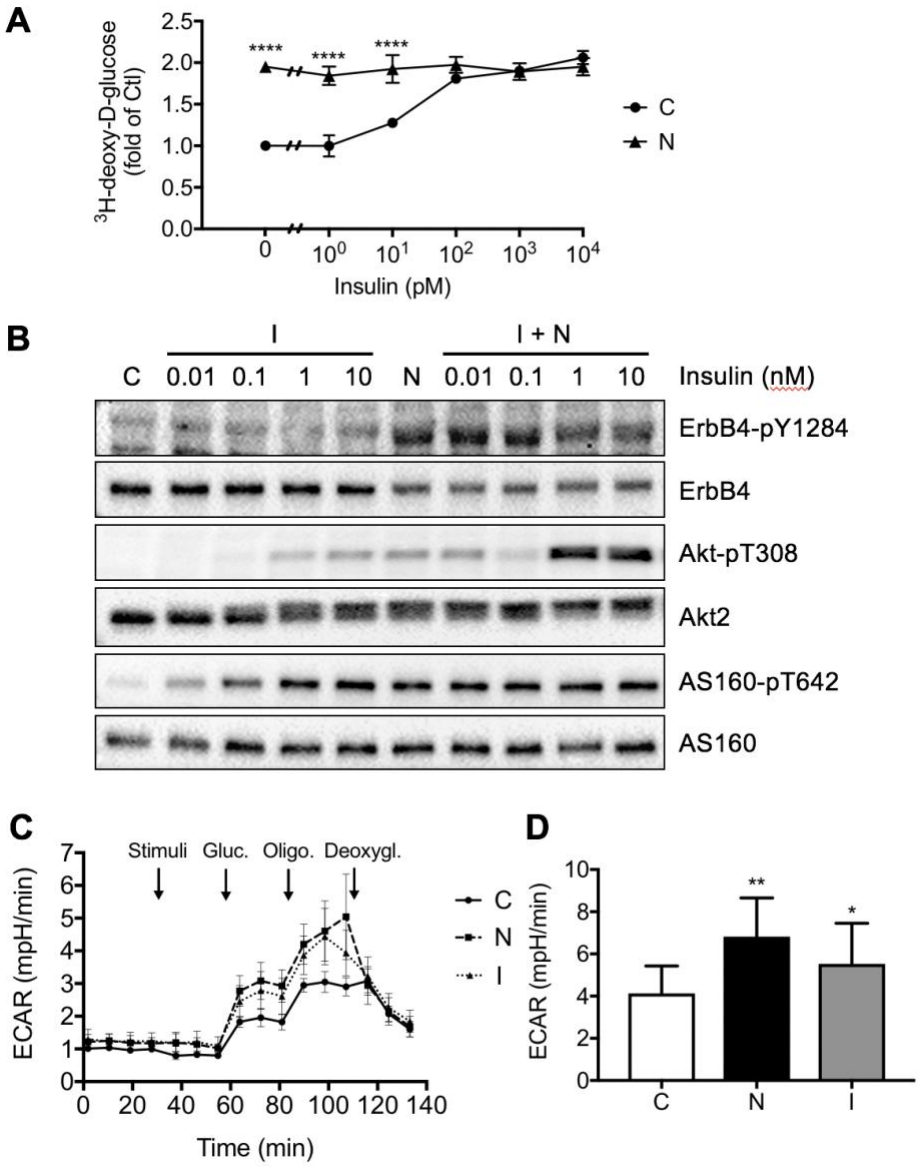


Figure 2. Nrg1β has no additive effect on glucose uptake in presence of insulin

A: NRVMs were stimulated for 30 min with an increasing dose of insulin in presence or absence of Nrg1β (20 ng/ml) or control vehicle. Glucose uptake was assessed. **B:** NRVMs were treated like in A and the phosphorylation status of the indicated proteins was detected by Western blot analysis. **C:** Extracellular acidification rate (ECAR) was measured by Seahorse XF24. The cells were stimulated with Nrg1β (10 ng/ml) or insulin (2.6 nM) and treated with D-glucose (10 mM), oligomycin (2 µM) and deoxyglucose (50 mM). **D:** The rate of glycolysis was determined according to the manufacturer's instructions (D, n = 6). *P < 0.05, **P < 0.01, ****P < 0.0001 Nrg1β/insulin vs. vehicle. Control vehicle (C), Nrg1β (N), insulin (I).

Figure 3

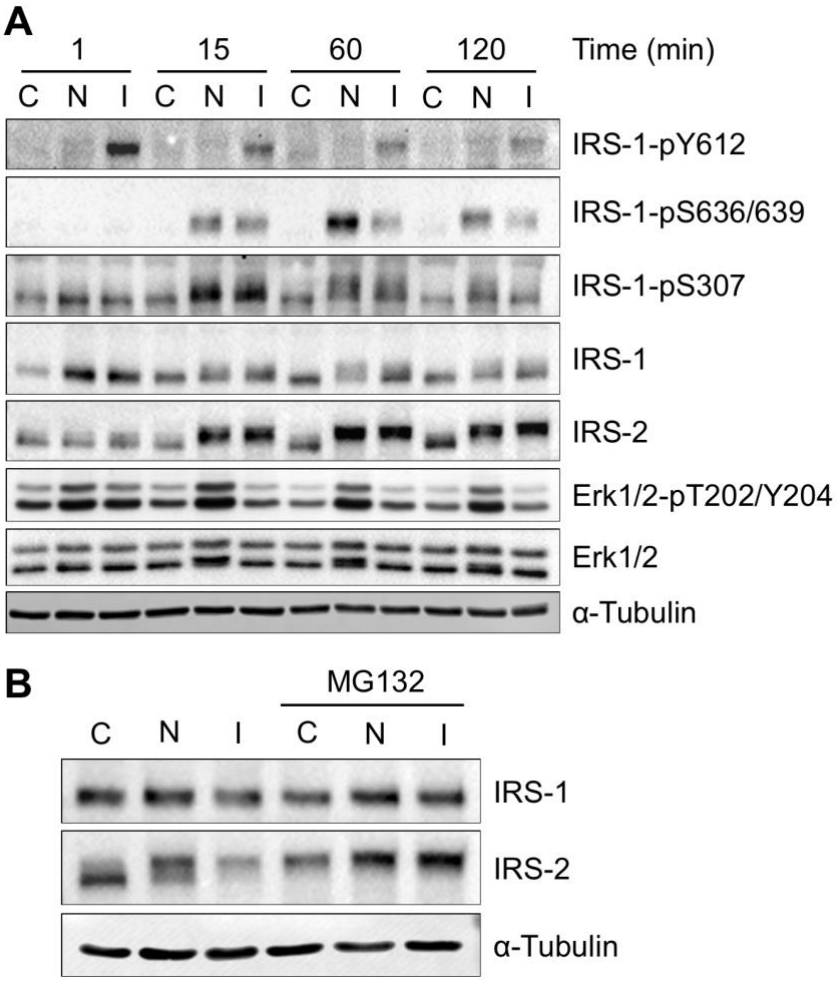


Figure 3. Total IRS-1 is reduced and IRS-1 Y612 is only phosphorylated in presence insulin

A: NRVMs were stimulated for 1, 15, 60 and 120 min with Nrg1β (10 ng/ml), insulin (2.6 nM) or control vehicle. By Western blot analysis, the phosphorylation status of the indicated proteins was detected. **B:** NRVMs were preincubated for 30 min with the proteasomal inhibitor MG132 (10 µM) or DMSO, followed by 24 h stimulation with Nrg1β or insulin (B). Total IRS proteins were detected by Western blot. Representative experiments shown; n = 3. Control vehicle (C), Nrg1β (N), insulin (I).

Figure 4

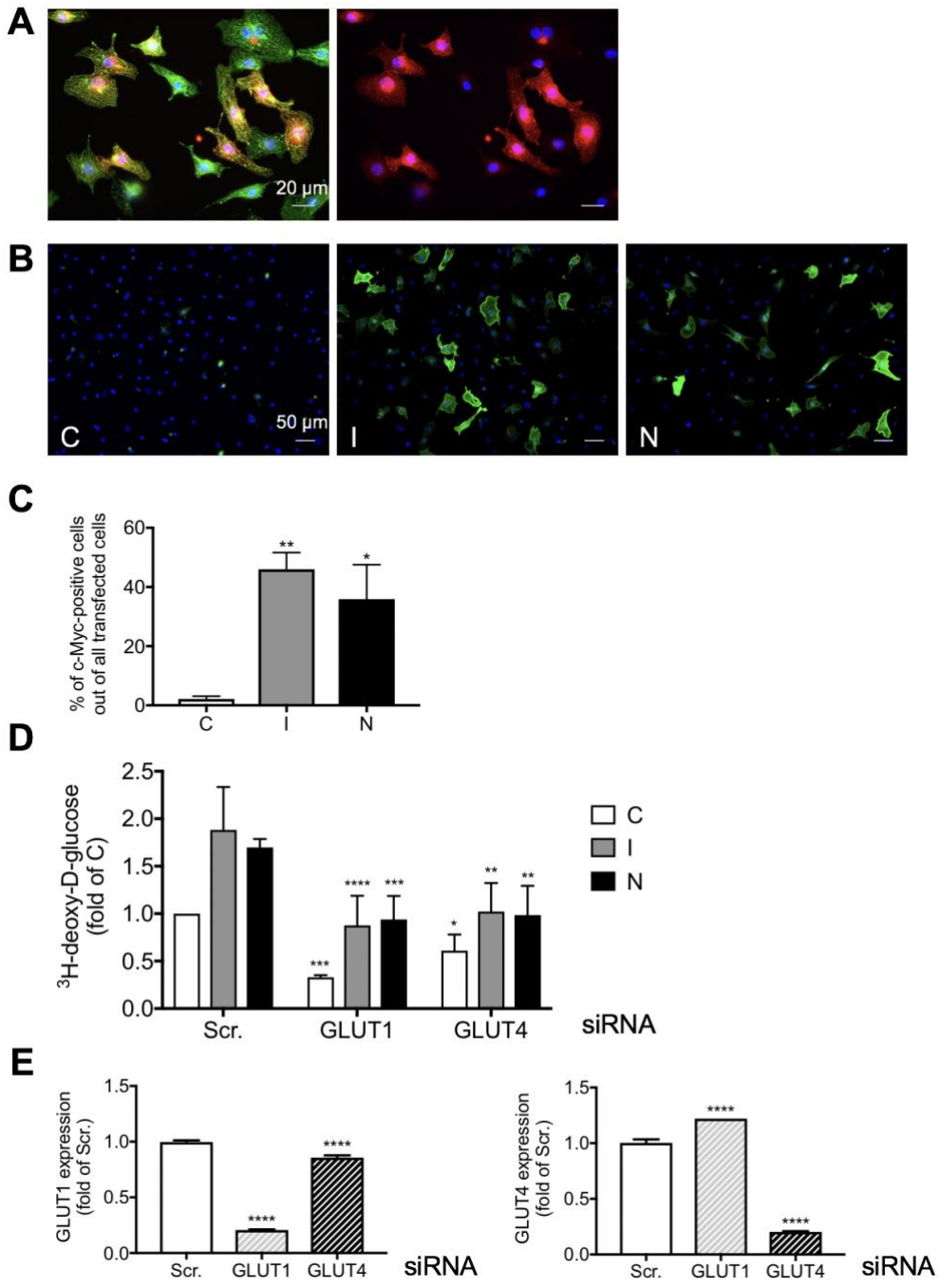


Figure 4. Nrg1β induces GLUT4 translocation

A: Representative picture of NRVMs that were transfected with pLenti-myc-GLUT4-mCherry plasmid (Addgene 64049, red). 72 h after transfection, the cells were immunostained against sarcomeric actinin (green) and the nuclei were stained with DAPI (blue). Left picture: all colors; right picture: only blue and red. Scale bar: 20 µm. **B:** Representative pictures of pLenti-myc-GLUT4-mCherry transfected NRVMs, stimulated with Nrg1β (N, 50 ng/ml), insulin (I, 13

nM) or control vehicle (C) and fixed after 30 min. With a c-Myc specific antibody, translocated GLUT4 was detected by immunofluorescence on non-permeabilized cells. Scale bar: 50 µm. **C:** Quantification of translocated GLUT4 induced by Nrg1β (n = 3, 583 cells), insulin (n = 3, 620 cells) or control vehicle (n = 2, 476 cells) stimulation. The percentage of c-Myc-positive cells out of all transfected GLUT4-mCherry-positive cells was determined. **D:** NRVMs were transfected with siRNA specific for GLUT1 or GLUT4. 48 h after transfection, NRVMs were stimulated for 30 min with Nrg1β (10 ng/ml), insulin (2.6 nM) or control vehicle and glucose uptake was assessed. Counts were normalized to total protein/well; n = 3. **E:** The expression mRNA of GLUT1 and GLUT4 of siRNA transfected NRVMs was detected by qRT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 GLUT1 or GLUT4 vs. scrambled. Control vehicle (C), Nrg1β (N), insulin (I), scrambled (Scr.).

Figure 5

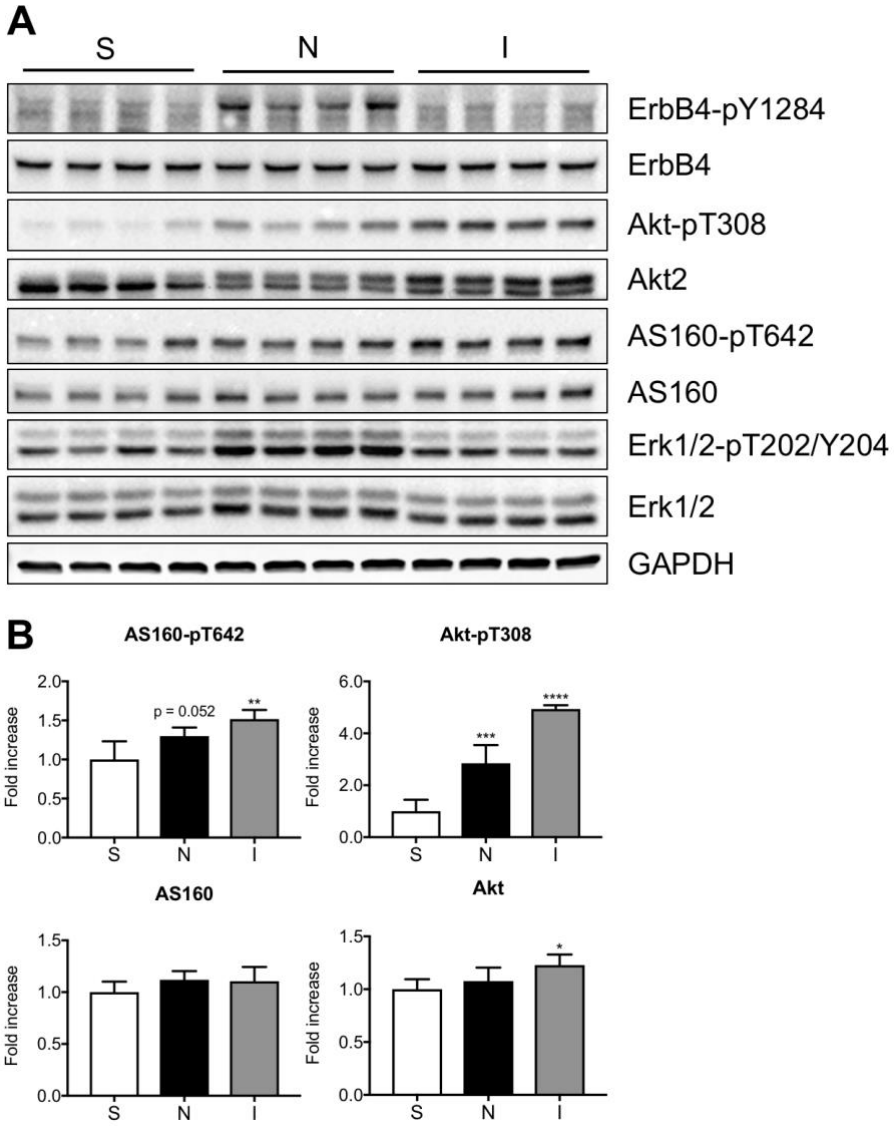


Figure 5. Neonatal rat hearts show a similar signaling response to Nrg1β like in vitro

A: 1-day-old neonatal rats (n = 4) were injected i.p. with Nrg1β (50 µ/kg body weight), insulin (1 U/kg body weight) or saline. 30 min later, animals were sacrificed and proteins were extracted from the whole heart. By Western blot analysis, the phosphorylation status of several signaling molecules were assessed. **B:** Quantification of AS160 and Akt, normalized to saline. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs. saline. Control saline (S), Nrg1β (N), insulin (I).

Figure 6

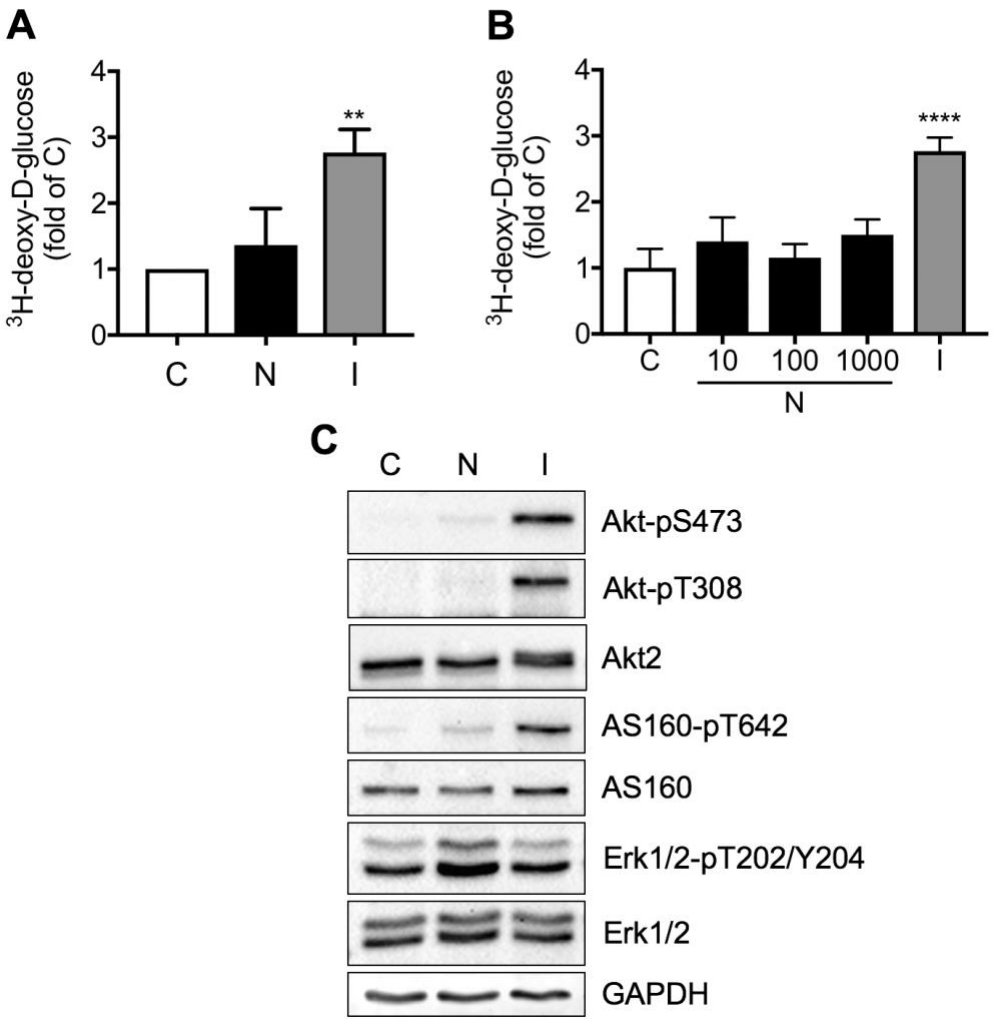


Figure 6. Adult cardiomyocytes differ in their response to Nrg1β stimulation

A: Adult cardiomyocytes were stimulated with Nrg1β (10 ng/ml) or insulin (1 µM) and glucose uptake was assessed. **B:** Adult cardiomyocytes were stimulated with 10, 100 and 1'000 ng/ml of Nrg1β. Insulin (1 µM) served as positive control and glucose uptake was assessed. **C:** In parallel, the phosphorylation status of several signaling proteins was detected by Western blot analysis. *P < 0.05, ***P < 0.001 and ****P < 0.0001 vs. control vehicle; n = 3. Control vehicle (C), Nrg1β (N), insulin (I).

Figure 7

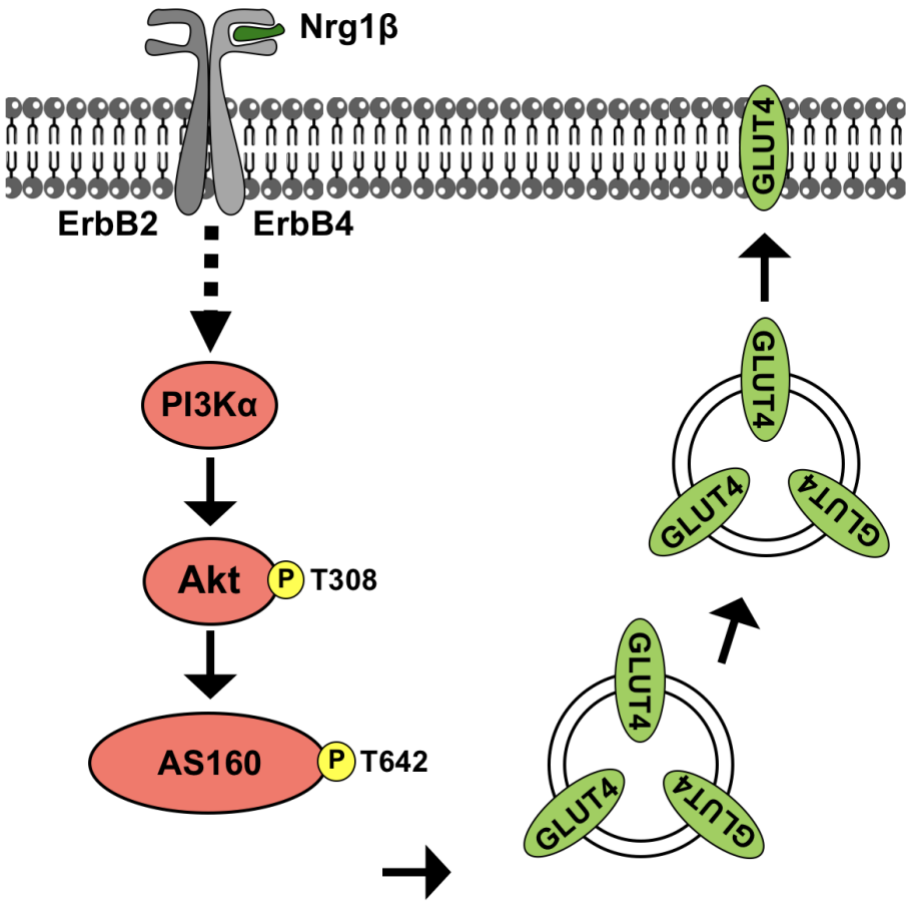
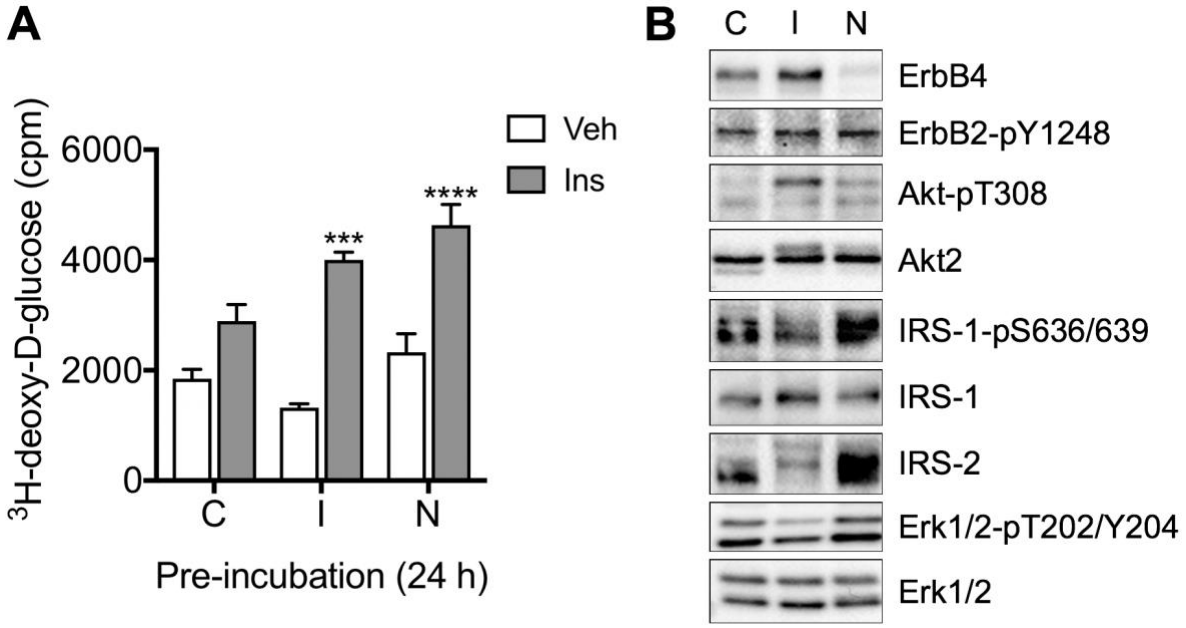


Figure 7. Working model for the pathway of Nrg1β-induced glucose uptake in neonatal NRVMs

Supplementary figure 1



Supplementary figure 1. Long-term stimulation with insulin and Nrg1β

A: After 24 h of stimulation with Nrg1β (10 ng/mL), insulin (2.6 nM) or vehicle (control), NRVMs were re-stimulated for 30 min with insulin or vehicle. Glucose uptake (cpm, counts per minute) was assessed as for Fig. 1. **B:** The phosphorylation status of the indicated proteins was detected after 24 h of stimulation by Western blot analysis. ***P < 0.001 and ****P < 0.0001 vs. control vehicle; n = 3. Control vehicle (C), Nrg1β (N), insulin (I).

3.3. Unpublished projects

The data presented in this chapter were not yet included in any manuscript, although the experiments were part of my PhD project. As my preliminary data provides important information for other studies of our lab and may lead to additional interesting findings, I would like here to highlight and discuss some of them. The aims of the experiments were:
(1) to analyze whether Nrg1β stimulates signaling pathways that regulate glucose metabolism using the STZ mouse model of diabetes.
(2) to test if Nrg1β can stimulate NRVM proliferation.
(3) to compare the ErbB receptors and glucose transporter expression in adult and neonatal whole hearts and NRVMs.

3.3.1. Effects of Nrg1β on glucose metabolism in a diabetic mouse model - preliminary data

Introduction

It was observed that the myocardial endothelium releases Nrg1 during ischemia-reperfusion (Kuramochi et al., 2004a) and more recently, ErbB3 was demonstrated to contribute to the recovery of the heart after an ischemic insult (Morano et al., 2017). Our *in vitro* experiments with NRVMs showed that Nrg can increase glucose uptake, based on which we hypothesized that the cardioprotective effects of Nrg1β may, at least in part, be related to enhanced glucose use by CMs. Indeed, it is known that glucose uptake increases acutely during ischemic events (Russell et al., 2004). As the effects of Nrg in cardiac ischemia models are already being investigated by several other groups (Fang et al., 2010; Galindo et al., 2014; Hill et al., 2013; Liu et al., 2006) and the technically difficult ischemia model was not established in our laboratory, we decided to explore the use of Nrg1β in a different clinical context, namely that of diabetes.

Nrg may be of therapeutic value in diabetic conditions, as suggested by pre-clinical studies in rodent models (Li et al., 2013; Li et al., 2011; Vandekerckhove et al., 2016). Diabetic patients are prone to develop diabetic cardiomyopathy, which is characterized by diastolic dysfunction. T1DM is associated with decreased insulin and hyperglycemia, whereas T2DM is associated with increased levels of fatty acids and inflammatory cytokines, impaired

responses to insulin and consequently hyperglycemia and hyperinsulinemia. Together, these factors change specific molecular pathways in CMs, leading to impaired cardiac contractility and cell death (Bugger and Abel, 2014; Kota et al., 2011). In the diabetic heart, the CMs are deprived of glucose and limited to fatty acids as energy source. Recently, it was shown that i.p. injection of Nrg1β enhances whole body glucose uptake in mice (Ennequin et al., 2015). Based on this study as well as our own finding of Nrg1β-stimulated glucose uptake in neonatal cardiomyocytes, our aim was to test whether Nrg1β stimulates cardiac glucose uptake *in vivo*. To this end, we started to establish an STZ mouse model of diabetes in our laboratory. STZ destroys primarily the beta cells of the islets of Langerhans and thereby reduces insulin secretion and consequently increases blood glucose levels (Ito et al., 2001). As a first step towards answering the question whether Nrg enhances glucose uptake and thereby contributes to the protection of the adult heart, we analyzed signaling pathway activation in the hearts of insulin- and Nrg1β-injected STZ mice, with focus on glucose-uptake related pathways.

Results

*Nrg1*β *and insulin lower blood glucose in STZ mice*

After STZ injection, the STZ animals reduced their body weight compared to non-STZ animals, which show a constant increase in body weight over time (Fig. 1A). Three weeks after the last STZ injection, blood glucose was measured and revealed elevated blood glucose levels in the STZ group (Fig. 1B). At 5 weeks after STZ, the hyperglycemic mice were treated with insulin or Nrg1β and blood glucose was measured over time (Fig. 2A). Whereas insulin rapidly reduced glucose levels to its lowest values (5.6 mM) within 30 min, Nrg1β started to lower blood glucose only after 30 min, reaching similarly low levels (7.9 mM) between 60 and 120 min. Thus, the kinetics of the two stimuli are clearly different. When the area under the curve (AUC) was analyzed, only the glucose-lowering effect of insulin was significant, whereas that of Nrg1β showed a trend (P=0.066) (Fig. 2B). The different kinetics suggests distinct mechanisms for the two factors. To test whether Nrg1β induces insulin release and thereby increases glucose clearance indirectly, we measured plasma insulin levels 30 min after the Nrg1β injection, the time point when Nrg1β started to lower blood glucose. As expected, plasma insulin was elevated in the insulin-treated mice, confirming that our ELISA assay

worked. Notably, Nrg1β did not influence plasma insulin at 30 min after injection (Fig. 2C), the time point at which plasma glucose started to decrease in this group of mice.

Analysis of cardiac signaling molecules

The mice were sacrificed 11 weeks after the last STZ injection and 30 min after i.p. injection with the insulin, Nrg1β or saline as a control. Western blot analysis of total protein extracts of the left ventricles demonstrated increased phosphorylation of p70S6K1 at Thr^{389} and Erk1/2 at $Thr^{202/204}$ after Nrg1β stimulation, but no changes in Akt phosphorylation (Fig. 3A). In contrast to Nrg1β, insulin triggered phosphorylation of Akt at Thr^{308}. For Nrg1β, AS160 phosphorylation was not changed, indicating that the GLUT4 translocation-related signaling mechanism that we found to be activated in neonatal cardiomyocytes, is not activated in the adult heart of STZ-treated mice. Notably, the effect of insulin on AS160 was also small and would require more animals. In non-STZ mice, we could not detect any increase in Akt or AS160 phosphorylation after Nrg1β stimulation either (data not shown).

Since we observed that Nrg1β lowered blood glucose, we decided to study another organ that could be responsible for blood glucose clearance. Next to fat and skeletal muscle, the liver is implicated in glucose uptake and known to increase glycogen synthesis after insulin stimulation (Benito, 2011). Therefore, we investigated glucose-metabolism-related signaling in the liver of our STZ mice. Indeed, Nrg1β elevated the phosphorylation level of glycogen synthase kinase 3β (GSK3β) at Ser^{9} (Fig. 3B), suggestive of increased glycogen synthesis (Cross et al., 1995). Increased phosphorylation of ErbB3 and InsR/IGF-IR after injection of Nrg1β and insulin, respectively, confirmed specific receptor activation. Interestingly, Nrg1β strongly increased hepatic phosphorylation of Akt at Thr^{308} and Ser^{473} and GSK3β at Ser^{9}, whereas the increases induced by insulin were less pronounced.

Finally, we also analyzed signaling in skeletal muscle, but found no differences on Akt phosphorylation after Nrg1β injection (data no shown).

Discussion

Our main finding in this chapter is that Nrg1β lowers blood glucose in STZ mice like insulin, however the blood glucose clearance response after Nrg1β and insulin stimulation shows different kinetics. We therefore analyzed the possibility that Nrg1β decreases plasma glucose as a secondary effect to enhanced insulin secretion. Our data show that this is not the case.

In addition, one could still measure plasma IGF-I, which is primarily produced and released by the liver and may also lower blood glucose (Schwander et al., 1983). Perhaps the distinct kinetics could be explained by the different receptor distributions in the whole body. The insulin receptor and the ErbB receptors are expressed in many organs (www.proteinatlas.org). Insulin is known to efficiently trigger glucose uptake in several tissues, like adipose tissue, liver and skeletal muscle, whereas Nrg1β was previously shown to induce glucose uptake only in skeletal muscle cells (Canto et al., 2004). Consistent with our results, Nrg1β was recently reported to lower blood glucose in Zucker diabetic fatty rats, mainly affecting the liver (Lopez-Soldado et al., 2016). Similarly, Nrg1 improved glucose tolerance by ErbB3 activation in the liver in db/db mice (Ennequin et al., 2015). Another important point is the route of administration of the hormones, which was done by i.p. injection. It could be worth to try an intravenous injection (i.v.), thereby reaching a higher concentration of hormone in the heart.

In summary, we could not detect an effect of Nrg1β on glucose uptake-related signaling events in the heart, but we observed that Nrg1β injections affect mainly the liver of STZ mice. In the future, it would be worth to test Nrg1β in a model, in which the heart is more stressed, because it is known that the Nrg1 and ErbB receptor expression is upregulated under certain stress conditions like hemodynamic overload or pregnancy (Lemmens et al., 2011; Morano et al., 2017).

Figure 1

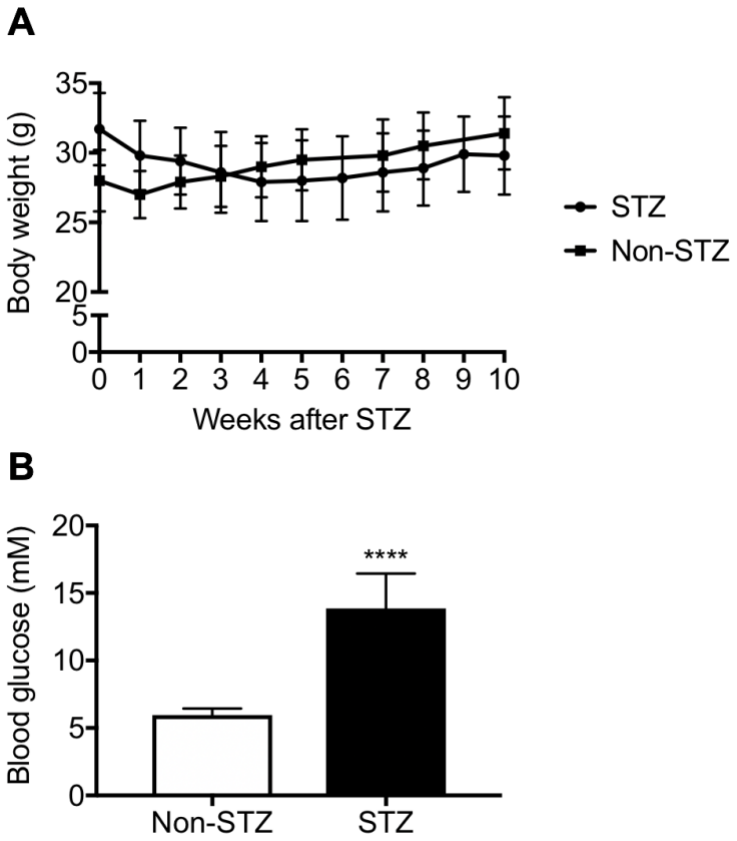


Figure 1. Effects of streptozotocin on body weight and blood glucose

Diabetes was induced by STZ administration. 14-16 weeks old C57BL/6N mice were injected i.p. with STZ (50 mg/kg body weight) to for 5 consecutive days. **A:** Body weight measurement at the indicated weeks after STZ injection. **B:** Blood glucose measurement 3 weeks after STZ injections (n = 20 and 6). ****P < 0.0001; mean +/- STDV.

Figure 2

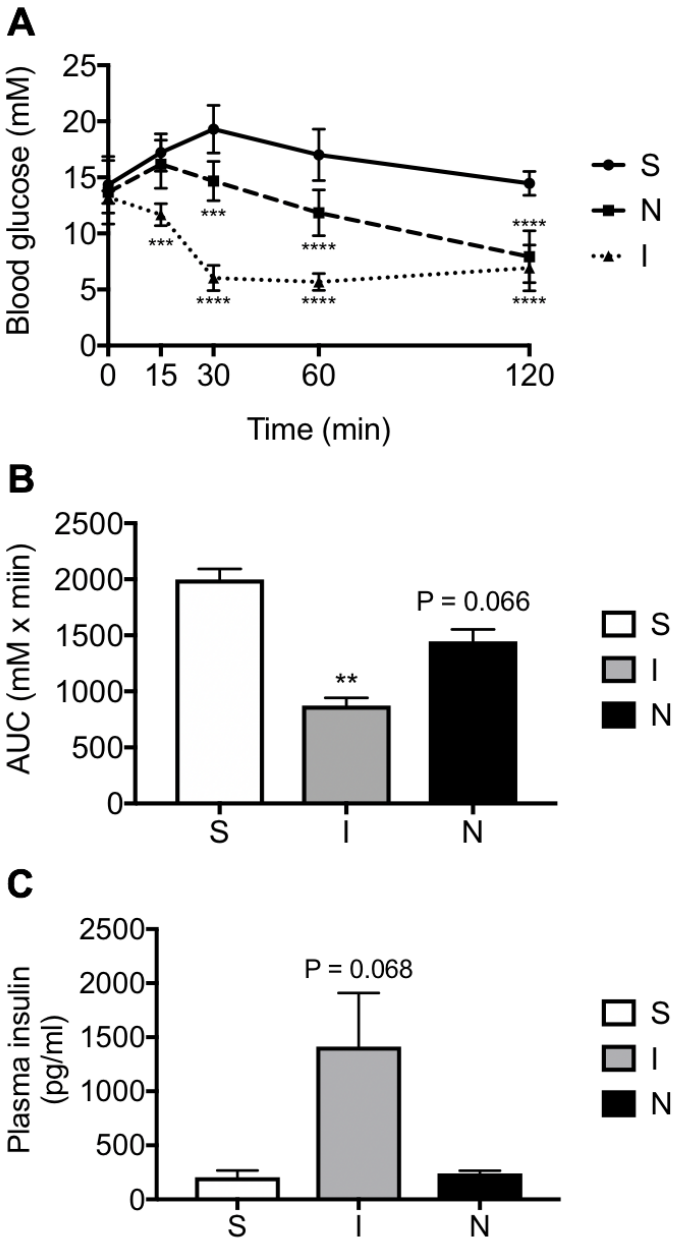


Figure 2. Effect of Nrg1β in streptozotocin mice

A: 5 weeks after STZ treatment, the mice were injected with Nrg1β (50 µg/kg body weight, n = 7), insulin (1 U/kg body weight, n = 7) or saline (n = 9) and blood glucose was measured after 15, 30, 60 and 120 min. **B:** Area under the curve (AUC) of A. **C:** The mice were sacrificed 11 weeks after STZ. Measurement of plasma insulin levels 30 min after Nrg1β and insulin injection. (n = 3-5). **P < 0.01, ***P < 0.001, ****P < 0.0001 Nrg1β or insulin vs. saline; mean +/- STDV. Saline (S), Nrg1β (N), insulin (I).

Figure 3

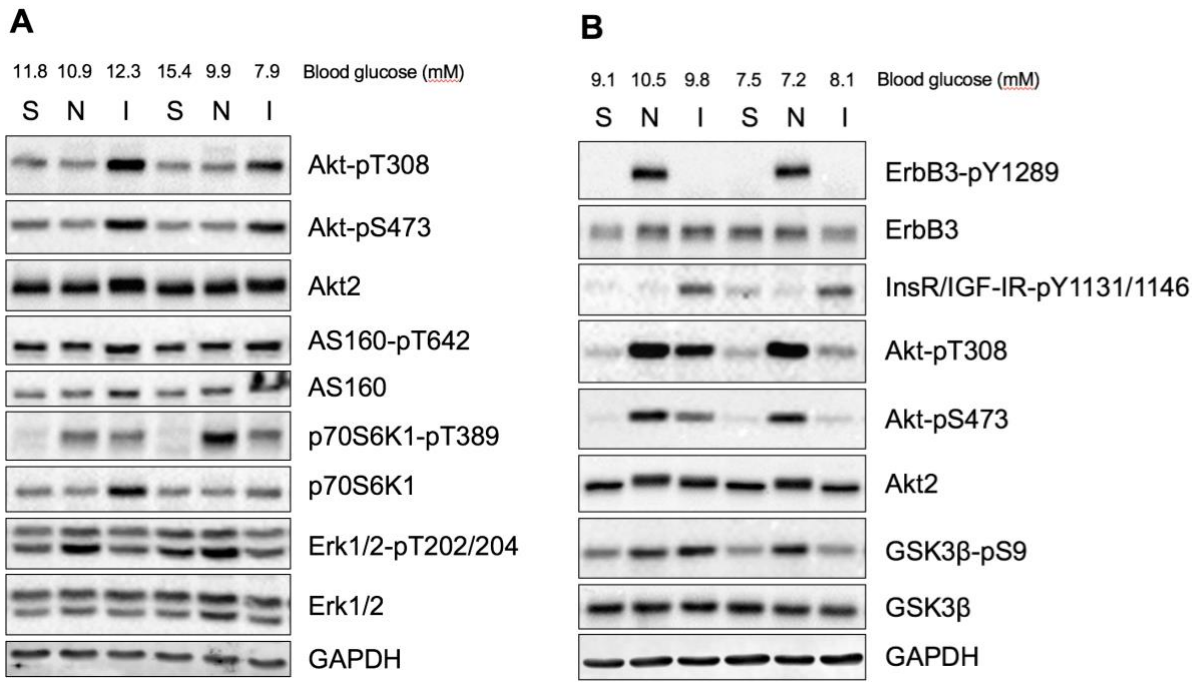


*Figure 3. Effect of Nrg1*β *on cardiac and hepatic signaling in streptozotocin mice*

All mice were sacrificed 11 weeks after STZ. **A:** Western blot analysis of heart protein extracts from STZ mice after 30 min of Nrg1β, insulin and saline injection. Levels of blood glucose after sacrifice are indicated. **B:** Western blot analysis of liver protein extracts from STZ mice after 30 min of Nrg1β, insulin and saline injection. Levels of blood glucose after sacrifice are indicated. Saline (S), Nrg1β (N), insulin (I).

3.3.2. Nrg1β and IGF-I induce proliferation of NRVMs - preliminary data

Since Nrg1β has been shown to induce CMs proliferation *in vivo* and thereby may contribute to improve heart function *(Polizzotti et al., 2015)*, we investigated the mitogenic effect of Nrg1β in NRVMs. To prove increased proliferation correctly, one should evaluate several markers. Therefore, I established the immunostaining of Histone H3-pSer^{10} (p-HH3), Ki67 and Aurora B in NRVMs (Fig. 4). All three targets are well accepted markers to analyze proliferation in CMs (Leone et al., 2015). The aim of my experiments was to test if Nrg1β can increase the number of proliferating CMs *in vitro*. To this end, NRVMs were stimulated for 48 h with Nrg1β. IGF-I was used as positive control since it was previously shown to increase proliferation in NRVMs (Kajstura et al., 1994). Immunofluorescent labeling of the three proliferation markers revealed positive cells in our cultures. Ki67 labeling detected the highest numbers of positive cells, followed by p-HH3 and finally Aurora B (Fig. 4A-C). To confirm that the positive cells were CMs and not fibroblasts or other cardiac cells that are always present in low quantities in our primary cultures, we performed double-labeling experiments with antibodies specific for sarcomeric actinin and p-HH3 (Fig. 4C). Sarcomeric actinin and p-HH3 were co-located in many of the cells, identifying these p-HH3-positive cells as cardiomyocytes. Some p-HH3-positive cells were also negative for sarcomeric actinin. We therefore next quantified only the p-HH3/sarcomeric actinin positive cells (Fig. 4D). First of all, this analysis revealed that IGF-I increases the number of p-HH3-positive CMs under the culture conditions that we established for our NRVMs. Furthermore, incubation with Nrg1β alone had no effect on proliferation. Interestingly however, when NRVMs were co-stimulated with Nrg1β and IGF-I, proliferation was significantly higher than after stimulation with IGF-I alone (Fig. 2D). Consistent with our results, increased DNA synthesis after a combined treatment with Nrg1 and IGF-I was observed (Hertig et al., 1999). These data indicate that Nrg1β contributes to the proliferative response in NRVMs, but only in the presence of IGF-I.

In the future, we want to investigate the mechanism whereby Nrg1β enhances the IGF-I-induced proliferation and test whether Nrg1β-induced glucose uptake contributes to the observed increase in proliferation in our NRVM model.

Figure 4

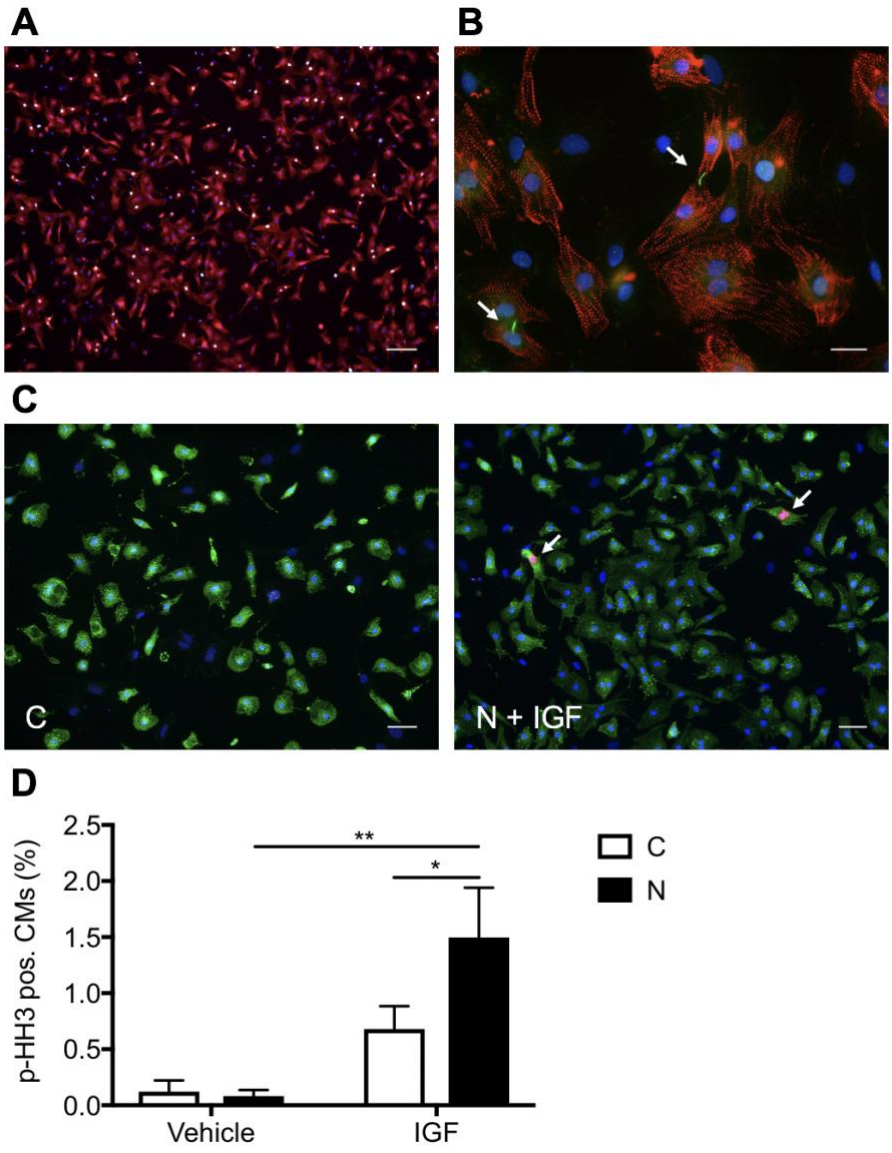


Figure 4. Analysis of Histone H3-pSer[10] (p-HH3) in NRVMs

A: Representative picture of Ki67 staining. DAPI (blue), sarcomeric actinin (red) and Ki67 (white). Scale bar: 100 µM. **B:** Representative picture of Aurora B staining. DAPI (blue), sarcomeric actinin (red) and Aurora B (green). Scale bar: 20 µM. **C:** NRVMs were stimulated for 48 h with Nrg1β (10 ng/ml), IGF-I (20 ng/ml) or vehicle. After fixation, immunostaining was performed. DAPI (blue), sarcomeric actinin (green) and p-HH3 (red). Two representative pictures of control and Nrg1β/IGF-I condition. Scale bar: 50 µM. **D:** Quantification of p-HH3 positive NRVMs (n=4, > 1'100 cells/sample counted, mean +/- SEM). *P < 0.05, IGF-I vs. Nrg1β/IGF-I; **P < 0.01, Nrg1β vs. Nrg1β/IGF-I. Control vehicle (C), Nrg1β (N), IGF-I (IGF).

3.3.3. Comparison of GLUT1/4 and ErbB receptors expression in our models

Since we observed different signaling and glucose uptake responses to Nrg1β stimulation in our models, we investigated the level of expression of the ErbB receptors and GLUT1/4.

To examine the neonatal rat model in more detail, we isolated proteins and RNA from the heart of P1, P2, P3, P4, P7 and P8 rats. By Western blot analysis, we detected that during the first week after birth, protein expression of ErbB2, ErbB4 and β-myosin heavy chain (β-MHC) were downregulated, whereas ErbB3 and GLUT4 were upregulated (Fig. 5A). β-MHC is known to be highly expressed in the developing heart and downregulated in the adult ventricle, matching our observation (Morkin, 2000). p-HH3, which is widely used as proliferation marker (Wang and Higgins, 2013), peaked at P2 and was reduced afterwards. This corresponds with literature, which demonstrated high proliferation rates after birth that are reduced within the first week of life in rats (de Carvalho et al., 2017).

Moreover, mRNA analysis by qRT-PCR revealed trends of lower GLUT1 and increased GLUT4 expression one week after birth (Fig. 5B). The mRNA expression levels of the ErbB receptors showed some minor tendencies as well. ErbB2 and ErbB4 were lower after one week, whereas ErbB1 and ErbB3 were increased compared to P1. In agreement with the literature, GLUT1, ErbB2 and ErbB4 are more expressed at birth and GLUT4 and ErbB3 expression gets upregulated after birth (Camprecios et al., 2011; Ma et al., 2016; Wang and Hu, 1991). For our cell culture model and the *in vivo* experiment with neonatal rats, we used P1-P2 old rats. These results showed that at this time point, the neonatal heart expresses all ErbB receptors, GLUT1 and GLUT4 and has very likely increased numbers of proliferating cells, indicated by elevated p-HH3.

In addition, we compared the mRNA expression of GLUT1, GLUT4 and all ErbB receptors in NRVMs, a neonatal heart from a P2 rat and an adult rat heart. Indeed, we could confirm that the adult heart has a higher expression of GLUT4 and ErbB3 and a lower expression of ErbB2, ErbB4 and GLUT1 (Fig. 6A), again matching the findings from literature (Camprecios et al., 2011; Wang and Hu, 1991). When comparing the isolated NRVMs with the whole neonatal heart tissue, which includes all cardiac cell types and not only CMs, a difference is revealed. ErbB2 mRNA is higher in isolated CMs than in whole hearts, indicating that ErbB2 density may be higher in CMs than in the other cell types of the heart. Similarly, such a comparison of

isolated NRVM with neonatal whole hearts indicates that GLUT1 is enriched in CMs. These conclusions remain to be confirmed at the protein level.

Protein lysates from ARVMs were compared with those from NRVMs. Interestingly, ARVMs showed much lower ErbB2, ErbB3 and ErbB4 protein levels than NRVMs, whereas GLUT1 and GLUT4 proteins were more abundant in ARVMs (Fig. 6B). Surprisingly, GLUT1 expression was higher in ARVMs than in NRVMs. This could be explained by the observation of Montessuit et al., which showed that ARVMs upregulate GLUT1 after isolation (Montessuit et al., 2004). Another explanation could be that it is an artefact of the antibody that might not properly detect GLUT1. Therefore, one should test additional antibodies and also measure the mRNA level of GLUT1 in ARVMs.

To conclude, in our models, we observed several differences of ErbB receptor and glucose transporter expression at the mRNA and protein level that might explain unequal responsiveness to Nrg1β stimulation. Expression of ErbB3 mRNA is much higher in the adult heart, whereas ErbB2 and ErbB4 are lower compared to the neonatal heart. Since we identified the ErbB2/4 heterodimer to be responsible to mediate Nrg1β-induced glucose uptake in NRVMs, this could be an explanation why the adult heart responded less to Nrg1β stimulation. In addition, the protein levels of all ErbB receptors were much lower in ARVMs compared to NRVMs, proposing that ARVMs may respond less to Nrg1β, which would match with our glucose uptake data from ARVMs. In summary, the different expression levels of ErbB receptors may explain the differing responses to Nrg1β stimulation between our models.

Figure 5

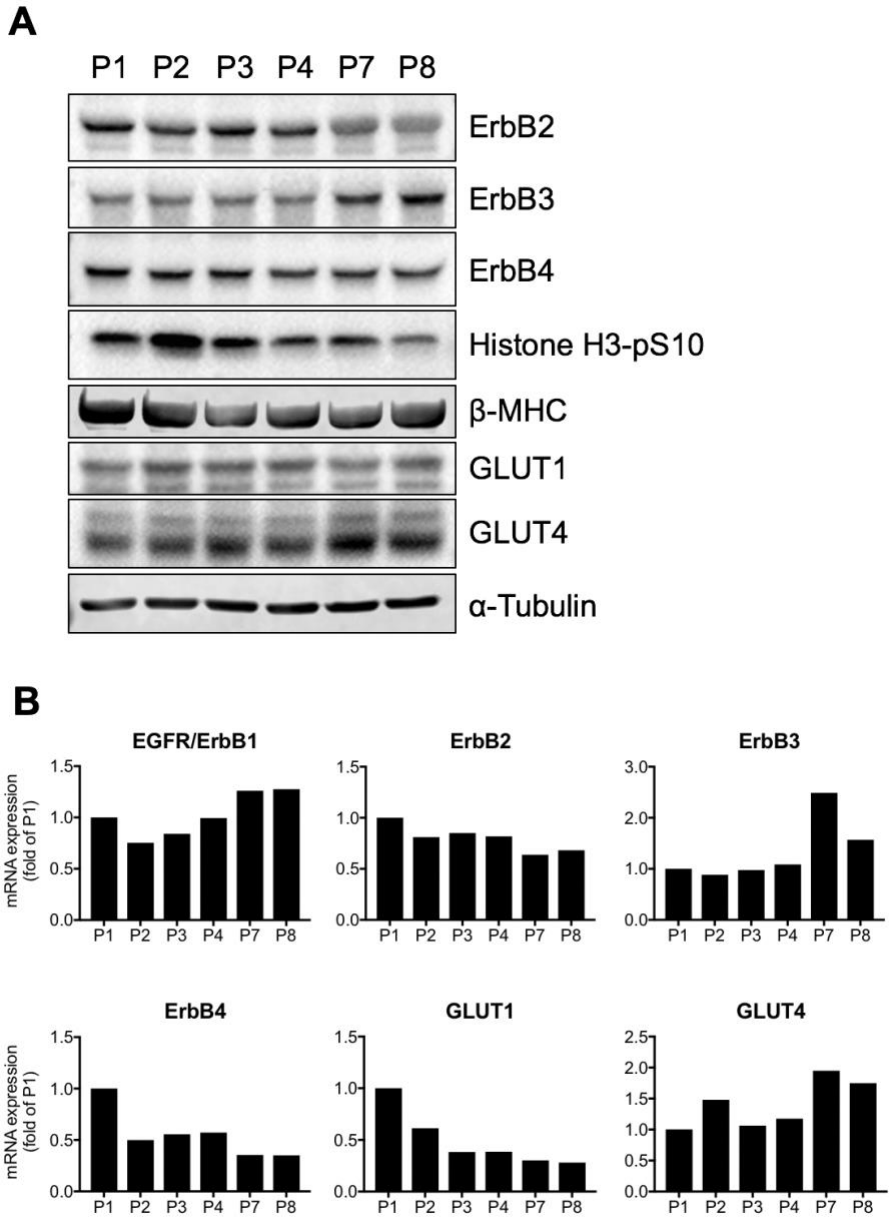


Figure 5. Analysis of the ErbB receptor and GLUT1/4 expression neonatal rats

A: The hearts from P1, P2, P3, P4, P7 and P8 rats were harvested and proteins were analyzed by Western blot. **B:** From the same rat hearts, RNA was isolated and the expression of several genes was measured and normalized to myoglobin (n=1).

Figure 6

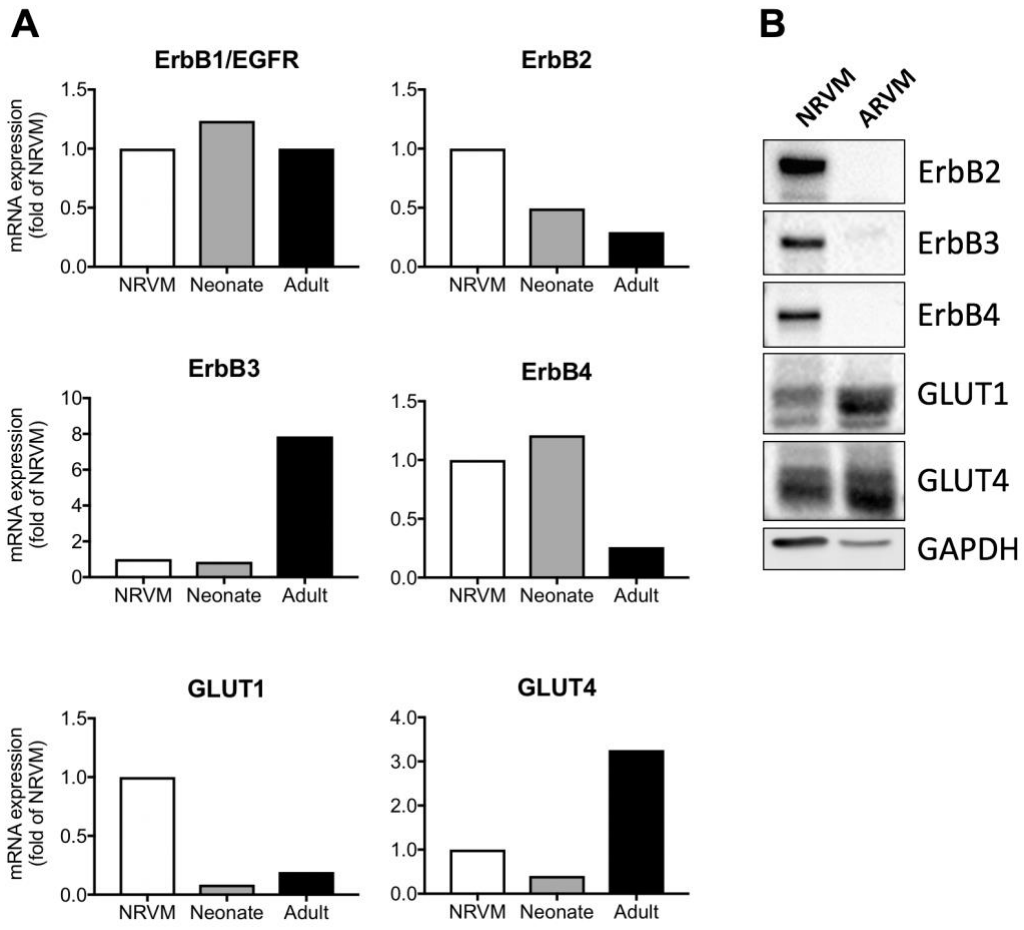


Figure 6. Comparison of the ErbB receptor and GLUT1/4 expression in our models

A: RNA was isolated from NRVMs, a P2 neonatal rat heart and an adult rat heart. The expression of several genes was analyzed and normalized to TATA-binding protein (n=1). **B:** The expression of several proteins from NRVM and ARVM protein lysates were compared by Western blot analysis.

4. Discussion

Clinical trials in chronic heart failure patients revealed positive effects of Nrg1β on heart function, although the exact mechanism is not understood (Gao et al., 2010; Jabbour et al., 2011a; Lenihan et al., 2016). Related to these beneficial observations of Nrg1β on the stressed heart, the aim of my thesis was to investigate molecular mechanisms of Nrg1β in CMs that could help to explain the positive outcomes in animal models and clinical trials. As previously mentioned, the stressed heart is able to adapt its source of energy to increased glucose consumption to have continuous energy supply for contraction. To understand the regulation of increased glucose consumption, one important factor that needs to be investigated is glucose uptake. Therefore, the question of my thesis was if Nrg1β influences glucose uptake in CMs and if so, what is the molecular mechanism.

4.1. Nrg1β/ErbB signaling increases glucose uptake in NRVMs

The role of the ErbB receptors was analyzed by using pharmacological inhibitors and receptor type-specific siRNAs. Our results show that the ErbB2/4 heterodimer is required for the induction of glucose uptake and protein synthesis by Nrg1β in NRVMs, whereas a role of ErbB3 was excluded. Moreover, in the unpublished projects chapter, I analyzed and discussed the different levels of ErbB receptor expression in our models, which could explain why the NRVMs responded stronger to Nrg1β stimulation than the ARVMs. Furthermore, the distinct activation of the PI3K and Erk1/2 pathway by Nrg1β stimulation in NRVMs and the absence of PI3K pathway activity in ARVMs needs to be further investigated.

4.2. Analysis of the role of c-Src and FAK in Nrg1β-induced glucose uptake

Downstream of the ErbB receptors, effector signaling molecules were investigated to identify a link between the receptors and the activated PI3K signaling pathway. Although ErbB4 was shown to have a binding site for the p85 subunit of PI3K (Jones et al., 2006), others claim that the heart expresses an isoform of ErbB4 without p85 binding sites (Elenius et al., 1999). Therefore, we decided to analyze the effect of Nrg1β on c-Src and FAK, out of which c-Src and

FAK have already been linked to ErbB signaling in CMs (Kuramochi et al., 2006). Moreover, FAK was observed to interact and increase PI3K activity (Chen and Guan, 1994). We applied Dasatinib or PP2 to inhibit c-Src, while FAK was blocked by PF-573228. Both Src inhibitors blocked Nrg1β-induced glucose uptake, however not completely, which may indicate pathway redundancy. In addition, we performed siRNA-based knockdown experiments to reduce c-Src and FAK expression, which revealed only an effect on basal glucose uptake. The knockdown of both targets was probably not complete enough to fully block c-Src and FAK actions or there is an alternative mechanism that still allowed Nrg1β to induce glucose uptake. Furthermore, a time course analysis of c-Src phosphorylation at Tyr^{215} and FAK at Tyr^{861} revealed that Akt was phosphorylated at the same time as FAK, whereas c-Src phosphorylation was detected much later. These results point to a contribution of FAK in NRVMs glucose uptake, although we do not know the exact mechanism yet. c-Src and FAK could serve as scaffolding proteins to attract other signaling molecules or they might promote cytoskeleton reorganization, allowing GLUT4 vesicle translocation (Brown and Cooper, 1996; Huang et al., 2006). Moreover, reduced levels of FAK protein have been associated with insulin resistance in high-fat diet-induced diabetic mice (Bisht et al., 2008). Therefore, Nrg1β may be a drug candidate to activate FAK signaling and restore insulin sensitivity in diabetic individuals.

4.3. Analysis of the role of IRS in Nrg1β signaling

Next to c-Src and FAK, IRS proteins were investigated in the context of Nrg1β stimulation. Previously, IRS-1 and IRS-2 have been shown to play an important role to maintain glucose homeostasis, since knockout animals develop mild metabolic disorders and growth retardation or T2DM and insulin resistance in peripheral tissues, respectively (Araki et al., 1994; Withers et al., 1998). Since ErbB3 (Knowlden et al., 2011) and FAK (Lebrun et al., 2000) were demonstrated to interact with IRS-1, we tested the role of IRS in Nrg1β signaling in NRVMs. Due to the fact that no good commercial phopho-IRS-2 antibodies were available, we focused on phospho-IRS-1. Therefore, we analyzed several phosphorylation sites on IRS-1 after insulin and Nrg1β stimulation in NRVMs. A time course analysis revealed obvious differences between Nrg1β- and insulin-induced IRS-1 phosphorylation. We observed that only insulin increased IRS-1-Tyr^{612}, a site that indicates activation of IRS-1 (Esposito et al.,

2001) and this increase in phosphorylation happened very early after stimulation. The other two Ser phosphorylation sites (Ser^{307} and $Ser^{636/639}$) were increased much later, but in contrast to Tyr^{612}, by both stimuli. Both Ser sites have been shown to contribute to a negative feedback loop mechanism via mTOR and p70S6K1 (Copps and White, 2012). Analysis of total IRS-1 and IRS-2 protein showed that long-term stimulation with insulin induced a reduction of both proteins, which was dependent on proteasomal degradation, whereas Nrg1β had no effect. This effect of insulin on IRS protein degradation has already been observed previously (Rui et al., 2001). Interestingly, Nrg1β rather stabilized IRS-1 and IRS-2, indicating no desensitization like it is the case for insulin stimulation. Knockdown of IRS-1 and IRS-2 did not show any effect on insulin- and Nrg1β-stimulated glucose uptake, likely due to the remaining levels of IRS proteins or redundancy by other IRS proteins. In summary, there is a clear impact of Nrg1β on IRS-1 and IRS-2 observable in NRVMs, however we did not find a link to Nrg1β-induced glucose uptake.

4.4. The PI3Kα and Akt, but not mTORC2, are implicated in the mechanism that mediates Nrg1β-induced glucose uptake

Nrg1β binding to the ErbB receptors is known to activate the PI3K pathway. Activation of PI3K could be mediated by a direct interaction with the ErbB3 or ErbB4 receptors or by FAK (Chen and Guan, 1994; Schulze et al., 2005). By pharmacological inhibition of several isoforms of PI3K, we identified PI3Kα as the responsible isoform mediating Nrg1β-induced glucose uptake. After inhibition of PI3Kα, Nrg1β stimulation of Akt phosphorylation at Thr^{308} and Ser^{473} was blocked. Both phosphorylation sites were shown to be needed for full activation of Akt (Alessi et al., 1996). Moreover, activation of Akt is crucial since the blockage of Akt with Akt inhibitor VIII completely abolished Nrg1β-induced glucose uptake. Taking into account that Akt is phosphorylated at Ser^{473} by mTORC2 (Hresko and Mueckler, 2005; Sarbassov et al., 2005), the role of mTORC2 in Nrg1β-induced glucose uptake was investigated by comparing the effects of the pharmacological inhibitors, Rapamycin and PP242, and siRNAs specific for rapamycin-insensitive companion of mTOR (rictor), an essential component of mTORC2 (Sarbassov et al., 2004). Our results indicated that mTORC2 and Ser^{473} phosphorylation of Akt are not required for AS160 phosphorylation at Thr^{642} and glucose uptake upon Nrg1β stimulation in NRVMs, matching with our previously published data in adult mice

(Pentassuglia et al., 2016; Shende et al., 2016). This is in contrast to insulin-induced glucose uptake in skeletal muscle and liver, which were demonstrated to depend on mTORC2 (Hagiwara et al., 2012; Kumar et al., 2008). Moreover, in brown adipose tissue, it was observed that mTORC2 contributes to glucose uptake during cold-induced glucose uptake (Albert et al., 2016). However, this is not happening in NRVMs stimulated with Nrg1β.

4.5. AS160 and GLUT4 mediate Nrg1β-induced glucose uptake

Downstream of Akt, AS160 is phosphorylated at Thr^{642} (Geraghty et al., 2007). AS160 is part of a well-studied mechanism *in vitro* and *in vivo* that leads upon activation to increased GLUT4 translocation (Chen et al., 2011; Rowland et al., 2011). After Nrg1β stimulation, we observed that AS160 showed increased phosphorylation at Thr^{642}, indicating activation of GLUT4 translocation (Sano et al., 2003). To test whether GLUT4 translocates to the sarcolemma, we overexpressed GLUT4 linked to a c-Myc tag on its extracellular part in NRVMs. Our data revealed that Nrg1β induces GLUT4 translocation to a similar extent as insulin. However, the disadvantage of this approach is the fact that the GLUT4 levels are much higher than under physiological conditions since its expression is regulated by the ubiquitin promoter. Next to this approach, we performed cell fractionation and immunostaining experiments to detect GLUT4 translocation of the endogenous GLUT4 protein. Preliminary data of the cell fractionation experiments indicate that endogenous GLUT4 translocates to the sarcolemma after Nrg1β stimulation (data not shown), whereas the immunostaining approach failed due to technical limitations. To further analyze the contribution of GLUT4 in Nrg1β-induced glucose uptake in NRVMs, we knocked down GLUT4 with specific siRNAs. Data from these experiments confirmed that GLUT4 contributes to Nrg1β-induced glucose uptake, although the increase was not fully blocked. Possibly, other GLUT transporters are contributing or the remaining GLUT4 protein is enough to trigger the small increase of glucose uptake, even though it was not significant, which was observed after Nrg1β and insulin stimulation.

4.6. Nrg1β increases AS160 phosphorylation in neonatal rat hearts

Since all signaling analysis and GLUT4 translocation studies were performed in NRVMs, we next aimed to confirm our findings using two *in vivo* models. The first model that we applied

was the one-day old neonatal rat. The rats were injected i.p. with Nrg1β or insulin and 30 min later the animals were sacrificed to harvest the heart for protein analysis by Western blot. Our results showed that Nrg1β increased Akt and AS160 phosphorylation, although to a less extent as insulin. These findings indicated that Nrg1β likely increases glucose uptake in the neonatal heart like in our NRVM model. To reach higher significance, more animals should be included into the study. Another factor that could be adapted is the dose of Nrg1β (50 µg/kg body weight), which was chosen according to a pilot experiment and previous studies in adult animal models (Caillaud et al., 2015; Ennequin et al., 2015).

4.7. Nrg1β lowers blood glucose of STZ mice

In addition to the neonatal rat model, we applied a diabetic mouse model. STZ was used to destroy the pancreatic beta cells of the islets of Langerhans to induce a hyperglycemic state. After Nrg1β injection, we measured a decrease of blood glucose to the same level as insulin, but with different kinetics. Insulin reduced blood glucose much faster than Nrg1β, indicating that the response to Nrg1β could be a secondary effect. Therefore, we measured the level of plasma insulin in the Nrg1β-treated mice, but we did not find an increase. Protein analysis of the heart revealed no effect on Akt or AS160 phosphorylation upon Nrg1β injection, only Erk1/2 showed elevated phosphorylation, confirming activation of ErbB signaling. In addition, we examined liver proteins and detected increased phosphorylation of the ErbB3 receptor, Akt and GSK3β, indicating raised glycogen synthesis. In the meantime, other groups have published similar results in mice and rats showing this effect of Nrg1β on the liver (Caillaud et al., 2015; Ennequin et al., 2015; Lopez-Soldado et al., 2016). In summary, Nrg1β lowered blood glucose in STZ mice, very likely due to the strong effect on liver ErbB3 activation. In the STZ model, the diabetic state likely changes the metabolism of the heart (Mansor et al., 2013), which could affect the glucose uptake-related response to Nrg1β. In addition, STZ is known to have side effects on other organs (Rerup, 1970; Weiss, 1982), which could as well influence the data obtained from this model. We injected our mice i.p., which for future studies could be reconsidered to change to another administration route. With an intra venous injection one could theoretically reach a higher concentration of Nrg1β in the heart and perhaps lower variability. To investigate the effect of Nrg1β on glucose uptake in the heart, one should

perform experiments in an *ex vivo* setup. Thereby, the concentration of glucose and Nrg1β in the through flow could be exactly defined.

4.8. ARVMs do not increase glucose uptake upon Nrg1β stimulation

Since we could not find an effect of Nrg1β in the adult heart of STZ mice, we measured glucose uptake in ARVMs. For these experiments, we collaborated with Dr. Christophe Montessuit from the University of Geneva, who performed the experiments. The data obtained revealed no effect on glucose uptake and also Western blot analysis did not show increased Akt or AS160 phosphorylation after Nrg1β stimulation. A possible explanation could be that these cells express lower levels of the ErbB receptors and therefore are less responsive to Nrg1β treatment. We did not measure mRNA levels of the ErbB receptors in ARVMs yet, however preliminary data from Western blot analysis indicate lower ErbB2, ErbB3 and ErbB4 protein levels compared to NRVMs.

4.9. The ErbB2/4 hetero dimer mediates protein synthesis

Next to glucose uptake, we investigated the mechanism whereby Nrg1β increases protein synthesis and analyzed if glucose uptake contributes to protein synthesis in NRVMs. About 20 years ago, it was already observed that rhGGF2 (soluble Nrg1) can increase protein synthesis in NRVMs and ARVMs (Baliga et al., 1999; Zhao et al., 1998). Based on receptor phosphorylation, the authors suggested that ErbB2 and ErbB4 could mediate the signal and in addition, they showed that Erk1/2 and p70S6K1 are involved in rhGGF2-induced protein synthesis. By knocking down ErbB2 and ErbB4, we could demonstrate that both receptors contribute to Nrg1β-induced protein synthesis as ErbB2/4 heterodimer. In our study, we confirmed increased phosphorylation of Erk1/2 and p70S6K1 after Nrg1β stimulation and in addition, we observed elevated phosphorylation of 4E-BP1 and mTOR. Furthermore, when we compared Nrg1β-induced protein synthesis in presence of Rapamycin or PP242, we showed that mTORC1 only partially contributes to Nrg1β-induced protein synthesis. Since mTORC1 inhibition resulted only in a partial protein synthesis inhibition, we propose pathway redundancy, which could be responsible for the remaining protein synthesis increase observed after Nrg1β stimulation. In consideration that Nrg1β activates the Erk1/2, RSK2

(p90S6K family) could mediate the downstream signal to trigger protein synthesis by S6 activation (Zhao et al., 1998). Indeed, when we blocked Erk1/2 with U0126, protein synthesis was as well only partially reduced. This indicates that both signaling pathways, mTOR and Erk1/2, contribute to Nrg1β-induced protein synthesis (Pentassuglia et al., 2016). Finally, our data from experiments with Indinavir show that glucose uptake contributes to overall protein synthesis in NRVMs, but not Nrg1β-induced protein synthesis. These results indicate that next to GLUT4 one or more additional glucose transporters are involved in insulin- and Nrg1β-stimulated glucose uptake in NRVMs.

4.10. Nrg1β and IGF-I together stimulate NRVM proliferation

Recently, we started to investigate the effect of Nrg1β on proliferation in NRVMs. Based on our data from p-HH3 immunostainings and preliminary data from DNA synthesis assays (data not shown), we concluded that Nrg1β by itself did not increase NRVM proliferation. This was in contrast to IGF-I, which was used as positive control. Interestingly, when Nrg1β was combined with IGF-I, we observed an additive effect on proliferation. This additive effect has already been observed previously in a DNA synthesis experiment (Hertig et al., 1999). Recently, Missinato et al. published that Nrg1β induces proliferation in NRVMs, which seems to be in contrast to our finding (Missinato et al., 2018). Actually, they cultured their cells in medium containing 10% FBS and in addition stimulated with Nrg1β. Probably, our condition with IGF-I in the medium mimics the 10% FBS, indicating that Nrg1β needs other growth factors to induce NRVM proliferation. However, increased p-HH3-Ser^{10} phosphorylation and DNA synthesis is only a hint for NRVM proliferation, since NRVMs can undergo binucleation instead of cytokinesis. To have full proof, one would need to check other markers like Aurora B and anillin as proposed by Leone et al. (Leone et al., 2015).

Interestingly, when we analyzed signaling after combined stimulation with Nrg1β and IGF-I, like for insulin, we detected an additive effect on Akt phosphorylation (data not shown). Both factors are known to activate the PI3K/Akt pathway. Furthermore, in contrast to IGF-I, Nrg1β activates the MAPK/Erk1/2 signaling, which could contribute to an increased proliferation rate (Hecquet et al., 2002; Kang and Sucov, 2005). Moreover, in pulmonary epithelial cells, Nrg1 was shown to activate the janus kinase/signal transducer and activators of transcription (JAK/STAT) pathway, which induced proliferation (Liu and Kern, 2002). If the JAK/STAT

pathway is as well involved and if Nrg1β-induced glucose uptake contributes to proliferation in NRVMs remains to be investigated. In conclusion, the potential of Nrg1β to increase proliferation of NRVMs could play an important role in heart regeneration.

4.11. Nrg1β, a drug candidate

Overall, our findings contribute to a better understanding of Nrg1β signaling in the heart, especially in CMs. In recent years, several positive observations of Nrg1β in the heart have been published. As already mentioned in the introduction, Nrg1β lowers levels of apoptosis, increases hypertrophy, lowers autophagy, lowers contractility and induces higher proliferation rates of CMs *in vitro* (Baliga et al., 1999; Bersell et al., 2009; Fukazawa et al., 2003; Lemmens et al., 2004; Zhao et al., 1998). In addition, many *in vivo* studies with cardiac disease models demonstrate beneficial effects such as reduced infarct size, reduced mitochondrial dysfunction, lowered CMs apoptosis, increased microvasculature, increased CMs proliferation, decreased fibrotic remodeling and overall improved cardiac function, which was review by Odiete et al. and Rupert and Coulombe (Odiete et al., 2012; Rupert and Coulombe, 2015). A situation in which glucose plays an important role is ischemia. As already mentioned, during an ischemic event, Nrg1β is released from the cardiac endothelium. Moreover, ErbB3 has been demonstrated to be upregulated after ischemia/reperfusion. Therefore, Nrg1β could be of interest in situations when the heart lacks the basal supply of glucose. Already about 55 years ago, clinicians started to treat myocardial-ischemia with glucose-insulin-potassium infusions to increase glycolysis in the heart, which was shown to improve recovery (Grossman et al., 2013). Therefore, Nrg1β might have a similar effect on glycolysis in the ischemic heart. So far, Nrg1β was observed to activate the PI3K pathway in the heart after ischemia/reperfusion injury (Fang et al., 2010), which might activate glycolysis. However, once Nrg1β is applied as drug, one needs to be aware of its effect on blood glucose levels. However, in regard to blood glucose handling, Huang et al. have shown that there are differences between species and observed that human subjects were not affected (Huang et al., 2017).

As follow-up of our *in vitro* studies, which analyzed the effects of Nrg1β on CMs, our *in vivo* studies will be very relevant since in the heart Nrg1β acts not only on CMs but also on other cell types. These other cells could as well influence the structure and function of the heart. For example, Kirabo et al. showed that cardiac fibroblasts respond to Nrg1β with enhanced

proliferation, increased viability and secretion of pro-reparative factors like angiopoietin-2, brain-derived neurotrophic factor and crypto-1 (Kirabo et al., 2017). Interestingly, Nrg1β was demonstrated to reduce fibrosis by increasing ErbB4 phosphorylation in macrophages, which reduced their inflammatory activity (Vermeulen et al., 2017). Moreover, Nrg1β was shown to increase angiogenesis by inducing vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) secretion (Gui et al., 2018; Russell et al., 1999). All these observations could be possible mechanisms, whereby Nrg1β improves heart function. However, it is still surprising that a short period of Nrg1β treatment had long-lasting effects on heart function in patient with chronic heart failure (Gao et al., 2010; Lenihan et al., 2016). Unfortunately, there could be a potential risk to development cancer when patients are treated with Nrg1β (Vermeulen et al., 2016). Although, Ganapathy et al. reported that there was almost no effect on somatic, organ or neoplastic growth in mice (Ganapathy et al., 2016), patients that have an elevated risk for cancer should be treated with Nrg1β only with caution or one needs to apply a targeted therapy to reduce side effects.

5. Conclusions and outlook

The major achievement of my PhD project is the elucidation of the molecular mechanism how Nrg1β induces glucose uptake in NRVMs. I demonstrated that the ErbB2/4 heterodimer mediates Nrg1β-induced glucose uptake via the activation of the PI3Kα, Akt, AS160 signaling pathway, which leads to GLUT4 translocation. If the observed glucose uptake contributes to the recovery of the heart during or after a stress condition remains to be seen and may depend on the stress stimulus.

As follow-up project, our laboratory will apply the transverse aortic constriction model to investigate the effect of Nrg1β in the hemodynamically stressed mouse heart *in vivo*. Cardiac function will be assessed by echocardiography and the heart will be analyzed by Western blot, immunohistochemistry and qRT-PCR. In another project, one could measure glucose uptake after Nrg1β stimulation in an *ex vivo* setup while mimicking ischemia-reperfusion injury. In addition, one could further examine whether causal links exist between glucose uptake and the observed increases in protein synthesis and proliferation *in vitro*. In this context, the role of Akt, mTOR and Erk1/2 should be analyze in more detail to elucidate their contribution in both processes. Moreover, Nrg1β and insulin/IGF-I showed an additive increase of Akt-pThr^{308} phosphorylation without affecting glucose uptake levels. This observation could be investigated in more detail as well.

In conclusion, our new findings contribute to a better understanding of the molecular mechanism of Nrg1β signaling in CMs and might help to improve the treatment of heart disease, in which Nrg1β could be applied as promising drug candidate. Although a lot has already been examined, we still need to continue research to further investigate the role of Nrg1β in the heart and aim to find ways to use its benefits to treat heart disease.

6. Material and methods

The majority of all chemical ingredients were purchased from SIGMA, except if another company is stated.

6.1. Cell culture

All media and supplements for cell culture were bought from GIBCO Life Technologies if nothing is indicated. Pentamycin (100 units/ml), streptomycin (100 µg/ml) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 25 mM was added to all cell culture media. The cells were cultured in a HERAcell 150 incubator at 37°C and 5% CO_2. They were kept in plastic ware from Sarstedt. The NRVMs cultured in complete or starvation medium indicated in the table below.

Complete Medium
DMEM 1 g/L Glucose, 10% FBS, 25 mM HEPES, 150 µM BrdU
Starvation Medium
DMEM 4.5 g/L Glucose, 25 mM HEPES, 150 µM BrdU, ACCT (albumin 2 g/l, L-carnitine 2 mM, creatine 5 mM and taurine 5 mM)

6.2. Isolation of neonatal rat ventricular myocytes (NRVMs)

1 to 2 days old neonatal rats were sacrificed by decapitation. The hearts were dissected, the atria and big arteries were removed, the ventricles were cut into two pieces and pre-digested with 0.05% trypsin-EDTA at 4°C overnight. On the next day, the trypsin solution was discarded and the ventricle pieces were washed with complete medium. Afterwards, the ventricles were further digested in the water bath at 37°C with 0.83 mg/ml collagenase (Worthington) dissolved in HBSS (Hank's balanced salt solution). Every 2 to 5 min, the cell suspension was collected, fresh collagenase solution was added and gently agitated at 37°C. The collected cell suspension was transferred into pure fetal bovine serum (FBS) at room temperature (RT). This was repeated up to 5 times. The collagenase was removed by pelleting the cells for 6 min at 60 g. The cell pellet was suspended in complete medium. In order to remove the majority of

fibroblasts, which adhere much faster to the plastic surface than NRVMs, the cell suspension was pre-plated two times for 1h at 37°C. Afterwards, the NRVMs were counted and plated in complete medium containing 150 µM bromodeoxyuridine (BrdU), which keeps the number of fibroblasts low. For proliferation experiments, no BrdU was added. Most experiments were performed two days after plating. The organ isolation procedure was carried out according to the guidelines for the care and use of laboratory animals and with approval of the Swiss authorities.

6.3. Transfection

NRVMs were transfected by nucleofection with AMAXA biosystems Nucleofector II. Before plating the cells on day 2, $3x10^6$ cells were transfected with plasmid DNA or siRNA in 100 µl of RTNucleoFector solution (AMAXA Lonza) with program G-009. Immediately after electroporation, pre-warmed RPMI medium with 10% FBS was added to the cells and incubated for 20 min at 37°C. Afterwards, the cells were transferred into a corresponding culture ware, coated with 10 µg/ml laminin, and incubated overnight. The next morning, the medium was replaced by fresh medium. On day 4, the cells were used for experiment.

6.4. Experimental procedure

One day before the experiment, NRVMs were calmed down overnight by exchanging the complete medium with ACCT medium. The following stimuli and inhibitors were used at final concentrations as indicated in the tables below.

Stimuli	Company	Conc.	Receptors
Neuregulin1β1	R&D	10 ng/ml	ErbB3 and ErbB4
Neuregulin1β1	PeproTech	10 ng/ml	ErbB3 and ErbB4
Insulin	Sigma	2.61 nM	Insulin receptor
IGF-I	Genentech	20 ng/ml	Insulin-like growth factor 1 receptor

Inhibitors	Company	Conc.	Target
Akt inhibitor VIII	Calbiochem	20 µM	Akt
AS605240	M. P. Wymann	1 µM	PI3Kγ
Byl-719	M. P. Wymann	1 µM	PI3Kα
Cal101	M. P. Wymann	1 µM	PI3Kδ
Dasatinib	LC Laboratories	1 µM	c-Src/BCR-ABL
Lapatinib	LC Laboratories	10 µM	ErbB2/EGFR
LY-294002	Calbiochem	10 µM	PI3K
MG132	APExBIO	10 µM	Proteasome complex 9
Oligomycin	Calbiochem	2 µM	ATP synthase
PF573228	Calbiochem	10 µM	FAK
PP2	Calbiochem	5 µM	FAK
PP242	Sigma	2 µM	mTOR
Rapamycin	Calbiochem	20 ng/ml	FK-binding protein 12
SB203580	Calbiochem	10 µM	p38 MAPK
TGX-221	M. P. Wymann	1 µM	PI3Kβ
U0126	Calbiochem	10 µM	MEK1/2 kinase

6.5. Protein extraction

Radioimmunoprecipitation assay (RIPA) lysis buffer, containing 50 mM TRIS-HCl pH 7.4 (MERK), 150 mM NaCl, 1% NP40, 0.25% deoxycholate, 1% protease inhibitor cocktail, 0.5% phosphatase inhibitor cocktail 2 and 3, 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% sodium dodecyl sulfate (SDS) was used for protein extraction. The cells were washed twice with ice-cooled phosphate buffered saline (PBS), lysis buffer was added, cells were scratched out of the dish, vortexed for 5 s and incubated for 10 min on ice. Afterwards, the extract was centrifuged at 16'000 g at 4°C for 10 min. The supernatant was frozen in liquid nitrogen and stored at -80°C.

6.6. Protein assay

Micro BCA Protein Assay Kit from Thermo Scientific was used according to the manufacturer's instructions. The optical measurement was done with a spectrophotometer from Bucher Biotec.

6.7. Western blot

For Western blot polyacrylamide gels were homemade and electrophoresis was performed with PowerPac Basic power supply (Bio Rad). The resolving gel consisted of 375 mM TRIS-HCl pH 8.8, 8% to 15% acrylamide/bis (Bio Rad), 0.1% SDS, 0.1% ammonium persulfate (Bio Rad) and 0.08% TEMED (Bio Rad). The stacking gel consisted of 125 mM TRIS-HCl pH 6.8, 5.1% acrylamide/bis, 0.1% SDS, 0.1% ammonium persulfate (APS) and 0.08% tetramethylethylendiamin (TEMED). Before adding TEMED, the solution was deaerated. The electrophoresis buffer consisted of 25 mM Tris base, 192 mM glycine and 0.1% SDS. The transfer buffer consisted of 25 mM Tris base, 192 mM glycine and 5% methanol (MERK). The protein extracts were thawed on ice and mixed with SDS sample buffer (Laemmli), containing 50 mM TRIS-HCl pH 6.8, 2% SDS, 0.016% bromophenol blue, 10% glycerol and 100 mM dithiothreitol (DTT). Afterwards, the samples were denatured by heating for 5 min at 95°C and loaded onto the gels. Electrophoretic separation was performed for 1.5 h at 120 V and proteins were blotted onto a methanol-pretreated polyvinylidenfluorid (PVDF) membrane (GE Healthcare) for 2 h at 350 A in ice-cooled transfer buffer. The proteins were stained with Ponceau S solution to check the efficiency of the transfer. The membrane was blocked with tris buffered saline (TBS, Tris base 250 mM + NaCl 1.5 M) + 0.1% TWEEN-20 + 5% BSA or 5% milk. Primary antibodies were diluted in TBS + 0.1% TWEEN-20 + 5% BSA or 5% milk and the membrane was incubated on a shaking platform at 4°C overnight. Horse Radish Peroxidase (HRP) secondary antibodies were used, when followed by detection with enhanced chemo luminescence (ECL), using SuperSignal West Pico or Dura Chemiluminescent Substrate (Thermo Scientific). The ECL signal was recorded with ChemiDoc imaging system from BioRad. The Secondary antibodies with an infrared signal were detected with Odyssey from LI-COR Bioscience. For quantification, the intensity of the bands was measured with Fiji and normalized to a housekeeping protein.

Following first antibodies were applied:

Target	Catalogue nr.	Information
Akt-pSer^{473}	9271S	rabbit, 60 kDa, 1:1000, Cell Signaling
Akt-pThr^{308}	9275	rabbit, 60 kDa, 1:1000, Cell Signaling
Akt 2	3063	rabbit, 60 kDa, 1:1000, Cell Signaling
Akt	9272	rabbit, 60 kDa, 1:1000, Cell Signaling
AS160-pThr^{642}	AB271	rabbit, 160 kDa, 1:500, Cell Signaling
AS160	2670	rabbit, 160 kDa, 1:1000, Cell Signaling
4E-BP1	9452	rabbit, 15 – 20 kDa, 1:2000, Cell Signaling
ErbB2-pTyr^{1248}	sc-12352-R	rabbit, 185 kDa, 1:500, Santa Cruz
ErbB2	sc-284	rabbit, 185 kDa, 1:500, Santa Cruz
ErbB3-pTyr1^{289}	4791	rabbit, 185 kDa, 1:1'000, Cell Signaling
ErbB3	12708	rabbit, 185 kDa, 1:500, Cell Signaling
ErbB4-pTyr^{1284}	ab61059	rabbit, 185 kDa, 1:500, Abcam
ErbB4	sc-283	rabbit, 185 kDa, 1:500, Santa Cruz
Erk1/2-pThr^{202}/Tyr^{204}	9101	rabbit, 42/44 kDa, 1:1000, Cell Signaling
Erk1/2	9102	rabbit, 42/44 kDa, 1:1000, Cell Signaling
FAK-pTyr^{397}	3283S	rabbit, 125 kDa, 1:1000, Cell Signaling
FAK-pTyr^{861}	ab38458	rabbit, 125 kDa, 1:1000, Abcam
FAK	sc-558	rabbit, 125 kDa, 1:500, Santa Cruz
GAPDH	sc-32233	mouse, 37 kDa, 1:1000, Santa Cruz
GLUT1	ab40084	mouse, 45/55 kDa, 1:500, Abcam
GLUT4	ab654	rabbit, 45/55 kDa, 1:500, Abcam
IRS-1-pSer^{307}	07-247	rabbit, 170 kDa, 1:500, Upstate
IRS-1-pSer$^{636/639}$	2388	rabbit, 180 kDa, 1:1000, Cell Signaling
IRS-1-pTyr^{612}	MBS624304	rabbit, 180 kDa, 1:500, MyBioSource
IRS-1	sc-559	rabbit, 170 kDa, 1:500, Santa Cruz
IRS-2	06-506	rabbit, 170-185 kDa, 1:500, Upstate
IR/IGF-IR-pTyr$^{1131/1146}$	3021	rabbit, 95 kDa, 1:1000, Cell Signaling
P70S6K1-pThr^{389}	9205	rabbit, 70 kDa, 1:1000, Cell Signaling
P70S6K1	9202	rabbit, 70 kDa, 1:1000, Cell Signaling
c-Src	36D10	rabbit, 60 kDa, 1:2'000, Cell Signaling
mTOR-pSer^{2448}	2971	rabbit, 289 kDa, 1:1000, Cell Signaling
mTOR-pSer^{2481}	2974	rabbit, 289 kDa, 1:1000, Cell Signaling
mTOR	2972	rabbit, 289 kDa, 1:1000, Cell Signaling
α-Tubulin	T-6199	mouse, 50-55 kDa, 1:1000, SIGMA
ULK-pSer^{757}	6888	rabbit, 140 kDa, 1:1000, Cell Signaling
ULK1	A-7481	rabbit, 150 kDa, 1:1000, SIGMA
Vinculin	sc-7649	goat, 117 kDa, 1:1'000, Santa Cruz

Following secondary antibodies were applied:

Target	Catalogue nr.	Information
α-rabbit IgG	111-035-003	HRP, 1:10’000, Jackson ImmunoResearch
α-mouse IgG	115-035-003	HRP, 1:10’000, Jackson ImmunoResearch
α-goat IgG	305-035-003	HRP, 1:10’000, Jackson ImmunoResearch
α-mouse IgG	926-32210	IR-Dye 800CW, 1:10’000, LI-COR Biosciences

6.8. RNA isolation

RNAse-free materials were used. The cells were washed twice with ice-cooled PBS and TRI reagent was added. The cells were scraped out of the dish, transferred into a micro tube and vortexed vigorously. Afterwards, the samples were incubated for 10 min at RT and centrifuged for 10 min at 16’100 g at 4°C. The supernatant was transferred into a new micro tube and chloroform (MERCK) was added. After vortexing, the samples were again centrifuged for 15 min at 9’300 g at 4°C. The aqueous phase was transferred into a new micro tube and the RNA was precipitated with isopropanol. The precipitate was centrifuged at 16’100 g at 4°C for 10 min and washed twice with 70% ethanol. The RNA pellet was first dried on air and then re-suspended in RNAse-free water. DNAse treatment with Ambion DNA-free DNase Treatment & Removal Reagents kit from life technologies was performed according to the manufacturer’s instructions. The RNA concentration was measured with NanoDrop 2000.

6.9. Reverse transcription and quantitative real-time polymerase chain reaction

With cDNA reverse transcription kit of Applied Biosystems, 1.5 µg of RNA was reverse transcribed into the complementary DNA (cDNA) according to the manufacturer’s instructions. A thermocycler from Biometra was used. For the quantitative real-time polymerase chain reaction reaction (qRT-PCR) a 7500 Fast Real-Time PCR System from Applied Biosystems and the GoTaq qPCR master mix from Promega was used according to the manufacturer’s instructions. The cDNA was diluted and added to the reaction mix. The measurements of all target genes were performed in triplicates and the housekeeping genes in duplicates. The following primers were purchased from Microsynth.

Gene	Forward primer	Reverse primer
ErbB1	5'-ACA GCA AGG CTT CTT CAA CAG C-3'	5'-GTC TTC TTT GAC ACG GCA GCT-3'
ErbB2	5'-TTC TGG ATG TCC GAG ACC-3'	5'-TCA TCC CCT TGG CAA TCT GA-3'
ErbB3	5'-CGA GAT GGG CAA CTC TCA GGC-3'	5'-AGG TTA CCC ATG ACC ACC TCA CAC-3'
ErbB4	5'-CGG GCC ATT CCA CTT TAC C-3'	5'-TGA CTC CGG CTG CAA TCA G-3'
GAPDH	5’-GAT GGT GAA GGT CGG TGT GAA-3’	5’-TTG AAC TTG CCG TGG GTA GAG-3’
GLUT1	5’-ATC AAC GCC CCC CAG AA-3'	5'-AAT CAT GCC CCC GAC AGA-3'
GLUT4	5'-CCC CCG ATA CCT CTA CAT-3'	5'-GCA TCA GAC ACA TCA GCC CAG-3'
Myoglobin	5'-CGC CAC CAA GCA CAA GAT C-3'	5'-TCA GGA CTT GGA TGA TGA CTT CTG-3'
TBP	5'-CAC AGG AGC CAA GAG TGA AGA AC-3'	5'-GCT TCT GCA CAA CTC TAG CGT ATT-3'

6.10. Immunofluorescence

For immunofluorescence, cells were cultured in P35 dishes. After treatment, the cells were washed twice with cold PBS, fixed and permeabilized for 20 min at 4°C with PBS containing 4% formaldehyde (Polyscience) and 0.1-0.3% Triton-X100. The samples were then blocked with PBS + 0.1% TWEEN + 3% BSA for 1 h at RT and incubated with the first antibody over night at 4°C. On the next day, the samples were washed 3 times for 5 min with PBS + 0.1% TWEEN-20 and incubated for 1 h at RT with a fluorescent secondary antibody and DAPI (1 µg/ml). The samples were washed again and mounted onto a glass slide. The mounting media consisted of 4.3 mM polyvinyl alcohol 4-88, 33% glycerol, 133.3 mM Tris-HCl pH 8.5 and 2.5% diazabicyclooctane.

Following first antibodies were used:

Target	Catalogue number	Information
Aurora B	A5102	α-rabbit, 1:200, SIGMA
Histone H3-pSer^{10}	06-570	α-rabbit, 1:200, Upstate
Ki67	15580	α-rabbit, 1:200, Abcam
Sarcomeric actinin	A2172	α-mouse, 1:200, Sigma-Aldrich

Following secondary antibodies were used:

Target	Catalogue number	Information
α-mouse IgG	A11017	Alexa 488, 1:800, Invitrogen
α-rabbit IgG	A21430	Alexa 555, 1:800, Invitrogen
α-rabbit IgG	A21070	Alexa 633, 1:800, Invitrogen

6.11. Microscopy

An inverted microscope (Zeiss Axiovert 40 C) was used for cell counting. For the detection of GFP/mCherry-transfected cells or immunofluorescence, a fluorescent microscope (Olympus BX63 or Nikon-Ti) was used. For quantification, the large image scanning function of the Nikon-Ti was applied and the images were analyzed with Fiji.

6.12. Seahorse assay

Freshly isolated NRVMs were seeded in a laminin (10 µg/ml) coated 24-well Seahorse plate (60'000 cells/well). After overnight incubation in ACCT medium, the medium was again changed to Seahorse base medium containing 2 mM L-glutamine and put for 1 h in a CO_2-free incubator. The hydrated sensor cartridge was loaded with stimuli and inhibitors and inserted into the Seahorse XF24 for calibration. Afterwards, the cell plate was put into the Seahorse XF24 and extra cellular acidification rate (ECAR) and oxygen consumption rate (OCR) was measured. The Seahorse XF24 protocol consisted of 11 min calibration/equilibration, injection of the stimuli/drugs, followed by 4 min of mixing, 2 min waiting and 2 min measuring. ECAR and OCR were measured 4 times at base line and 3 times after each injection.

6.13. Diabetic STZ mouse model

After 4 h of fasting, STZ was administered by i.p. injection (50 mg/kg body weight) to 14-15 weeks old C57BL/6N mice for 5 consecutive days. STZ was freshly dissolved in 20 mM sodiumcitrate at pH 4.5 and kept on ice. Blood glucose was measured with Freestyle Papillon Vision from Abbott. For experiments, animals were i.p. injected with Nrg1β (50 µg/kg body weight) or insulin (1 U or 34.7 µg/kg body weight) diluted in saline. Body weight was recorded over time. For sacrifice, animals were anesthetized with isoflurane and blood was collected from the right ventricle. The heart and other organs were harvested and processed for Western blot analysis and immunohistochemistry. All animal experiments were carried out

according to guidelines for the care and use of laboratory animals and with approval of the Swiss authorities.

7. References

Aerni-Flessner, L., Abi-Jaoude, M., Koenig, A., Payne, M., and Hruz, P.W. (2012). GLUT4, GLUT1, and GLUT8 are the dominant GLUT transcripts expressed in the murine left ventricle. Cardiovasc Diabetol *11*, 63.

Albert, V., Svensson, K., Shimobayashi, M., Colombi, M., Munoz, S., Jimenez, V., Handschin, C., Bosch, F., and Hall, M.N. (2016). mTORC2 sustains thermogenesis via Akt-induced glucose uptake and glycolysis in brown adipose tissue. EMBO Mol Med *8*, 232-246.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J *15*, 6541-6551.

Allard, M.F. (2004). Energy substrate metabolism in cardiac hypertrophy. Curr Hypertens Rep *6*, 430-435.

Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B., 3rd, Johnson, R.S., and Kahn, C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature *372*, 186-190.

Asrih, M., Pellieux, C., Papageorgiou, I., Lerch, R., and Montessuit, C. (2011). Role of ERK1/2 activation in microtubule stabilization and glucose transport in cardiomyocytes. Am J Physiol Endocrinol Metab *301*, E836-843.

Baliga, R.R., Pimental, D.R., Zhao, Y.Y., Simmons, W.W., Marchionni, M.A., Sawyer, D.B., and Kelly, R.A. (1999). NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70(S6K), and MEK-MAPK-RSK. Am J Physiol *277*, H2026-2037.

Bao, J.X., Lin, H., Ouyang, Y., Lei, D.B., Osman, A., Kim, T.W., Mei, L., Dai, P.G., Ohlemiller, K.K., and Ambron, R.T. (2004). Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. Nat Neurosci *7*, 1250-1258.

Beguinot, F., Kahn, C.R., Moses, A.C., and Smith, R.J. (1985). Distinct biologically active receptors for insulin, insulin-like growth factor I, and insulin-like growth factor II in cultured skeletal muscle cells. J Biol Chem *260*, 15892-15898.

Benito, M. (2011). Tissue-specificity of insulin action and resistance. Arch Physiol Biochem *117*, 96-104.

Bergmann, O., Bhardwaj, R.D., Bernard, S., Zdunek, S., Barnabe-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B.A., Druid, H., et al. (2009). Evidence for cardiomyocyte renewal in humans. Science *324*, 98-102.

Bersell, K., Arab, S., Haring, B., and Kuhn, B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell *138*, 257-270.

Bing, R.J. (1965). Cardiac Metabolism. Physiol Rev *45*, 171-213.

Bisht, B., Srinivasan, K., and Dey, C.S. (2008). In vivo inhibition of focal adhesion kinase causes insulin resistance. J Physiol *586*, 3825-3837.

Bouzakri, K., Roques, M., Gual, P., Espinosa, S., Guebre-Egziabher, F., Riou, J.P., Laville, M., Le Marchand-Brustel, Y., Tanti, J.F., and Vidal, H. (2003). Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes *52*, 1319-1325.

Brown, M.T., and Cooper, J.A. (1996). Regulation, substrates and functions of src. Biochim Biophys Acta *1287*, 121-149.

Bugger, H., and Abel, E.D. (2014). Molecular mechanisms of diabetic cardiomyopathy. Diabetologia *57*, 660-671.

Burgess, A.W., Cho, H.S., Eigenbrot, C., Ferguson, K.M., Garrett, T.P., Leahy, D.J., Lemmon, M.A., Sliwkowski, M.X., Ward, C.W., and Yokoyama, S. (2003). An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell *12*, 541-552.

Caillaud, K., Boisseau, N., Ennequin, G., Chavanelle, V., Etienne, M., Li, X., Denis, P., Dardevet, D., Lacampagne, A., and Sirvent, P. (2015). Neuregulin 1 improves glucose tolerance in adult and old rats. Diabetes Metab.

Camprecios, G., Lorita, J., Pardina, E., Peinado-Onsurbe, J., Soley, M., and Ramirez, I. (2011). Expression, localization, and regulation of the neuregulin receptor ErbB3 in mouse heart. J Cell Physiol *226*, 450-455.

Canto, C., Chibalin, A.V., Barnes, B.R., Glund, S., Suarez, E., Ryder, J.W., Palacin, M., Zierath, J.R., Zorzano, A., and Guma, A. (2006). Neuregulins mediate calcium-induced glucose transport during muscle contraction. J Biol Chem *281*, 21690-21697.

Canto, C., Pich, S., Paz, J.C., Sanches, R., Martinez, V., Orpinell, M., Palacin, M., Zorzano, A., and Guma, A. (2007). Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells. Diabetes *56*, 2185-2193.

Canto, C., Suarez, E., Lizcano, J.M., Grino, E., Shepherd, P.R., Fryer, L.G., Carling, D., Bertran, J., Palacin, M., Zorzano, A., et al. (2004). Neuregulin signaling on glucose transport in muscle cells. J Biol Chem *279*, 12260-12268.

Carraway, K.L., 3rd, Soltoff, S.P., Diamonti, A.J., and Cantley, L.C. (1995). Heregulin stimulates mitogenesis and phosphatidylinositol 3-kinase in mouse fibroblasts transfected with erbB2/neu and erbB3. J Biol Chem *270*, 7111-7116.

Carruthers, A., and Helgerson, A.L. (1991). Inhibitions of Sugar-Transport Produced by Ligands Binding at Opposite Sides of the Membrane - Evidence for Simultaneous Occupation of the Carrier by Maltose and Cytochalasin-B. Biochemistry-Us *30*, 3907-3915.

Chang, L., Chiang, S.H., and Saltiel, A.R. (2004). Insulin signaling and the regulation of glucose transport. Mol Med *10*, 65-71.

Chen, H.C., and Guan, J.L. (1994). Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A *91*, 10148-10152.

Chen, S., Wasserman, D.H., MacKintosh, C., and Sakamoto, K. (2011). Mice with AS160/TBC1D4-Thr649Ala knockin mutation are glucose intolerant with reduced insulin sensitivity and altered GLUT4 trafficking. Cell Metab *13*, 68-79.

Cho, H.S., Mason, K., Ramyar, K.X., Stanley, A.M., Gabelli, S.B., Denney, D.W., Jr., and Leahy, D.J. (2003). Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature *421*, 756-760.

Citri, A., Skaria, K.B., and Yarden, Y. (2003). The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res *284*, 54-65.

Clemmons, D.R. (2006). Involvement of insulin-like growth factor-I in the control of glucose homeostasis. Curr Opin Pharmacol *6*, 620-625.

Codony-Servat, J., Albanell, J., Lopez-Talavera, J.C., Arribas, J., and Baselga, J. (1999). Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. Cancer Res *59*, 1196-1201.

Cooper, R., Cutler, J., Desvigne-Nickens, P., Fortmann, S.P., Friedman, L., Havlik, R., Hogelin, G., Marler, J., McGovern, P., Morosco, G., et al. (2000). Trends and disparities in coronary heart disease, stroke, and other cardiovascular diseases in the United States: findings of the national conference on cardiovascular disease prevention. Circulation *102*, 3137-3147.

Copps, K.D., and White, M.F. (2012). Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia *55*, 2565-2582.

Cote, G.M., Miller, T.A., Lebrasseur, N.K., Kuramochi, Y., and Sawyer, D.B. (2005). Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. Exp Cell Res *311*, 135-146.

Crone (2002). ErbB2 is essential in the prevention of dilated cardiomyopathy. npg.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

D'Uva, G., Aharonov, A., Lauriola, M., Kain, D., Yahalom-Ronen, Y., Carvalho, S., Weisinger, K., Bassat, E., Rajchman, D., Yifa, O., et al. (2015). ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. Nat Cell Biol *17*, 627-638.

de Carvalho, A., Bassaneze, V., Forni, M.F., Keusseyan, A.A., Kowaltowski, A.J., and Krieger, J.E. (2017). Early Postnatal Cardiomyocyte Proliferation Requires High Oxidative Energy Metabolism. Sci Rep *7*, 15434.

Del Monte-Nieto, G., Ramialison, M., Adam, A.A.S., Wu, B., Aharonov, A., D'Uva, G., Bourke, L.M., Pitulescu, M.E., Chen, H., de la Pompa, J.L., et al. (2018). Control of cardiac jelly dynamics by NOTCH1 and NRG1 defines the building plan for trabeculation. Nature *557*, 439-445.

Depre, C., Vanoverschelde, J.L., and Taegtmeyer, H. (1999). Glucose for the heart. Circulation *99*, 578-588.

Dimitriadis, G., Mitrou, P., Lambadiari, V., Maratou, E., and Raptis, S.A. (2011). Insulin effects in muscle and adipose tissue. Diabetes Res Clin Pract *93 Suppl 1*, S52-59.

Elenius, K., Choi, C.J., Paul, S., Santiestevan, E., Nishi, E., and Klagsbrun, M. (1999). Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase. Oncogene *18*, 2607-2615.

Ennequin, G., Boisseau, N., Caillaud, K., Chavanelle, V., Etienne, M., Li, X., and Sirvent, P. (2015). Neuregulin 1 Improves Glucose Tolerance in db/db Mice. PLoS One *10*, e0130568.

Erickson, S.L., O'Shea, K.S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L.H., and Moore, M.W. (1997). ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. Development *124*, 4999-5011.

Esposito, D.L., Li, Y., Cama, A., and Quon, M.J. (2001). Tyr(612) and Tyr(632) in human insulin receptor substrate-1 are important for full activation of insulin-stimulated phosphatidylinositol 3-kinase activity and translocation of GLUT4 in adipose cells. Endocrinology *142*, 2833-2840.

Falls, D.L. (2003). Neuregulins: functions, forms, and signaling strategies. Exp Cell Res *284*, 14-30.

Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S., and Fischbach, G.D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell *72*, 801-815.

Fang, S.J., Wu, X.S., Han, Z.H., Zhang, X.X., Wang, C.M., Li, X.Y., Lu, L.Q., and Zhang, J.L. (2010). Neuregulin-1 preconditioning protects the heart against ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism. Chin Med J (Engl) *123*, 3597-3604.

Fleck, D., van Bebber, F., Colombo, A., Galante, C., Schwenk, B.M., Rabe, L., Hampel, H., Novak, B., Kremmer, E., Tahirovic, S., et al. (2013). Dual Cleavage of Neuregulin 1 Type III by BACE1 and ADAM17 Liberates Its EGF-Like Domain and Allows Paracrine Signaling. Journal of Neuroscience *33*, 7856-7869.

Fonseca, V.A. (2009). Defining and characterizing the progression of type 2 diabetes. Diabetes Care *32 Suppl 2*, S151-156.

Fukazawa, R., Miller, T.A., Kuramochi, Y., Frantz, S., Kim, Y.D., Marchionni, M.A., Kelly, R.A., and Sawyer, D.B. (2003). Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of PI3-kinase/Akt. J Mol Cell Cardiol *35*, 1473-1479.

Fuller, S.J., Sivarajah, K., and Sugden, P.H. (2008). ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. J Mol Cell Cardiol *44*, 831-854.

Galindo, C.L., Kasasbeh, E., Murphy, A., Ryzhov, S., Lenihan, S., Ahmad, F.A., Williams, P., Nunnally, A., Adcock, J., Song, Y., et al. (2014). Anti-remodeling and anti-fibrotic effects of the neuregulin-1beta glial growth factor 2 in a large animal model of heart failure. J Am Heart Assoc *3*, e000773.

Ganapathy, B., Nandhagopal, N., Polizzotti, B.D., Bennett, D., Asan, A., Wu, Y., and Kuhn, B. (2016). Neuregulin-1 Administration Protocols Sufficient for Stimulating Cardiac Regeneration in Young Mice Do Not Induce Somatic, Organ, or Neoplastic Growth. PLoS One *11*, e0155456.

Gao, R., Zhang, J., Cheng, L., Wu, X., Dong, W., Yang, X., Li, T., Liu, X., Xu, Y., Li, X., et al. (2010). A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. J Am Coll Cardiol *55*, 1907-1914.

Garbern, J.C., Mummery, C.L., and Lee, R.T. (2013). Model systems for cardiovascular regenerative biology. Cold Spring Harb Perspect Med *3*, a014019.

Garcia-Rivello, H., Taranda, J., Said, M., Cabeza-Meckert, P., Vila-Petroff, M., Scaglione, J., Ghio, S., Chen, J., Lai, C., Laguens, R.P., et al. (2005). Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. Am J Physiol Heart Circ Physiol *289*, H1153-1160.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature *378*, 390-394.

Gemberling, M., Karra, R., Dickson, A.L., and Poss, K.D. (2015). Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. Elife *4*.

Geraghty, K.M., Chen, S., Harthill, J.E., Ibrahim, A.F., Toth, R., Morrice, N.A., Vandermoere, F., Moorhead, G.B., Hardie, D.G., and MacKintosh, C. (2007). Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. Biochem J *407*, 231-241.

Graham, Z.A., Gallagher, P.M., and Cardozo, C.P. (2015). Focal adhesion kinase and its role in skeletal muscle. J Muscle Res Cell Motil *36*, 305-315.

Greene, M.W., Sakaue, H., Wang, L., Alessi, D.R., and Roth, R.A. (2003). Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by Serine 312 phosphorylation. J Biol Chem *278*, 8199-8211.

Grossman, A.N., Opie, L.H., Beshansky, J.R., Ingwall, J.S., Rackley, C.E., and Selker, H.P. (2013). Glucose-insulin-potassium revived: current status in acute coronary syndromes and the energy-depleted heart. Circulation *127*, 1040-1048.

Gui, C., Zeng, Z.Y., Chen, Q., Luo, Y.W., Li, L., and Chen, L.L. (2018). Neuregulin-1 Promotes Myocardial Angiogenesis in the Rat Model of Diabetic Cardiomyopathy. Cell Physiol Biochem *46*, 2325-2334.

Gui, C., Zhu, L., Hu, M., Lei, L., and Long, Q. (2012). Neuregulin-1/ErbB signaling is impaired in the rat model of diabetic cardiomyopathy. Cardiovasc Pathol *21*, 414-420.

Guma, A., Martinez-Redondo, V., Lopez-Soldado, I., Canto, C., and Zorzano, A. (2010). Emerging role of neuregulin as a modulator of muscle metabolism. Am J Physiol Endocrinol Metab *298*, E742-750.

Hagiwara, A., Cornu, M., Cybulski, N., Polak, P., Betz, C., Trapani, F., Terracciano, L., Heim, M.H., Ruegg, M.A., and Hall, M.N. (2012). Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. Cell Metab *15*, 725-738.

Harrington, L.S., Findlay, G.M., and Lamb, R.F. (2005). Restraining PI3K: mTOR signalling goes back to the membrane. Trends Biochem Sci *30*, 35-42.

Harvey, R.P., Wystub-Lis, K., Del Monte-Nieto, G., Graham, R.M., and Tzahor, E. (2015). Cardiac Regeneration Therapies - Targeting Neuregulin 1 Signalling. Heart Lung Circ.

Hecquet, C., Lefevre, G., Valtink, M., Engelmann, K., and Mascarelli, F. (2002). Activation and role of MAP kinase-dependent pathways in retinal pigment epithelial cells: ERK and RPE cell proliferation. Invest Ophthalmol Vis Sci *43*, 3091-3098.

Hedhli, N., Huang, Q., Kalinowski, A., Palmeri, M., Hu, X., Russell, R.R., and Russell, K.S. (2011). Endothelium-derived neuregulin protects the heart against ischemic injury. Circulation *123*, 2254-2262.

Heilig, C.W., Saunders, T., Brosius, F.C., 3rd, Moley, K., Heilig, K., Baggs, R., Guo, L., and Conner, D. (2003). Glucose transporter-1-deficient mice exhibit impaired development and deformities that are similar to diabetic embryopathy. Proc Natl Acad Sci U S A *100*, 15613-15618.

Hellyer, N.J., Kim, H.H., Greaves, C.H., Sierke, S.L., and Koland, J.G. (1995). Cloning of the rat ErbB3 cDNA and characterization of the recombinant protein. Gene *165*, 279-284.

Hemmings, B.A., and Restuccia, D.F. (2012). PI3K-PKB/Akt pathway. Cold Spring Harb Perspect Biol *4*, a011189.

Hertig, C.M., Kubalak, S.W., Wang, Y., and Chien, K.R. (1999). Synergistic roles of neuregulin-1 and insulin-like growth factor-I in activation of the phosphatidylinositol 3-kinase pathway and cardiac chamber morphogenesis. J Biol Chem *274*, 37362-37369.

Hill, M.F., Patel, A.V., Murphy, A., Smith, H.M., Galindo, C.L., Pentassuglia, L., Peng, X., Lenneman, C.G., Odiete, O., Friedman, D.B., et al. (2013). Intravenous glial growth factor 2 (GGF2) isoform of neuregulin-1beta improves left ventricular function, gene and protein expression in rats after myocardial infarction. PLoS One *8*, e55741.

Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., et al. (1992). Identification of heregulin, a specific activator of p185erbB2. Science *256*, 1205-1210.

Hopf, A.E., Andresen, C., Kotter, S., Isic, M., Ulrich, K., Sahin, S., Bongardt, S., Roll, W., Drove, F., Scheerer, N., et al. (2018). Diabetes-Induced Cardiomyocyte Passive Stiffening Is Caused by Impaired Insulin-Dependent Titin Modification and Can Be Modulated by Neuregulin-1. Circulation Research *123*, 342-355.

Horiuchi, K., Zhou, H.M., Kelly, K., Manova, K., and Blobel, C.P. (2005). Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. Dev Biol *283*, 459-471.

Hresko, R.C., and Hruz, P.W. (2011). HIV Protease Inhibitors Act as Competitive Inhibitors of the Cytoplasmic Glucose Binding Site of GLUTs with Differing Affinities for GLUT1 and GLUT4. Plos One *6*.

Hresko, R.C., and Mueckler, M. (2005). mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J Biol Chem *280*, 40406-40416.

Huang, D., Khoe, M., Ilic, D., and Bryer-Ash, M. (2006). Reduced expression of focal adhesion kinase disrupts insulin action in skeletal muscle cells. Endocrinology *147*, 3333-3343.

Huang, Z., Sawyer, D.B., Troy, E.L., McEwen, C., Cleator, J.H., Murphy, A., Caggiano, A.O., Eisen, A., and Parry, T.J. (2017). Species-specific effects of neuregulin-1beta (cimaglermin alfa) on glucose handling in animal models and humans with heart failure. Toxicol Appl Pharmacol.

Ito, M., Kondo, Y., Nakatani, A., Hayashi, K., and Naruse, A. (2001). Characterization of low dose streptozotocin-induced progressive diabetes in mice. Environ Toxicol Pharmacol *9*, 71-78.

Jabbour, A., Gao, L., Kwan, J., Watson, A., Sun, L., Qiu, M.R., Liu, X., Zhou, M.D., Graham, R.M., Hicks, M., et al. (2011a). A recombinant human neuregulin-1 peptide improves preservation of the rodent heart after prolonged hypothermic storage. Transplantation *91*, 961-967.

Jabbour, A., Hayward, C.S., Keogh, A.M., Kotlyar, E., McCrohon, J.A., England, J.F., Amor, R., Liu, X., Li, X.Y., Zhou, M.D., et al. (2011b). Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. Eur J Heart Fail *13*, 83-92.

Jaldin-Fincati, J.R., Pavarotti, M., Frendo-Cumbo, S., Bilan, P.J., and Klip, A. (2017). Update on GLUT4 Vesicle Traffic: A Cornerstone of Insulin Action. Trends Endocrinol Metab.

Jones, R.B., Gordus, A., Krall, J.A., and MacBeath, G. (2006). A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature *439*, 168-174.

Kaburagi, Y., Yamauchi, T., Yamamoto-Honda, R., Ueki, K., Tobe, K., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1999). The mechanism of insulin-induced signal transduction mediated by the insulin receptor substrate family. Endocr J *46 Suppl*, S25-34.

Kajstura, J., Cheng, W., Reiss, K., and Anversa, P. (1994). The IGF-1-IGF-1 receptor system modulates myocyte proliferation but not myocyte cellular hypertrophy in vitro. Exp Cell Res *215*, 273-283.

Kang, J.O., and Sucov, H.M. (2005). Convergent proliferative response and divergent morphogenic pathways induced by epicardial and endocardial signaling in fetal heart development. Mech Dev *122*, 57-65.

Kirabo, A., Ryzhov, S., Gupte, M., Sengsayadeth, S., Gumina, R.J., Sawyer, D.B., and Galindo, C.L. (2017). Neuregulin-1beta induces proliferation, survival and paracrine signaling in normal human cardiac ventricular fibroblasts. J Mol Cell Cardiol *105*, 59-69.

Knowlden, J.M., Gee, J.M., Barrow, D., Robertson, J.F., Ellis, I.O., Nicholson, R.I., and Hutcheson, I.R. (2011). erbB3 recruitment of insulin receptor substrate 1 modulates insulin-like growth factor receptor signalling in oestrogen receptor-positive breast cancer cell lines. Breast Cancer Res *13*, R93.

Knowlden, J.M., Jones, H.E., Barrow, D., Gee, J.M., Nicholson, R.I., and Hutcheson, I.R. (2008). Insulin receptor substrate-1 involvement in epidermal growth factor receptor and insulin-like growth factor receptor signalling: implication for Gefitinib ('Iressa') response and resistance. Breast Cancer Res Treat *111*, 79-91.

Kota, S.K., Kota, S.K., Jammula, S., Panda, S., and Modi, K.D. (2011). Effect of diabetes on alteration of metabolism in cardiac myocytes: therapeutic implications. Diabetes Technol Ther *13*, 1155-1160.

Kraegen, E.W., Sowden, J.A., Halstead, M.B., Clark, P.W., Rodnick, K.J., Chisholm, D.J., and James, D.E. (1993). Glucose transporters and in vivo glucose uptake in skeletal and cardiac muscle: fasting, insulin stimulation and immunoisolation studies of GLUT1 and GLUT4. Biochem J *295 (Pt 1)*, 287-293.

Kumar, A., Harris, T.E., Keller, S.R., Choi, K.M., Magnuson, M.A., and Lawrence, J.C., Jr. (2008). Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. Mol Cell Biol *28*, 61-70.

Kuramochi, Y., Cote, G.M., Guo, X., Lebrasseur, N.K., Cui, L., Liao, R., and Sawyer, D.B. (2004a). Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. J Biol Chem *279*, 51141-51147.

Kuramochi, Y., Guo, X., and Sawyer, D.B. (2006). Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. J Mol Cell Cardiol *41*, 228-235.

Kuramochi, Y., Lim, C.C., Guo, X., Colucci, W.S., Liao, R., and Sawyer, D.B. (2004b). Myocyte contractile activity modulates norepinephrine cytotoxicity and survival effects of neuregulin-1beta. Am J Physiol Cell Physiol *286*, C222-229.

Laplante, M., and Sabatini, D.M. (2009). mTOR signaling at a glance. J Cell Sci *122*, 3589-3594.

Lavin, D.P., White, M.F., and Brazil, D.P. (2016). IRS proteins and diabetic complications. Diabetologia *59*, 2280-2291.

Lawson, J.W., and Uyeda, K. (1987). Effects of insulin and work on fructose 2,6-bisphosphate content and phosphofructokinase activity in perfused rat hearts. J Biol Chem *262*, 3165-3173.

Lebrun, P., Baron, V., Hauck, C.R., Schlaepfer, D.D., and Van Obberghen, E. (2000). Cell adhesion and focal adhesion kinase regulate insulin receptor substrate-1 expression. J Biol Chem *275*, 38371-38377.

Lee, K.F., Simon, H., Chen, H., Bates, B., Hung, M.C., and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature *378*, 394-398.

Lemmens, K., Doggen, K., and De Keulenaer, G.W. (2011). Activation of the neuregulin/ErbB system during physiological ventricular remodeling in pregnancy. Am J Physiol Heart Circ Physiol *300*, H931-942.

Lemmens, K., Fransen, P., Sys, S.U., Brutsaert, D.L., and De Keulenaer, G.W. (2004). Neuregulin-1 induces a negative inotropic effect in cardiac muscle: role of nitric oxide synthase. Circulation *109*, 324-326.

Lemmens, K., Segers, V.F., Demolder, M., and De Keulenaer, G.W. (2006). Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk. J Biol Chem *281*, 19469-19477.

Lenihan, D.J., Anderson, S.A., Lenneman, C.G., Brittain, E., Muldowney, J.A.S., Mendes, L., Zhao, P.Z., Iaci, J., Frohwein, S., Zolty, R., et al. (2016). A Phase I, Single Ascending Dose Study of Cimaglermin Alfa (Neuregulin 1β3) in Patients With Systolic Dysfunction and Heart Failure. JACC: Basic to Translational Science *1*, 576-586.

Leone, M., Magadum, A., and Engel, F.B. (2015). Cardiomyocyte proliferation in cardiac development and regeneration: a guide to methodologies and interpretations (vol 309, pg H1237, 2015). Am J Physiol-Heart C *309*, H1997-H1997.

Leto, D., and Saltiel, A.R. (2012). Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol *13*, 383-396.

Li, B., Xiao, J., Li, Y., Zhang, J., and Zeng, M. (2013). Gene transfer of human neuregulin-1 attenuates ventricular remodeling in diabetic cardiomyopathy rats. Exp Ther Med *6*, 1105-1112.

Li, B., Zheng, Z., Wei, Y., Wang, M., Peng, J., Kang, T., Huang, X., Xiao, J., Li, Y., and Li, Z. (2011). Therapeutic effects of neuregulin-1 in diabetic cardiomyopathy rats. Cardiovasc Diabetol *10*, 69.

Lim, C.Y., Bi, X.Z., Wu, D.H., Kim, J.B., Gunning, P.W., Hong, W.J., and Han, W.P. (2015). Tropomodulin3 is a novel Akt2 effector regulating insulin-stimulated GLUT4 exocytosis through cortical actin remodeling. Nat Commun *6*.

Liu, J., and Kern, J.A. (2002). Neuregulin-1 activates the JAK-STAT pathway and regulates lung epithelial cell proliferation. Am J Respir Cell Mol Biol *27*, 306-313.

Liu, X., Gu, X., Li, Z., Li, X., Li, H., Chang, J., Chen, P., Jin, J., Xi, B., Chen, D., et al. (2006). Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. J Am Coll Cardiol *48*, 1438-1447.

Lopaschuk, G.D., and Jaswal, J.S. (2010). Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. J Cardiovasc Pharmacol *56*, 130-140.

Lopez-Soldado, I., Niisuke, K., Veiga, C., Adrover, A., Manzano, A., Martinez-Redondo, V., Camps, M., Bartrons, R., Zorzano, A., and Guma, A. (2016). Neuregulin improves response to glucose tolerance test in control and diabetic rats. Am J Physiol Endocrinol Metab *310*, E440-451.

Ma, H., Yin, C., Zhang, Y., Qian, L., and Liu, J. (2016). ErbB2 is required for cardiomyocyte proliferation in murine neonatal hearts. Gene *592*, 325-330.

Mansor, L.S., Gonzalez, E.R., Cole, M.A., Tyler, D.J., Beeson, J.H., Clarke, K., Carr, C.A., and Heather, L.C. (2013). Cardiac metabolism in a new rat model of type 2 diabetes using high-fat diet with low dose streptozotocin. Cardiovascular Diabetology *12*.

Marchionni, M.A., Goodearl, A.D., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature *362*, 312-318.

Mei, L., and Nave, K.A. (2014). Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. Neuron *83*, 27-49.

Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. Nature *378*, 386-390.

Miki, T., Yuda, S., Kouzu, H., and Miura, T. (2013). Diabetic cardiomyopathy: pathophysiology and clinical features. Heart Fail Rev *18*, 149-166.

Missinato, M.A., Saydmohammed, M., Zuppo, D.A., Rao, K.S., Opie, G.W., Kuhn, B., and Tsang, M. (2018). Dusp6 attenuates Ras/MAPK signaling to limit zebrafish heart regeneration. Development *145*.

Mollova, M., Bersell, K., Walsh, S., Savla, J., Das, L.T., Park, S.Y., Silberstein, L.E., dos Remedios, C.G., Graham, D., Colan, S., et al. (2013). Cardiomyocyte proliferation contributes to heart growth in young humans. P Natl Acad Sci USA *110*, 1446-1451.

Montessuit, C., Papageorgiou, I., and Lerch, R. (2008). Nuclear receptor agonists improve insulin responsiveness in cultured cardiomyocytes through enhanced signaling and preserved cytoskeletal architecture. Endocrinology *149*, 1064-1074.

Montessuit, C., Rosenblatt-Velin, N., Papageorgiou, L., Campos, L., Pellieux, C., Palma, T., and Lerch, R. (2004). Regulation of glucose transporter expression in cardiac myocytes: p38 MAPK is a strong inducer of GLUT4. Cardiovascular Research *64*, 94-104.

Morano, M., Angotti, C., Tullio, F., Gambarotta, G., Penna, C., Pagliaro, P., and Geuna, S. (2017). Myocardial ischemia/reperfusion upregulates the transcription of the Neuregulin1 receptor ErbB3, but only postconditioning preserves protein translation: Role in oxidative stress. Int J Cardiol *233*, 73-79.

Morkin, E. (2000). Control of cardiac myosin heavy chain gene expression. Microsc Res Tech *50*, 522-531.

Mueckler, M., and Thorens, B. (2013). The SLC2 (GLUT) family of membrane transporters. Mol Aspects Med *34*, 121-138.

Nakamura, M., and Sadoshima, J. (2018). Mechanisms of physiological and pathological cardiac hypertrophy. Nat Rev Cardiol *15*, 387-407.

Neely, J.R., Rovetto, M.J., and Oram, J.F. (1972). Myocardial utilization of carbohydrate and lipids. Prog Cardiovasc Dis *15*, 289-329.

Nishimura, H., Pallardo, F.V., Seidner, G.A., Vannucci, S., Simpson, I.A., and Birnbaum, M.J. (1993). Kinetics of GLUT1 and GLUT4 glucose transporters expressed in Xenopus oocytes. J Biol Chem *268*, 8514-8520.

Odiete, O., Hill, M.F., and Sawyer, D.B. (2012). Neuregulin in cardiovascular development and disease. Circ Res *111*, 1376-1385.

Onay-Besikci, A. (2006). Regulation of cardiac energy metabolism in newborn. Mol Cell Biochem *287*, 1-11.

Opie, L.H. (1990). Myocardial ischemia--metabolic pathways and implications of increased glycolysis. Cardiovasc Drugs Ther *4 Suppl 4*, 777-790.

Ozcelik, C., Erdmann, B., Pilz, B., Wettschureck, N., Britsch, S., Hubner, N., Chien, K.R., Birchmeier, C., and Garratt, A.N. (2002). Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. Proc Natl Acad Sci U S A *99*, 8880-8885.

Palfreyman, R.W., Clark, A.E., Denton, R.M., Holman, G.D., and Kozka, I.J. (1992). Kinetic resolution of the separate GLUT1 and GLUT4 glucose transport activities in 3T3-L1 cells. Biochem J *284 (Pt 1)*, 275-282.

Parodi, E.M., and Kuhn, B. (2014). Signalling between microvascular endothelium and cardiomyocytes through neuregulin. Cardiovasc Res *102*, 194-204.

Pearson, G., Robinson, F., Gibson, T.B., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev *22*, 153-183.

Peles, E., Bacus, S.S., Koski, R.A., Lu, H.S., Wen, D.Z., Ogden, S.G., Benlevy, R., and Yarden, Y. (1992). Isolation of the Neu/Her-2 Stimulatory Ligand - a 44kd Glycoprotein That Induces Differentiation of Mammary-Tumor Cells. Cell *69*, 205-216.

Peng, J., and He, L. (2018). IRS posttranslational modifications in regulating insulin signaling. J Mol Endocrinol *60*, R1-R8.

Pentassuglia, L., Heim, P., Lebboukh, S., Morandi, C., Xu, L., and Brink, M. (2016). Neuregulin-1beta promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes. Am J Physiol Endocrinol Metab *310*, E782-794.

Pentassuglia, L., and Sawyer, D.B. (2009). The role of Neuregulin-1beta/ErbB signaling in the heart. Exp Cell Res *315*, 627-637.

Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B.J., and Yarden, Y. (1996). Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. J Biol Chem *271*, 19029-19032.

Polizzotti, B.D., Ganapathy, B., Walsh, S., Choudhury, S., Ammanamanchi, N., Bennett, D.G., Dos Remedios, C.G., Haubner, B.J., Penninger, J.M., and Kuhn, B. (2015). Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. Science translational medicine *7*, 281ra245.

Porrello, E.R., Mahmoud, A.I., Simpson, E., Hill, J.A., Richardson, J.A., Olson, E.N., and Sadek, H.A. (2011). Transient regenerative potential of the neonatal mouse heart. Science *331*, 1078-1080.

Qi, Y.J., Xu, Z.H., Zhu, Q.L., Thomas, C., Kumar, R., Feng, H., Dostal, D.E., White, M.F., Baker, K.M., and Guo, S.D. (2013). Myocardial Loss of IRS1 and IRS2 Causes Heart Failure and Is Controlled by p38 alpha MAPK During Insulin Resistance. Diabetes *62*, 3887-3900.

Razeghi, P., Young, M.E., Alcorn, J.L., Moravec, C.S., Frazier, O.H., and Taegtmeyer, H. (2001). Metabolic gene expression in fetal and failing human heart. Circulation *104*, 2923-2931.

Rentschler, S., Zander, J., Meyers, K., France, D., Levine, R., Porter, G., Rivkees, S.A., Morley, G.E., and Fishman, G.I. (2002). Neuregulin-1 promotes formation of the murine cardiac conduction system. Proc Natl Acad Sci U S A *99*, 10464-10469.

Rerup, C.C. (1970). Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol Rev *22*, 485-518.

Riehle, C., and Abel, E.D. (2016). Insulin Signaling and Heart Failure. Circ Res *118*, 1151-1169.

Rohrbach, S., Yan, X.H., Weinberg, E.O., Hasan, F., Bartunek, J., Marchionni, M.A., and Lorell, B.H. (1999). Neuregulin in cardiac hypertrophy in rats with aortic stenosis - Differential expression of erbB2 and erbB4 receptors. Circulation *100*, 407-412.

Roux, P.P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev *68*, 320-344.

Rowland, A.F., Fazakerley, D.J., and James, D.E. (2011). Mapping insulin/GLUT4 circuitry. Traffic *12*, 672-681.

Rui, L., Fisher, T.L., Thomas, J., and White, M.F. (2001). Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J Biol Chem *276*, 40362-40367.

Rupert, C.E., and Coulombe, K.L. (2015). The roles of neuregulin-1 in cardiac development, homeostasis, and disease. Biomarker insights *10*, 1-9.

Russell, K.S., Stern, D.F., Polverini, P.J., and Bender, J.R. (1999). Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis. Am J Physiol *277*, H2205-2211.

Russell, R.R., 3rd, Li, J., Coven, D.L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F.J., Mu, J., Birnbaum, M.J., and Young, L.H. (2004). AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. J Clin Invest *114*, 495-503.

Sanchis-Gomar, F., Perez-Quilis, C., Leischik, R., and Lucia, A. (2016). Epidemiology of coronary heart disease and acute coronary syndrome. Ann Transl Med *4*, 256.

Sano, H., Kane, S., Sano, E., Miinea, C.P., Asara, J.M., Lane, W.S., Garner, C.W., and Lienhard, G.E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem *278*, 14599-14602.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol *14*, 1296-1302.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Schulze, W.X., Deng, L., and Mann, M. (2005). Phosphotyrosine interactome of the ErbB-receptor kinase family. Mol Syst Biol *1*, 2005 0008.

Schwander, J.C., Hauri, C., Zapf, J., and Froesch, E.R. (1983). Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. Endocrinology *113*, 297-305.

Shao, D., and Tian, R. (2015). Glucose Transporters in Cardiac Metabolism and Hypertrophy. Compr Physiol *6*, 331-351.

Shao, D., Villet, O., Zhang, Z., Choi, S.W., Yan, J., Ritterhoff, J., Gu, H., Djukovic, D., Christodoulou, D., Kolwicz, S.C., Jr., et al. (2018). Glucose promotes cell growth by suppressing branched-chain amino acid degradation. Nat Commun *9*, 2935.

Shende, P., Xu, L., Morandi, C., Pentassuglia, L., Heim, P., Lebboukh, S., Berthonneche, C., Pedrazzini, T., Kaufmann, B.A., Hall, M.N., et al. (2016). Cardiac mTOR complex 2 preserves ventricular function in pressure-overload hypertrophy. Cardiovasc Res *109*, 103-114.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. New Engl J Med *344*, 783-792.

Stanley, W.C., Recchia, F.A., and Lopaschuk, G.D. (2005). Myocardial substrate metabolism in the normal and failing heart. Physiol Rev *85*, 1093-1129.

Strom, C.C., Aplin, M., Ploug, T., Christoffersen, T.E.H., Langfort, J., Viese, M., Galbo, H., Haunso, S., and Sheikh, S.P. (2005). Expression profiling reveals differences in metabolic gene expression between exercise-induced cardiac effects and maladaptive cardiac hypertrophy. Febs J *272*, 2684-2695.

Suarez, E., Bach, D., Cadefau, J., Palacin, M., Zorzano, A., and Guma, A. (2001). A novel role of neuregulin in skeletal muscle. Neuregulin stimulates glucose uptake, glucose transporter translocation, and transporter expression in muscle cells. J Biol Chem *276*, 18257-18264.

Szablewski, L. (2017). Glucose transporters in healthy heart and in cardiac disease. Int J Cardiol *230*, 70-75.

Szczepaniak, L.S., Victor, R.G., Orci, L., and Unger, R.H. (2007). Forgotten but not gone: the rediscovery of fatty heart, the most common unrecognized disease in America. Circ Res *101*, 759-767.

Taegtmeyer, H., Sen, S., and Vela, D. (2010). Return to the fetal gene program: a suggested metabolic link to gene expression in the heart. Ann N Y Acad Sci *1188*, 191-198.

Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol *13*, 513-609.

Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N.L., Yayon, A., Wen, D.Z., et al. (1994). Erbb-3 and Erbb-4 Function as the Respective Low and High-Affinity Receptors of All Neu Differentiation Factor Heregulin Isoforms. Journal of Biological Chemistry *269*, 25226-25233.

Vandekerckhove, L., Vermeulen, Z., Liu, Z.Z., Boimvaser, S., Patzak, A., Segers, V.F., and De Keulenaer, G.W. (2016). Neuregulin-1 attenuates development of nephropathy in a type 1 diabetes mouse model with high cardiovascular risk. Am J Physiol Endocrinol Metab *310*, E495-504.

Vermeulen, Z., Hervent, A.S., Dugaucquier, L., Vandekerckhove, L., Rombouts, M., Beyens, M., Schrijvers, D.M., De Meyer, G.R.Y., Maudsley, S., De Keulenaer, G.W., et al. (2017). Inhibitory actions of the NRG-1/ErbB4 pathway in macrophages during tissue fibrosis in the heart, skin, and lung. Am J Physiol Heart Circ Physiol *313*, H934-H945.

Vermeulen, Z., Segers, V.F., and De Keulenaer, G.W. (2016). ErbB2 signaling at the crossing between heart failure and cancer. Basic Res Cardiol *111*, 60.

Walker, C.A., and Spinale, F.G. (1999). The structure and function of the cardiac myocyte: a review of fundamental concepts. J Thorac Cardiovasc Surg *118*, 375-382.

Wang, C., and Hu, S.M. (1991). Developmental regulation in the expression of rat heart glucose transporters. Biochem Biophys Res Commun *177*, 1095-1100.

Wang, F., and Higgins, J.M. (2013). Histone modifications and mitosis: countermarks, landmarks, and bookmarks. Trends Cell Biol *23*, 175-184.

Waring, C.D., Vicinanza, C., Papalamprou, A., Smith, A.J., Purushothaman, S., Goldspink, D.F., Nadal-Ginard, B., Torella, D., and Ellison, G.M. (2014). The adult heart responds to increased workload with physiologic hypertrophy, cardiac stem cell activation, and new myocyte formation. Eur Heart J *35*, 2722-2731.

Weber, K.T., Sun, Y., Bhattacharya, S.K., Ahokas, R.A., and Gerling, I.C. (2013). Myofibroblast-mediated mechanisms of pathological remodelling of the heart. Nat Rev Cardiol *10*, 15-26.

Weiss, R.B. (1982). Streptozocin: a review of its pharmacology, efficacy, and toxicity. Cancer Treat Rep *66*, 427-438.

Willem, M. (2016). Proteolytic processing of Neuregulin-1. Brain Res Bull *126*, 178-182.

Wilson, K.J., Gilmore, J.L., Foley, J., Lemmon, M.A., and Riese, D.J., 2nd (2009). Functional selectivity of EGF family peptide growth factors: implications for cancer. Pharmacol Ther *122*, 1-8.

Withers, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., Ren, J.M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G.I., et al. (1998). Disruption of IRS-2 causes type 2 diabetes in mice. Nature *391*, 900-904.

Xin, M., Olson, E.N., and Bassel-Duby, R. (2013). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol *14*, 529-541.

Ye, L., D'Agostino, G., Loo, S.J., Wang, C.X., Su, L.P., Tan, S.H., Tee, G.Z., Pua, C.J., Pena, E.M., Cheng, R.B., et al. (2018). Early Regenerative Capacity in the Porcine Heart. Circulation.

Yester, J.W., and Kuhn, B. (2017). Mechanisms of Cardiomyocyte Proliferation and Differentiation in Development and Regeneration. Curr Cardiol Rep *19*, 13.

Zhao, Y.Y., Sawyer, D.R., Baliga, R.R., Opel, D.J., Han, X., Marchionni, M.A., and Kelly, R.A. (1998). Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. J Biol Chem *273*, 10261-10269.

Zhu, W., Zhang, E., Zhao, M., Chong, Z., Fan, C., Tang, Y., Hunter, J.D., Borovjagin, A.V., Walcott, G.P., Chen, J.Y., et al. (2018). Regenerative Potential of Neonatal Porcine Hearts. Circulation.

Zipes, D.P., Libby, P., Bonow, R.O., and Braunwald, E. (2005). Heart disease a textbook of cardiovascular medicine *7th Edition*.

Acknowledgments

First of all, I want to thank a lot Marijke, who gave me the opportunity to do my master thesis and later on also my PhD thesis in her laboratory. She taught me scientific thinking and how basic research works. She supported me a lot, she was always around to discuss my latest results and answer my questions. Moreover, she was great in turning my “weird” results into “interesting” results. Marijke encouraged me to visit scientific meetings and present my results in front of a big scientific audience.

I also want to thank my lab mates Christian Morandi, Lifen Xu, Sonia Lebboukh, Laura Pentassuglia, Gian Brouwer, Joseph Iharinjaka Randriamboavonjy and Hagena Thuraisingam. We always had a very good working atmosphere, but we also had a great time outside the laboratory and shared many nice experiences besides doing research. Whenever I needed support, they were ready to help me.
Already during my time as master student and afterwards as PhD student, Christian Morandi taught me many techniques and he was always very patient to answer my questions. Especially, I want to thank him for performing many valuable experiments for my PhD project.
I also want to acknowledge Laura Pentassuglia, who brought the Nrg1β research to our lab and introduced me to the topic when I started my PhD thesis.

I would like to thank Christophe Montessuit from the University of Geneva for his collaboration. He performed several experiments with the ARVM model for our study.

Moreover, I want to thank Markus Rüegg and Marc Donath, who were part of my PhD committee.

Finally, my greatest thank goes to my wife Barbara. She always supported me, we could discuss our scientific problems and at the same time she successfully managed to finish her own PhD thesis.

CURRICULUM VITAE

"I am a highly reliable person, well-organized and I am ready to face a new challenge. I would appreciate to learn new skills and get exposed to an exciting industrial environment."

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WORK AND PRACTICAL EXPERIENCE

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2007 – 2014	Host, Messe Schweiz, Basel
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P. Shende, L. Xu, C. Morandi, L. Pentassuglia, **P. Heim**, S. Lebboukh, C. Berthonneche, T. Pedrazzini, B. A. Kaufmann, M. N. Hall, M. A. Rüegg, M. Brink (2016). *"Cardiac mTOR complex 2 preserves ventricular function in pressure-overload hypertrophy"*, Cardiovascular Research