Influence of thyroid hormones on myocardial ischaemia/reperfusion injury in isolated perfused mouse hearts

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Zusammenfassung

Schilddrüsenhormone (THs) sind substanziell für eine normale Herzfunktion. Im Myokardinfarkt muss die chronische und akute TH-Wirkung noch geklärt werden, insbesondere die Bedeutung der Schilddrüsenhormonrezeptor (TR) abhängigen Signaltransduktion am Ischämie/Reperfusions (IR) Schaden.

Um die Auswirkungen einer chronischen TH-Wirkung auf den IR-Schaden zu untersuchen, wurden isoliert druckkonstant perfundierte Herzen von 3 und 20 Monate alten Euthyreose- (Kontrolle), Hypothyreose- (Hypo) und Hyperthyreose- (Hyper) Mäusen im Langendorff-Modell untersucht und die Infarktgröße, sowie funktionelle Parameter analysiert. Im Vergleich zu Kontrollen war eine chronische Hypothyreose in beiden Altersklassen kardioprotektiv. Dies war mit einer geringeren Infarktgröße und einer verbesserten Wiederherstellung des linksventrikulären Drucks (LVDP) verbunden. Die Kardioprotektion war zudem unabhängig von der hämodynamischen Ausgangssituation der Hypothyreose mit vorangegangener Bradykardie, sowie einem reduzierten Baseline LVDP. Eine chronische Hyperthyreose beeinflusste den IR-Schaden hingegen nachteilig und führte zu größeren Infarkten und einer reduzierten Wiederherstellung des LVDP im Vergleich zu Kontrollen. Interessanterweise zeigten Mäuse ohne TRα ($TR\alpha^0$), genau wie hypothyreote Mäuse, auch einen geringeren IR-Schaden im Herzen. Im Vergleich zu den hyperthyreoten Mäusen war die systemische Hyperthyreose in $TR\alpha^0$-Mausherzen allerdings ohne nachteiligen Effekt und die Infarktgröße blieb unverändert klein. Bemerkenswerterweise war speziell das Fehlen des kanonischen TRα-Signals mit der Kardioprotektion verbunden, da auch die Herzen von Mäusen mit einem mutierten TRα ($TR\alpha^{GS}$), der nicht-kanonische TRα-Wirkung ausüben, aber nicht an die DNA binden kann, eine geringe Infarktgröße und eine verbesserte Erholung des LVDP zeigten.

Die akute Wirkung von THs auf den IR-Schaden wurde untersucht, indem verschiedene Dosen von exogen verabreichtem Triiodthyronin (T3) zu verschiedenen Zeitpunkten gegeben wurden. Die Infarktgröße konnte durch akute T3-Gabe vor Ischämie dosisabhängig reduziert werden. Die Infarktgröße war ebenso verringert, wenn T3 erst nach der Ischämie verabreicht wurde, was auf einen schnellen nicht-kanonischen Effekt hindeutete, der auf den Reperfusionsschaden abzielte. Darüber hinaus ergaben erste Versuche Hinweise auf eine mögliche Entkopplung der endothelialen Stickstoffmonoxidsynthase als Ursache des verringerten IR-Schadens.

Zusammenfassend liefern diese Untersuchungen Anhaltspunkte dafür, dass THs die Größe des Myokardinfarkts unterschiedlich beeinflussen, je nachdem, ob die Wirkung am Herzen chronisch vor akutem Myokardinfarkt (AMI) oder akut (kurz vor/nach AMI) erfolgt. Die Kardioprotektion unter Hypothyreose konnte als ein altersunabhängiger Effekt gezeigt werden, welcher – unabhängig von einer vorteilhaften hämodynamischen Ausgangssituation – auf das Fehlen einer kanonischen TH-TRα-Wirkung zurückgeführt werden konnte. Die akute T3-Verabreichung reduzierte die Infarktgröße unabhängig vom Vor- oder Nachkonditionierungsmodus, über eine schnelle nicht-kanonische Wirkung. Diese Ergebnisse deuten also auf unterschiedliche Effekte der TH-Wirkung im Kontext einer akuten Myokardischämie hin und legen ein hohes therapeutisches Potential nahe, das in weiterführenden Untersuchungen geklärt werden muss.

Abstract

Thyroid hormones (THs) are crucial for normal cardiac function. In myocardial infarction, the precise role of chronic and acute TH action still requires clarification, in particular the importance of thyroid hormone receptor (TR) signalling on ischaemia/reperfusion (IR) injury.

To delineate the role of chronic TH action on myocardial IR injury, isolated pressure constant perfused hearts of 3 and 20 months old euthyroid (control), hypothyroid (hypo) and hyperthyroid (hyper) mice were investigated in the Langendorff model and myocardial infarct size and functional performance were analysed. Compared to controls chronic hypothyroidism was cardioprotective with smaller infarct size and improved recovery of left ventricular developed pressure (LVDP) in both age groups. Cardioprotection was independent of favorable hemodynamics such as decreased heart rate and LVDP at baseline. On the other hand, chronic hyperthyroidism was detrimental in IR injury and led to larger infarcts and reduced functional recovery of LVDP compared to controls. Interestingly, hearts of mice lacking TRα ($TR\alpha^0$) were protected against IR injury, resembling the cardioprotective effect observed in hypothyroid hearts. Moreover, in contrast to the hyper group, systemic hyperthyroidism did not increase infarct size or reduce recovery of LVDP in $TR\alpha^0$ hearts. Strikingly, specifically the lack of canonical TRα signalling confers cardioprotection, because hearts from mice expressing a mutant TRα ($TR\alpha^{GS}$) that is capable of noncanonical TRα signalling but incapable of binding to the DNA, also showed a small infarct size and an improved recovery of LVDP.

Acute effect of THs on IR injury was investigated by addressing the role of different doses and time points of exogenously administered triiodothyronine (T3) on myocardial infarct size. Notably, in contrast to chronic hyperthyroid condition, an acute treatment with T3 reduced infarct size dose-dependently and improved recovery of LVDP after IR injury. In fact, infarct size was also reduced when T3 was given after ischaemia, suggesting a rapid non-canonical effect targeting reperfusion damage. Furthermore, possible causal involvement of endothelial nitric oxide synthase uncoupling was suggested in first experiments.

In conclusion, this investigation provides evidence that THs impact myocardial infarct size differently, depending on whether they are administered chronically before acute myocardial infarction (AMI) or acutely in/after AMI. Thus, cardioprotection under hypothyroidism was an age-independent effect that could be attributed to the lack of canonical TRα signalling. Acute T3 administration reduced infarct size regardless of pre- or post-conditioning mode demonstrating a rapid noncanonical effect. Therefore, these results indicate different effects of

TH action on myocardial IR injury. Taken together these findings open up therapeutic avenues with regard to modulation of intracardiac TH status in cardiovascular risk patients and in situation of acute myocardial ischaemia, which should be pursued in further studies.

Introduction

Thyroid hormones

Thyroid hormone synthesis and secretion

Thyroid hormones (THs), T3 (3,5,3'-triiodothyronine; thyronine) and T4 (3,5,3',5'-tetraiodothyronine; thyroxine), are crucial for organ development and maintenance (Brix, Fuhrer, and Biebermann 2011). Their synthesis and secretion take place in the thyroid gland. The thyroid gland is a butterfly shaped organ located at the anterior side of the trachea (Fig. 1) and consists of two lobes that are connected by a cellular belt called *isthmus glandularis thyroidae*. The synthesis of THs takes place in the thyroid follicles, which are formed by thyrocytes and comprice a lumen (Yen 2001). TH synthesis and secretion is tightly regulated by the hypothalamic-pituitary-thyroid (HPT) axis (Fig. 1). The thyrotropin-releasing hormone (TRH) is produced and released by the hypothalamus. It is transported through the pituitary-portal vasculature to the anterior pituitary gland where it induces the expression of thyroid-stimulating hormone (TSH; thyrotropin). Via the blood stream, TSH is transported to the thyroid gland, where it binds to TSH receptors on thyrocytes stimulating TH synthesis and secretion (Rousset et al. 2000). A negative feedback loop tightly controls TH production (Fig. 1). High TH concentrations in the blood result in a reduction of TRH production and secretion in the hypothalamus as well as a reduction in TSH production in the pituitary. *Vice versa*, low circulating TH concentrations increase TRH and TSH secretion and thereby TH production (Shupnik, Ridgway, and Chin 1989; Hashimoto et al. 1991). Free rather than bound TH is relevant for the homeostatic control of the HPT axis (Schussler 2000). With a molar ratio of T4 to T3 of around 14:1 in humans (Pilo et al. 1990), T4 is the main hormone released from the thyroid gland (Mondal et al. 2016). In rodents the ratio is only around 6:1 (Wiersinga et al. 2012).

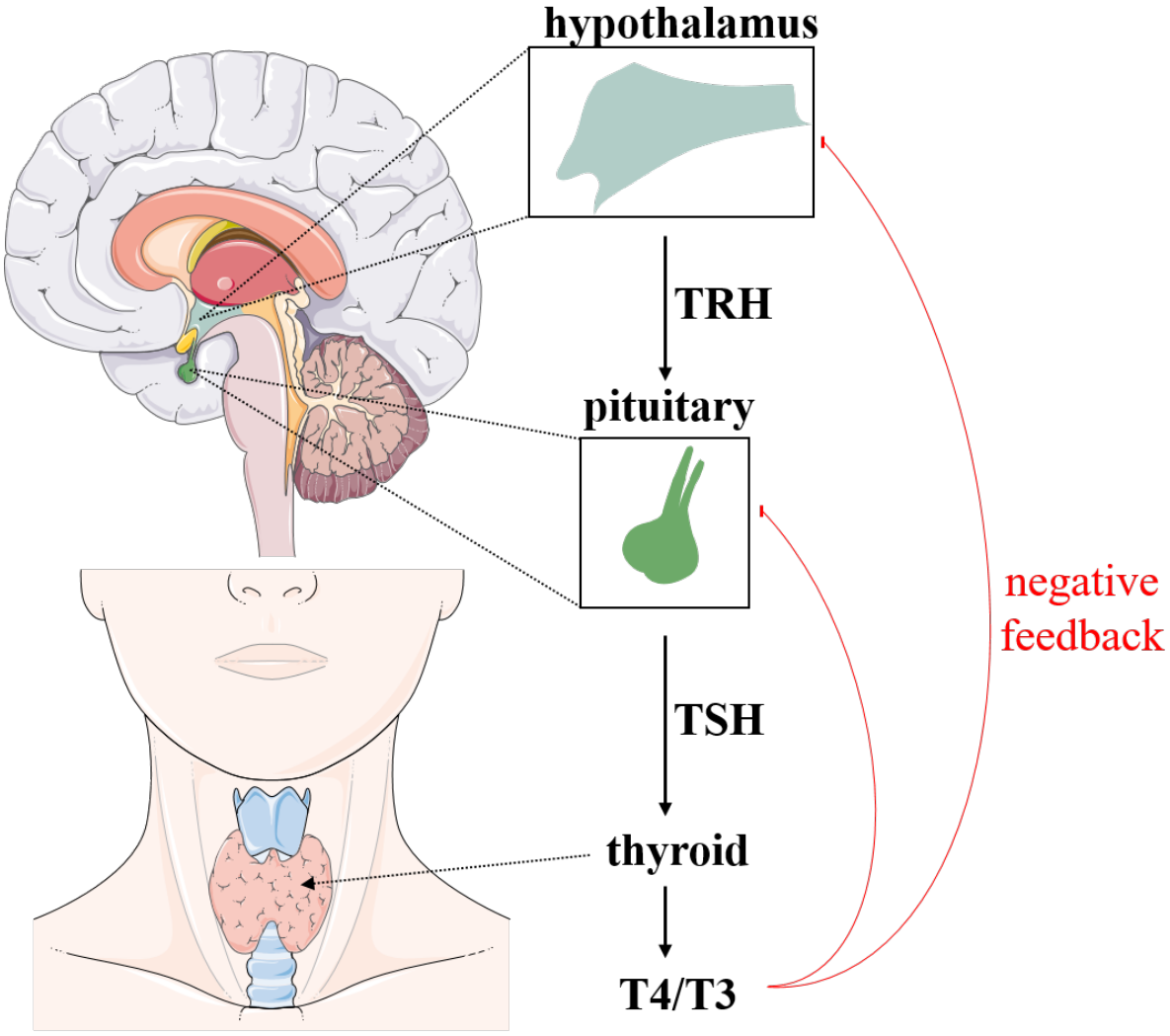

Figure 1: Hypothalamic-pituitary-thyroid axis. Thyrotropin releasing hormone (TRH) is produced and released by the hypothalamus and transported to the anterior pituitary gland, where it induces production of thyroid stimulating hormone (TSH). TSH binds to the TSH receptor on thyrocytes leading to synthesis and secretion of thyroid hormones T4 and T3. High concentrations of circulating T4/T3 decrease TSH and TRH expression and low circulating T4/T3 concentrations increase TSH and TRH expression (negative feedback loop).

Thyroid hormone transport and metabolism

THs are lipophilic and thus require specialized serum transport proteins to reach their target organs. The three serum transport proteins are thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA)/transthyretin, and albumin, with TBG alone binding 75% of serum T4 and T3 (Refetoff 2000). However, only a small fraction of approximately 0.5% unbound THs can be taken up by their target cells. Specific TH transporters like monocarboxylate transporter 8 (MCT8) and 10 (MCT10) or organic anion-transporting polypeptide 1C1 in mice (OATP1C1; (Hennemann et al. 2001; Visser et al. 2016; Groeneweg et al. 2019)) have been identified for TH uptake across the plasma membrane of cells.

THs belong to the class of iodothyronines. They consist of thyronine amino acids, which are iodinated on the aromatic ring at three (T3) or four positions (T4) (Camerman and Camerman 1972). Since T3 is the biologically active hormone, T4 needs to be deiodinated intracellularly via 5'deiodinases (DIOs) (Fig. 2) (Mondal et al. 2016; Kohrle 1996). DIOs can either activate or inactivate THs. There are three different types of DIOs, differing in their mode of action (Dumitrescu and Refetoff 2007) and organ- and cell-specific expression. Deiodinase type 1 (DIO1) is mainly expressed in liver, kidney and intestine and is responsible for conversion from T4 to T3 with liver DIO1 representing a major regulator of circulating T3 concentration. DIO2 expression is primarily found in the central nervous system (CNS), the pituitary gland, the heart and in brown adipose tissue, where it converts T4 to T3. DIO3 is highly expressed in placenta, heart and CNS and inactivates T4 to reverse T3 (rT3) or T3 to 3,3'-diiodothyronine (T2) (Fig. 2) (Gereben et al. 2008; van der Spek, Fliers, and Boelen 2017; Mondal et al. 2016). DIO3 expression has a particular role in pathological tissue conditions. Thus, under TH deprivation (hypothyroidism) DIO3 activity is reduced, whereas TH overload (hyperthyroidism) increases DIO3 activity to protect tissues from excess TH levels (Bianco et al. 2002). Furthermore, in an experimental model of acute myocardial infarction a low TH tissue state was reported that was induced by upregulation of cardiac DIO3 and resulted in reduced T3 availability in the myocardium (Simonides et al. 2008).

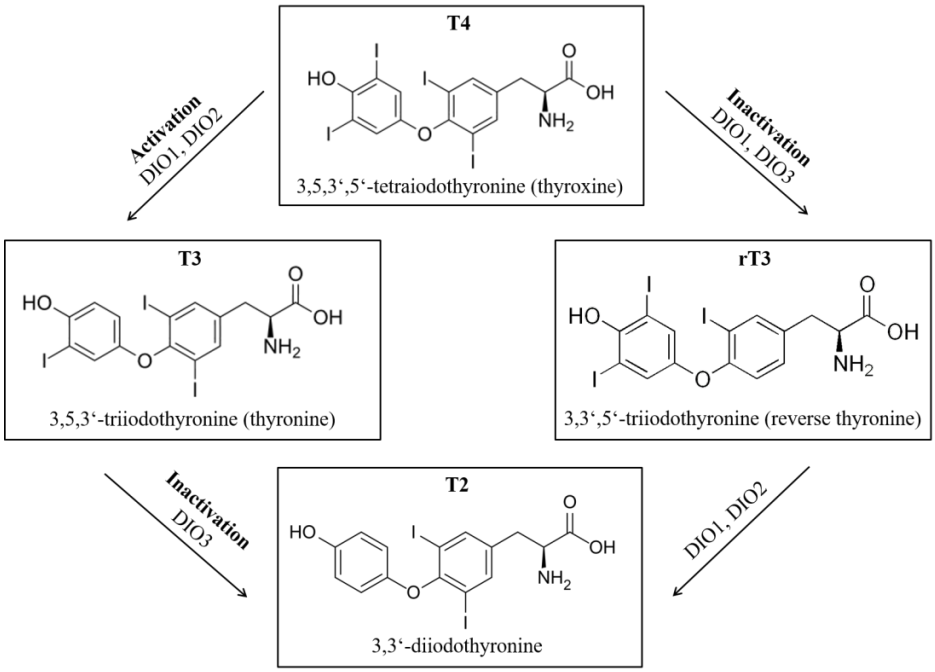

Figure 2: TH activation or inactivation. Deiodinases (DIOs) DIO1 or DIO2 convert T4 to T3 or it is inactivated via DIO1 or DIO3 to reverse T3 (rT3). DIO3 inactivates T3 to T2, while DIO1 or DIO2 convert rT3 to T2.

Thyroid hormone action

THs mainly exert their action upon binding to TH receptors (TRs). Since the 80s it is known that two TR isoforms exist (Vennstrom and Bishop 1982; Weinberger et al. 1986; Sap et al. 1986): TRα and TRβ (Yen 2001; Mondal et al. 2016). TRα and TRβ are encoded by the genes *THRA* and *THRB*, located on human chromosome 17 and 3, respectively (Laudet et al. 1991; Kanaka, Eble, and Mullis 1991). Similar to DIOs, TRs are also expressed in an organ- and cell-specific manner (Flamant and Gauthier 2013). While TRα is mainly expressed in heart, brain (cortex), bone or skeletal muscle, TRβ is mainly expressed in kidney, liver and distinct brain regions such as hippocampus or pituitary (Brent 2012). TRs belong to the superfamily of nuclear receptors and can act as ligand-modulated transcriptional factors (Beato, Herrlich, and Schutz 1995; Yen 2001). They are constitutively bound to TH responsive elements (TREs) and form either homo- or heterodimers with retinoic acid receptors (RXR) (Williams et al. 1991). Via binding of T3 to TRs, gene expression of different target genes is stimulated or repressed, which is called type 1 or canonical TR signalling (Fig. 3) (Chassande et al. 1997).

Type 2 signalling indicates an indirect binding of THs and TRs to the DNA via other proteins or multiprotein complexes (Flamant et al. 2017; Desbois et al. 1991).

While canonical TR action occurs via stimulated or repressed gene expression and subsequent protein synthesis (Yen 2001), type 3 signalling or noncanonical TR action happens rapidly (within minutes) and is independent from DNA binding and protein synthesis (Fig. 3) (Segal, Buckley, and Ingbar 1985). The proposed mechanism of noncanonical action includes an interaction of TRs with phosphoinositide 3-kinases (PI3K) and a downstream phosphorylation of proteinkinase B (AKT) (Cao et al. 2009; Martin et al. 2014). Consequently this might result in transcription of PI3K dependent genes and in the induction of noncanonically regulated TH target genes, respectively (Moeller and Broecker-Preuss 2011). However, there is little information about the precise mechanisms of noncanonical TR action. To distinguish between canonical and noncanonical TR signalling, the group of L.C. Möller in Essen has generated a mouse model with a mutation in the TRα DNA-binding domain ($TR\alpha^{GS}$) which completely abrogates canonical type 1 signalling while noncanonical type 3 signalling is preserved (Fig. 3) (Hones et al. 2017). Consequently the comparison of wildtype, $TR\alpha^{0}$ (mice being deficient of TRα) and $TR\alpha^{GS}$ mice allows differentiation between canonical and noncanonical TRα effects (Fig. 3) (Hones et al. 2017). We and others have shown that TRs can also act independent of TH-target gene expression via rapid activation of cellular signalling pathways and voltage-activated potassium channels (Storey et al. 2006; Hones et al. 2017; Martin et al.

2014; Flamant et al. 2017). Unbound TRs can also suppress transcription of target genes by interacting with corepressors, e.g. SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) (Chen and Evans 1995) or NCoR1 (nuclear receptor co-repressor 1) (Horwitz et al. 1996). Finally, type 4 signalling refers to extracellular TH action independent of TR binding and integrin αvβ3 has been proposed to serve as a membrane receptor of T4 and T3, particularly in the context of cancer. Binding of TH to integrin αvβ3 may then activate intracellular PI3K or extracellular related kinase (ERK) 1/2 (Davis, Goglia, and Leonard 2016; Flamant et al. 2017; Shinderman-Maman et al. 2016) and may propose tumor-driven neoangiogenesis (Latteyer et al. 2019).

Ultimately, canonical (type 1 signalling) and noncanonical TR signalling (type 3 signalling) have already been shown to exert physiological relevance in the heart (Hones et al. 2017; Wikstrom et al. 1998). Thus, regulation of heart rate is known to be mediated by TRα (Macchia et al. 2001; Wikstrom et al. 1998). Recently, we could show that noncanonical TRα signalling contributes to physiological regulation of heart rate, since *in vivo* heart rate, measured by noninvasive electrocardiography, was decreased in mice without TRα but preserved in $TR\alpha^{GS}$ mice (Hones et al. 2017). In contrast, *ex vivo* heart rate that was measured in the isolated heart according to Langendorff (Langendorff 1895) revealed a decreased heart rate in mice lacking TRα and also in $TR\alpha^{GS}$ mice, suggesting that noncanonical TRα signalling regulates heart rate via autonomous nervous system (Hones et al. 2017). However, the assignment to canonical or noncanonical TR signalling still remains open for many other physiological and also pathophysiological functions in the heart and requires further investigations (Martin et al. 2014; Cao et al. 2009). Taken together, TH signalling ensures physiological cardiac TH action. However, under pathophysiological conditions such as under thyroid dysfunction, the signalling can be disturbed, which then in turn can also affect heart health.

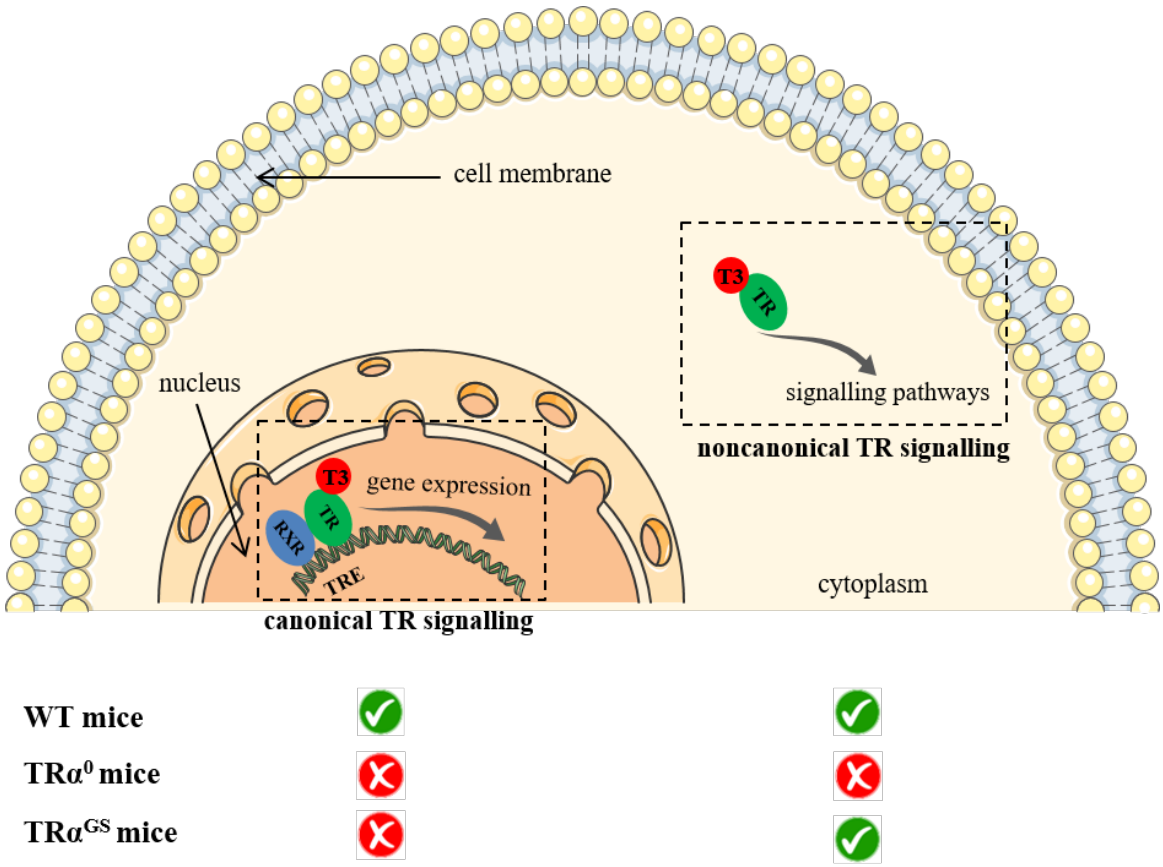

Figure 3: TH receptor signalling within a cell. TH receptors (TR) are constitutively bound to TH responsive elements (TREs) and form either homo- or heterodimers with retinoic acid receptors (RXR). Via binding of T3 to TR, gene expression of different target genes is stimulated or repressed (canonical TR signalling or type 1 signalling). Noncanonical TR action (type 3 signalling) happens rapidly and is independent from DNA binding and protein synthesis. The comparison of wildtype (WT), TRα^0 (mice being deficient of TRα) and TRα^{GS} mice allows differentiation between canonical and noncanonical TRα effects.

Thyroid dysfunction and the risk for cardiovascular disease

Thyroid dysfunction is characterized by abnormal TSH concentrations in the serum and may involve abnormal circulating TH levels. The definition of thyroid dysfunction is based on statistical reference ranges of the relevant biochemical parameters. Thus, the distinction between overt and subclinical thyroid dysfunction is purely a biochemical definition. Under hypothyroidism insufficient levels of THs are synthesized and secreted by the thyroid gland. Overt hypothyroidism is defined by increased TSH concentrations and decreased T4 levels (Chaker et al. 2017). On the other hand, hyperthyroidism is a pathological condition, in which excess of THs is synthesized and secreted by the thyroid gland. Overt hyperthyroidism is defined by decreased TSH concentrations and increased concentrations of T4 and/or T3 (De Leo, Lee, and Braverman 2016). Subclinical hypothyroidism is defined by increased TSH concentrations, while T4 levels remain normal. *Vice versa*, subclinical hyperthyroidism

is characterized by decreased TSH concentrations and reference range T4/T3 levels (Cooper and Biondi 2012). Taken together, overt thyroid dysfunction relates to more severe thyroid derangements, in which serum concentrations of free T4 (FT4) or T3 (FT3) are outside of their reference ranges.

The heart is a prominent TH target organ. Thus, thyroid dysfunction can lead to altered TH action in the heart, emerging in e.g. changed expression of myocardial TH target genes being relevant for cardiac function (Dillmann 1990). Hypothyroidism in mice decreases cardiac expression of alpha-myosin heavy chain *Myh6* (Rakov et al. 2016), which has a function in cardiac muscle contraction (Kim et al. 1999). Sarcoplasmic reticulum calcium ATPase 2a (SERCA2a/*Atp2a2*) which has a key role in regulating cardiac calcium dependent contraction and relaxation is decreased in hypothyroid rat hearts. Consistently, these rats demonstrated decreased left ventricular peak systolic pressure and a slowed relaxation (Vetter et al. 2011). Hyperpolarization activated cyclic nucleotide gated potassium channel 4 (*Hcn4*), being one of the two pacemaker channels in the myocardium (Ludwig et al. 1999), was also decreased in hypothyroid mice (Rakov et al. 2016). In line with this, noninvasive electrocardiography measurements in hypothyroid mice showed a decrease in *in vivo* heart rate (Rakov et al. 2016; Minerath et al. 2019) and also a reduction in cardiac ejection fraction (Minerath et al. 2019).

In contrast, T3 is known to positively regulate the expression of *Hcn2* (Engels et al. 2016), *Myh6* and *Atp2a2* (Rohrer and Dillmann 1988; Klein and Danzi 2007; Dillmann 2010), which is in line with an enhancement in cardiac contractility under hyperthyroidism (Trivieri et al. 2006; Carr and Kranias 2002; Seara et al. 2018). On the other hand, T3 negatively regulates *Myh7* and *Atp2a2* antagonist phospholamban (*Pln)* (Klein and Danzi 2007; Carr and Kranias 2002; Engels et al. 2016). Positive effects of THs on cardiac growth (Ojamaa 2010) or cardiac hypertrophy (Barreto-Chaves et al. 2020; Seara et al. 2018), as well as angiogenesis (Chen et al. 2012) have also been described on an experimental level. Patients may experience hyperthyroidism in form of palpitations or tachycardia (De Leo, Lee, and Braverman 2016; Dillmann 2010) and bradycardia can also manifest as a symptom in patients with severe hypothyroidism (Chaker et al. 2017). Furthermore, hyperthyroid patients show an increased risk of all-cause mortality with heart failure as a main cause (Selmer et al. 2014). Atrial fibrillation and subsequent development of congestive heart failure are also attributed to hyperthyroidism (Siu et al. 2007; Baumgartner et al. 2017). Strikingly, old hyperthyroid patients show less pronounced clinical symptoms compared to younger patients, however they are more likely to develop cardiovascular complications (De Leo, Lee, and Braverman 2016). Thus, the risk for atrial fibrillation in old hyperthyroid patients is three times higher than in

younger people (Sawin et al. 1994). All in all, thyroid dysfunction can lead to altered TH action in the heart, ultimately affecting cardiac contractile function, heart rate and growth. These changes can adversely affect heart health.

The cardiovascular system

Heart function

The central function of the heart is to sustain the circulation for oxygen supply in the entire body. Functionally, the heart consists of two separate pump systems with ventricle and atrium on each side (right and left heart) (Fig. 4) (Zimmerman 1966; Shoja et al. 2008; Mori et al. 2019). Electric signals from the sinoatrial node (SA node) initiate and coordinate heart contractions (Christoffels et al. 2010). Pumping of the heart is based on the rhythmic sequence of contraction (systole) and relaxation (diastole) (Ferrer and Harvey 1964). Physiologically the cardiac output is adjusted based on the tissue`s needs. In order to meet higher oxygen demands, the stroke volume can be increased by elevating myocardial preload, a phenomenon called *Frank-Starling-mechanism* (Frank 1895; Patterson and Starling 1914; Patterson, Piper, and Starling 1914). This results in an increased contractility, which is also observed under administration of noradrenaline (Furnival, Linden, and Snow 1971). Overall the pumping capacity of the heart is enormous and amounts to approximately 8000 L per day (Tzahor and Poss 2017). Diseases of heart and vessels have been the main cause of death in all industrialized countries since the beginning of the 20th century. One of the most common cardiovascular diseases is myocardial infarction (MI) (Stanley, Recchia, and Lopaschuk 2005).

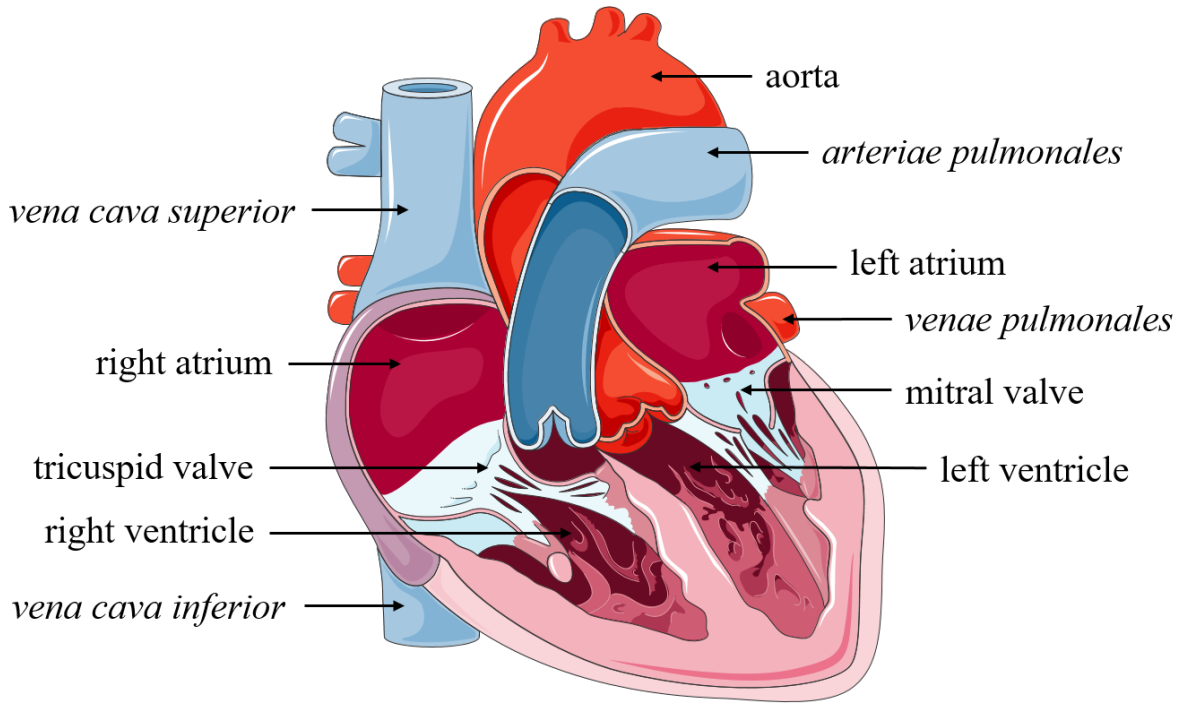

Figure 4: An anatomical model of the human heart. The heart is separated into the right and left heart, with atrium and ventricle on each side (right atrium, right ventricle, left atrium, left ventricle). The tricuspid valve divides the right atrium from the right ventricle, while the mitral valve separates left atrium from left ventricle. Venous blood from the *vena cava superior* and *inferior* flows through the right atrium and right ventricle through the *arteriae pulmonales* into pulmonary circulation where oxygen enrichment occurs. Oxygenated blood is transported through *venae pulmonales* into left atrium and left ventricle. During heart contraction the oxygenated blood is pumped through the aorta in the body circulation.

Myocardial infarction

The heart itself is supplied with oxygen and nutrients by two main coronary arteries (right and left coronary artery) (Fig. 5). The right and left coronary arteries are located epicardially and from there, many supply vessels extend into the endocardium. Thus, the myocardium is extremely well capillarized (Mercadante and Raja 2019; Mori et al. 2019). Pathologically, MI is defined as myocardial cell death due to prolonged ischaemia in the vessels that are responsible for heart nutrient supply (Thygesen et al. 2018). Myocardial ischaemia can be caused by atherosclerosis and thrombosis leading to plaque rupture or erosion (Fig. 5) or by an imbalance of oxygen supply and demand, e.g. due to severe hypertension. Mitochondrial abnormalities start after 10 min of coronary occlusion (Virmani, Forman, and Kolodgie 1990). Ten to 15 min after onset of ischaemia, declined cellular glycogen levels, relaxed myofibrils and sarcolemmal disruption are noted (Jennings and Ganote 1974). As an indicator of myocardial injury and infarction cardiac troponin is the most sensitive and pivotal biomarker, since it is exclusively expressed in the heart (Thygesen et al. 2010). Blood cardiac

troponin levels above the 99th percentile upper reference limit as well as rising concentrations, caused by myocardial ischaemia, define acute myocardial infarction (AMI) (Thygesen et al. 2018). The extent of myocyte death, progressing from subendocardium to subepicardium, can be influenced by collateral flow, myocardial oxygen consumption or preconditioning of the heart, e.g. by brief cycles of occlusion/reperfusion (Reimer, Jennings, and Tatum 1983). Ultimately, timely restoration of blood flow to the ischaemic myocardium (reperfusion) is the prime goal of MI treatment to reduce myocardial injury (Ibanez et al. 2015). While *post mortem* identification of necrosis can take hours in humans (Ooi, Isotalo, and Veinot 2000), myocardial cell death due to apoptosis can be analysed within 10 min of induced ischaemia in the experimental setting (Reimer, Jennings, and Tatum 1983).

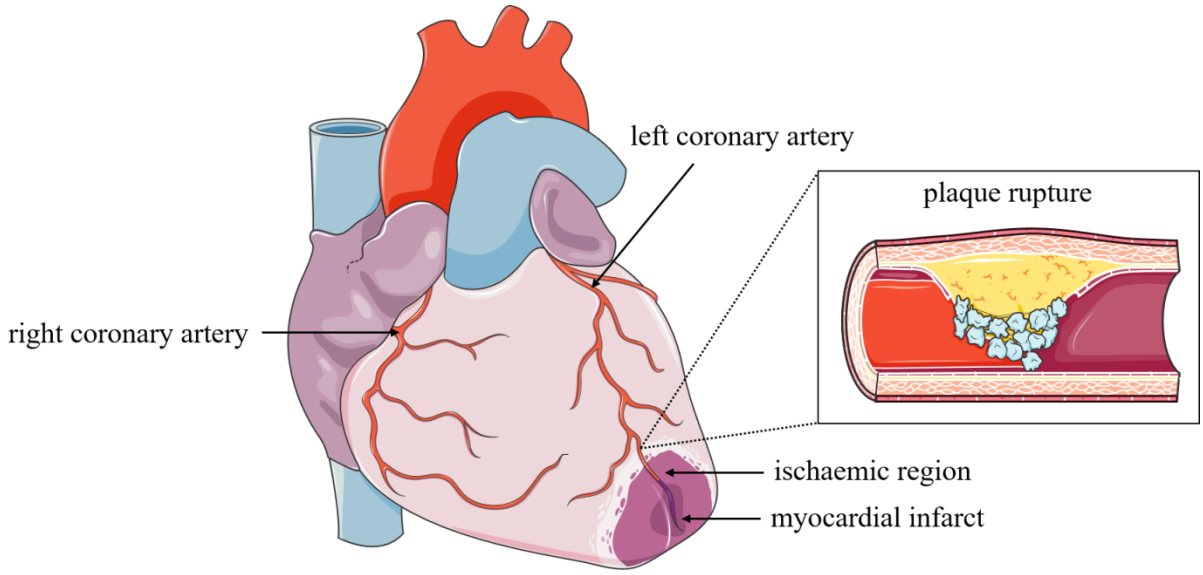

Figure 5: Myocardial infarction. The heart itself is supplied with oxygen and nutrients via the coronary arteries (right and left coronary artery). Myocardial ischaemia (ischaemic region or *area at risk*) caused by e.g. plaque rupture in the left coronary artery can result in myocardial infarction.

Myocardial energy metabolism during ischaemia/reperfusion

Under physiological and thus aerobic conditions, the heart has sufficient oxygen to burn its main substrates: Free fatty acids (50-60%), glucose (20-30%) and lactate (20%) (Stanley, Recchia, and Lopaschuk 2005). Oxidative phosphorylation in mitochondria provides enough ATP for muscle contraction. However, under pathophysiological conditions, e.g. during ischaemia, anaerobic conditions predominate and tissue hypoxia is the consequence. Oxidative energy generation is stopped and there is a change to anaerobic glycolysis. However, ATP requirement is greater than ATP synthesis generated by anaerobic glycolysis. Consequently, ATP content further decreases resulting in acidification due to

lactate dehydrogenase enzyme forming lactate and hydrogen atoms (Das et al. 1987). Decrease of pH also inhibits activity of the key enzyme of glycolysis (phosphofructokinase) leading to a further decrease in ATP content (Das et al. 1987). Additionally, ATP deficiency stops sodium/potassium exchange (Pierce and Czubryt 1995). Consequently sodium/calcium exchange is activated, leading to an intracellular calcium excess (Jacobshagen and Maier 2013; Tani and Neely 1989). However, these harmful processes that are calcium-dependent are counteracted by the overacidification during ischaemia. In reperfusion, oxygen supply is restored, neutralizing the acidification (Kalogeris et al. 2012). The intracellular calcium excess now leads to activation of the contractile apparatus SERCA2a (Akaike et al. 2017), causing hypercontracture that can result in mechanical damage (reperfusion damage) (Nishida et al. 1993). Furthermore, since calcium storage capacity of SERCA is limited, calcium accumulation might also affect mitochondria and may lead to mitochondrial swelling and mitochondrial permeability transition pore (mPTP) opening (Halestrap and Richardson 2015). Recently, the role of oxidants and the formation of reactive oxygen species (ROS) in the post-ischaemic heart has gained increasing attention (Zweier and Talukder 2006; Zweier, Flaherty, and Weisfeldt 1987; Marczin et al. 2003). Production of nitric oxide (NO) and NO levels might also be excessive and harmful under ischaemic and post-ischaemic conditions, which can result from NOS dependent NO formation or from NOS independent nitrite reductions (Totzeck, Hendgen-Cotta, and Rassaf 2017; Schulz, Kelm, and Heusch 2004). How to limit IR injury (cardioprotection) is still a hot topic in cardiovascular research, and, the underlying mechanisms of cardioprotection are still not fully understood. However, this is pivotal to develop novel therapy options.

Research on ischaemia/reperfusion injury

In 1895, Oskar Langendorff developed the Langendorff perfused heart model, in which an excised heart is cannulated on the aorta in order to enable retrograde perfusion via coronary artery (Langendorff 1895). A Krebs-Henseleit-buffer (KHB) is used for perfusion, mimicking the nutrient supply in the blood (Botker et al. 2018). This procedure allows a global analysis of heart function independent from the rest of the body as well as the nervous system, while still preserving the advantage of studying cardiac cells in their native myocardial structure and environment (Botker et al. 2018). While the Langendorff model is relatively reproducible and has the capacity for high throughput, tissue edema is often a consequence of protein-free KHB perfusion with glucose as sole substrate (Lindsey et al. 2018). Nevertheless, Langendorff preparation has been fundamental to understand physiology of the heart such as its contractile function, coronary flow (CF) regulation and cardiac metabolism (Bell, Mocanu,

and Yellon 2011) and is still in use for examining the pathophysiology in IR injury (Bell, Mocanu, and Yellon 2011; Botker et al. 2018). Mouse, rat, guinea pig, rabbit, dog, primate and even human hearts have already been analysed (Botker et al. 2018; Kadipasaoglu et al. 1993), however, in most studies isolated rodent hearts are used. Usually, rat hearts are preferred, as they have the advantage of being larger and therefore easier to cannulate compared to mouse hearts (Botker et al. 2018). Mouse hearts have the main advantage of allowing the use of genetically modified strains (Botker et al. 2018) which are more widely available than rat models. However, it should be noted that infarct size should always be the end parameter for assessing the IR injury, especially in preclinical studies on cardioprotection (Lindsey et al. 2018), since recovery of left ventricular function also depends on reversible injury (stunning) and the function of remote myocardium (Kloner and Jennings 2001; Gelpi et al. 2002).

Impact of thyroid hormones on ischaemia/reperfusion injury

Although it is known that thyroid dysfunction affects transcriptional homeostasis and functional parameters of the heart, such as contractility or cardiac output (Dillmann 1990), the outcome after MI with thyroid dysfunction has not yet been sufficiently investigated in randomized and controlled trials (Jabbar et al. 2017) (Fig. 6). At the experimental level, several groups investigated the outcome of myocardial IR injury in hypo- or hyperthyroid rodents, mainly in rats, and focused on the evaluation of post-ischaemic left ventricular function, however with inconsistent findings. On the one hand, a cardioprotective phenotype under hyperthyroidism with an improved recovery of left ventricular developed pressure (LVDP) (Pantos et al. 2002; Pantos et al. 2006) and decreased myocardial infarct size after IR has been described (Kumar, Taliyan, and Sharma 2012). On the other hand, a decreased functional recovery of hyperthyroid isolated rat hearts has been reported (Venditti, Agnisola, and Di Meo 2002). For hypothyroidism, there has also been evidence for a cardioprotective phenotype after IR injury, since functional tolerance to IR was improved in isolated rat hearts (Pantos, Malliopoulou, Mourouzis, et al. 2003; Mourouzis et al. 2009). Recently, hypothyroidism has also been shown to reduce infarct size in rats (Seara et al. 2018). Taken together, experimental studies show inconsistent effects of chronic thyroid dysfunction on myocardial IR injury (Fig. 6). In addition, published experimental studies on IR injury under thyroid dysfunction have so far not addressed an ageing society, with the elderly being even more vulnerable to cardiovascular diseases than younger people (Fig. 6).

Furthermore, it is unknown how TR signalling affects myocardial IR injury. A possible involvement of TRα has been suggested, since the *in vivo* inhibition of TRα after MI impaired

post-ischaemic performance in mice (Mourouzis et al. 2013). Nonetheless it remains unclear, which type of TRα signalling (canonical or noncanonical) is involved (Fig. 6).

An influence of THs in the acute setting has also been suggested. In experimental AMI a low TH tissue state, induced by upregulation of cardiac DIO3 resulting in reduced T3 availability in the myocardium, has been observed (Simonides et al. 2008). Contrary to the concept that this is an adaptive, energy-preserving effect (Galli, Pingitore, and Iervasi 2010), some clinical and experimental data suggest that TH intervention in AMI could in fact improve outcome (Gerdes and Iervasi 2010; Jabbar et al. 2017; Pingitore et al. 2019). Thus, a number of studies, mostly in rats, suggested that T3 administration not only reduces hypoxia/reoxygenation injury in cardiomyocytes (Nicolini et al. 2016; Zeng et al. 2019; Forini et al. 2018; Sabatino et al. 2016; da Silva et al. 2018; Bi et al. 2019) but also improves post-ischaemic functional recovery in isolated rat hearts (Pantos et al. 2007; Pantos et al. 2009; Bi et al. 2019). However, the precise role of acute TH intervention on myocardial infarct size still requires clarification, particularly with regard to the relevant target cells and causally involved molecular pathways (Fig. 6) (Jabbar et al. 2017; Cappola et al. 2019).

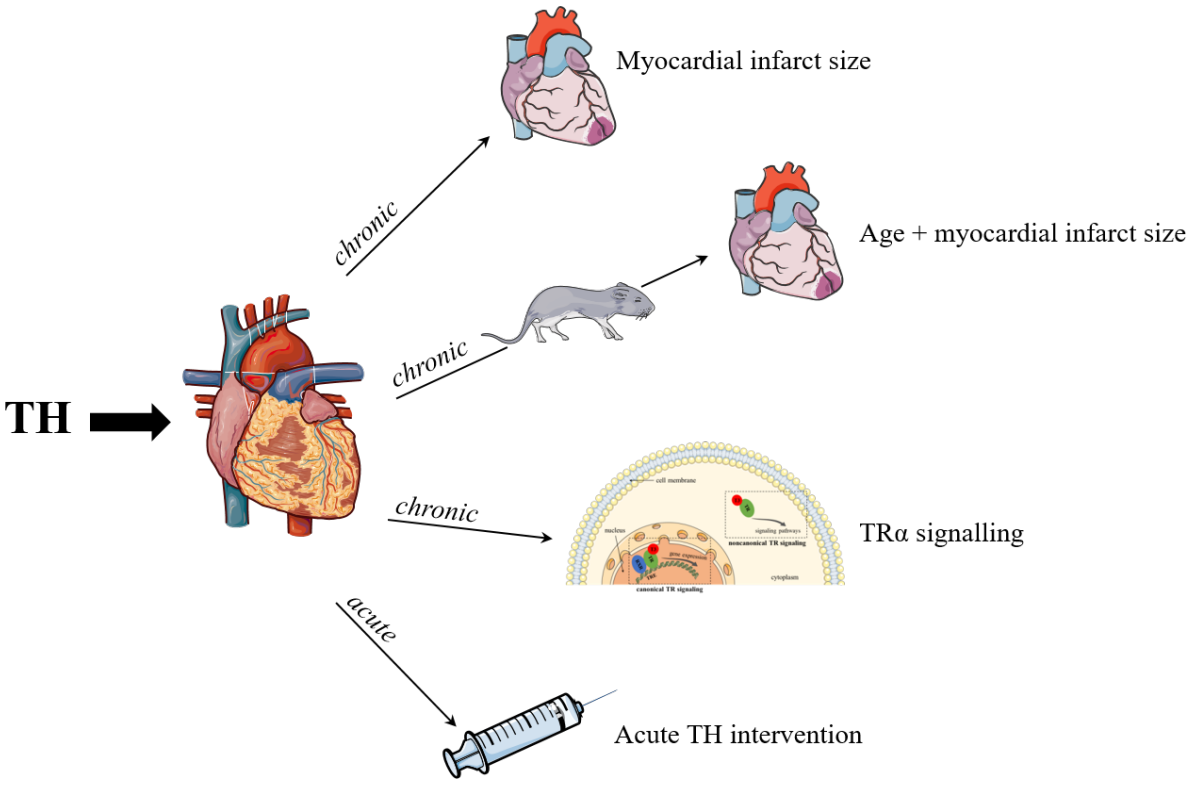

Figure 6: Possible influence of TH on myocardial ischaemia/reperfusion (IR) injury in the chronic and acute setting. Chronic TH action may affect myocardial infarct size after IR injury. This influence can also change with age. Canonical or noncanonical TRα signalling, as well as acute TH intervention may also play a role in the outcome after IR injury.

Aims of the study

1) Clinical data suggest that thyroid dysfunction affects cardiac homeostasis *per se* and can therefore also have a negative impact on cardiovascular risk factors. However, the outcome after MI with thyroid dysfunction has not yet been investigated in great detail. Experimental data indicate inconsistent findings on the outcome of hypo- and hyperthyroidism in IR injury. Thus, the first goal of this thesis was to investigate the role of thyroid dysfunction on myocardial infarct size.

2) Secondly, experimental studies on IR injury under thyroid dysfunction have not addressed an ageing society, with older people being even more vulnerable to cardiovascular diseases than younger people. Therefore, the second goal was to investigate IR injury outcome in the aged organism.

3) The mechanism of chronic TH action, including the role of TRα signalling during IR, remains elusive. Thus, a third purpose of this thesis was to clarify the role of canonical or noncanonical TRα signalling on myocardial infarct size.

4) According to clinical trials, TH treatment after MI may improve the outcome, however on the experimental level it remains unknown, whether T3 can acutely modify infarct size. The fourth goal of this thesis was to verify the acute effect of T3 administration on myocardial infarct size and which mode of TH signalling is implicated.

Material & Methods

Chemicals

Table 1: List of chemicals and reagents.

Chemicals and Reagents	Manufacturer/Supplier
(6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH_4)	Fisher Scientific, New Hampshire, USA
2-Mercapto-ethanol	Sigma-Aldrich, St. Louis, USA
2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline (c-PTIO)	Enzo Life Science, Antwerpen, Belgium
3,5,3'-triiodothyronine	Sigma-Aldrich, St. Louis, USA
3,5,3',5'-tetraiodothyronine	Sigma-Aldrich, St. Louis, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, USA
Calciumchlorid ($CaCl_2$)	AppliChem GmbH, Darmstadt, Germany
D(+)-glucose-monohydrate ($C_6H_{12}O_2$)	Merck, Darmstadt, Germany
Disodiumhydrogensulfate (Na_2HPO_4)	Sigma-Aldrich, St. Louis, USA
ECL Western Blotting Substrate	Pierce Biotechnology, Waltham, MA, USA
Ethylendiamintetraacetate (EDTA)	Sigma-Aldrich, St. Louis, USA
Ethanol (technical grade)	Pharmacy, UK Essen, Essen Germany
Fat free milk (blotting grade blocker)	BioRad, Munich, Germany
Heparin-Natrium-2500	Ratiopharm, Ulm, Germany
Isopropanol (2-propanol)	Sigma-Aldrich, St. Louis, USA
Magnesiumsulphate-heptahydrate ($MgSO_4$ x 7 (H_2O))	Merck, Darmstadt, Germany
Methanol	Sigma-Aldrich, St. Louis, USA
Methimazole (MMI)	Sigma-Aldrich, St. Louis, USA
Mucasol alkaline quick cleaner	Merz, Frankfurt, Germany
N-Acetyl-L-Cysteine (NAC)	Sigma-Aldrich, St. Louis, USA
Ponceau S solution	SERVA, Heidelberg, Germany
Potassiumchloride (KCl)	Sigma-Aldrich, St. Louis, USA
Potassiumhydrogenphosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Proteinase K	Qiagen, Hilden, Germany
Saccharine	Sigma-Aldrich, St. Louis, USA
Saline, sterile	Braun Melsungen AG, Melsungen, Germany

Sekusept™ active	Ecolab, Monheim am Rhein, Germany
Sodium Chloride (NaCl)	AppliChem GmbH, Darmstadt, Germany
Sodium Chloride 0.9% 1000 ml	Braun, Melsungen, Germany
Sodium dodecyl sulfate (SDS)	SERVA, Heidelberg, Germany
Sodium hydroxide (NaOH)	Sigma-Aldrich, St. Louis, USA
Sodium perchlorate (ClO_4^-)	Sigma-Aldrich, St. Louis, USA
Sodium pyruvate ($C_3H_3NaO_3$)	Sigma-Aldrich, St. Louis, USA
Suprarenin ampoules 1 mg/ml (epinephrine)	Sanofi, Paris, France
Triphenyltetrazoliumchloride (TTC)	Sigma-Aldrich, St. Louis, USA
Tris-Base (Tris)	Sigma-Aldrich, St. Louis, USA
Tween 20	Bio-Rad, Hercules, CA, USA

Technical devices

Table 2: List of technical devices.

Device	**Manufacturer/Supplier**
Aortic cannula for mouse hearts IH-SR	Hugo Sachs, March Hugstetten, Germany
Cassettes for pump tubing ISMCIS3510A	VWR International, Darmstadt, Germany
ChemCam/LabImage1D software	INTAS, Göttingen, Germany
Circulator Lauda Ecco	Omnilab, Bremen, Germany
Curved tweezers	Fine Science Tools, Heidelberg, Germany
Fine tweezers	Dumont, Switzerland
Flowmeter T402-PB	AD Instruments, Spechbach, Germany
Flowprobe HSE for TS420 + adapter	Hugo Sachs, March Hugstetten, Germany
Gel and chamber blotting system	Bio-Rad, Hercules, CA, USA
Heating block MHR 13	HLC, Pforzheim, Germany
Interface for data recording and software LabChart Pro PL3156/P	AD Instruments, Spechbach, Germany
ImageJ 1.48v	National Institutes of Health, Bethesda, Maryland
Light Cycler® 480 II	Roche, Berlin, Germany
Magnetic stirrer IKA RH digital	IKA, Staufen, Germany
Micro spring scissors	Aesculab AG, Tuttlingen, Germany
Micro tweezers	Aesculab AG, Tuttlingen, Germany

NanoDrop 2000	Thermo Fischer Scientific Inc. Waltham, USA
pH-electrode Blue Line 15	Schott Instruments, Mainz, Germany
Pressure transducer DPT-6000	CODAN, Forstinning, Germany
Quad-bridge Amplifier FE224	AD Instruments, Spechbach, Germany
Roller pump Ismatec	VWR International, Darmstadt, Germany
Stereomicroscope LS 6000IC	Beckman Coulter, Krefeld, Germany
Thermometer P77-L	ATP, Ettenheim, Germany
Temperature probes	ATP, Ettenheim, Germany
Ultrapure water system Milli-Q Advantage A 10	Milipore, Schwalbach, Germany
Ultra-Turrax	Janke & Kunke IKA, Staufen, Germany
Ultrasonic Flow Probe TS410	Transonic Systems Inc., Ithaca, New York, USA
Vacuum pump	Oehmen, Essen, Germany
Versadoc 4000MP	Bio-Rad, Munich, Germany
Vortex 4 Basic	IKA, Staufen, Germany
Water bath 1008	GFL Gesellschaft für Labortechnik, Bergwedel, Germany

Consumables

Table 3: List of consumables.

Consumable	**Manufacturer/Supplier**
0.45 µm Filter Type HWAP	Merck, Darmstadt, Deutschland
1.5 ml reaction tubes	Biozym Biotech Trading, Wien, Austria
2 ml reaction tubes	Biozym Biotech Trading, Wien, Austria
15 ml reaction tubes	Greiner Vacuette, Essen, Germany
1 ml Syringe	Terumo Europe N.V., Leuven, Belgien
5 ml Syringe	Terumo Europe N.V., Leuven, Belgien
10 ml Syringe	Terumo Europe N.V., Leuven, Belgien
20 ml Syringe	Terumo Europe N.V., Leuven, Belgien
Control diet TD.95007 (with added potassium iodide (0.0012 g/kg): TD.97350)	Harlan Laboratories, USA

Filter paper for Western Blot	Schleicher & Schuell, Dassel, Germany
Low iodine diet TD.95007	Harlan Laboratories, USA
Microvette	Sarstedt, Nümbrecht, Germany
Pipette tips 100-1000 µl	Eppendorf, Hamburg, Germany
Pipette tips 2-200 µl	Sarstedt, Nümbrecht, Germany
Pipette tips 0.1-10 µl	Sarstedt, Nümbrecht, Germany
Polyvinylidene fluoride membranes	BioRad, Munich, Germany
SDS-polyacrylamide gels	BioRad, Munich, Germany
Tubing LMT55 3 Stopp	Tygon, Charny, France
Tubing Silicon Masterflex	Cole Parmer, Illinois, USA
Tubing Tygon ST	Tygon, Charny, France
Compresses sterile, 10 cm x 10 cm	Fuhrmann, München, Germany

Antibodies

Table 4: List of primary and secondary antibodies.

Antibody	Dilution	Catalog#	Supplier
Anti-AKT(Ser)$_{473}$	1:150	9271	Cell Signalling
Anti-ERK(Thr/Tyr)$_{202/204}$	1:500	9101	Cell Signalling
Anti-eNOS(Ser)$_{1177}$	1:250	81510	Santa Cruz
Anti-eNOS(Thr)$_{495}$	1:750	9574	Cell Signalling
Anti-STAT3(Tyr)$_{705}$	1:500	9138	Cell Signalling
Anti-p38 MAPK(Thr/Tyr)$_{180/182}$	1:200	9211	Cell Signalling
Anti-total-AKT	1:200	9272	Cell Signalling
Anti-total-eNOS	1:500	610296	BD Biosciences
Anti-total-ERK	1:200	9102	Cell Signalling
Anti-total-STAT3	1:500	9139	Cell Signalling
Anti-total-p38	1:500	9212	Cell Signalling
Anti-mouse IgG (HRP linked)	1:5000	7076	Cell Signalling
Anti-rabbit IgG (HRP linked)	1:5000	7074	Cell Signalling

Kits

Table 5: List of kits.

Kit	Catalog#	Manufacturer/Supplier
Free T3 ELISA	EIA-2385	DRG Diagnostics GmbH, Marburg, Germany
Free T4 ELISA	EIA-2386	DRG Diagnostics GmbH, Marburg, Germany
Pierce™ BCA Protein Assay Kit	23225	Thermo Fischer Scientific Inc. Waltham, USA
QIAshredder	79654	Qiagen, Hilden, Germany
RNeasy Mini Kit	74106	Qiagen, Hilden, Germany
Superscript™ III Reverse Transcriptase	18080-051	Thermo Fischer Scientific Inc. Waltham, USA
Total T4 ELISA	EIA-1781	DRG Diagnostics GmbH, Marburg, Germany

Buffer

Table 6: Modified Krebs-Henseleit-buffer for isolated mouse hearts.

Chemicals	mmol/L
$CaCl_2$	1.6
$C_3H_3NaO_3$	2
$C_6H_{12}O_2$	5.55
EDTA	0.07
KCl	4.7
KH_2PO_4	1.18
$MgSO_4$ x 7 H_2O	1.64
NaCl	118
$NaHCO_3$	24.88
± T3	50-200 µg/L
± BH_4	10 mg/L
± c-PTIO	0.1 mmol/L
± NAC	60 µmol/L

Before use, the modified Krebs-Henseleit-buffer (KHB) was oxygenated with 95% O_2 and 5% CO_2 and then equilibrated to 37° C and a pH of 7.4. 1.6 mmol/L $CaCl_2$ was added after 30 min of oxygenation. T3 and/or tetrahydrobiopterin (BH_4) were added following the addition of $CaCl_2$. BH_4 was always given from the start of the experiment to enable rescue of eNOS coupling. KHB for isolated mouse hearts was filtered through a 0.45 µm membrane filter. Inhibitor experiments were also established in presence of NO scavenger carboxyphenyl-tetramethylimidazoline (c-PTIO) and ROS scavenger N-acetyl-L-cysteine (NAC), however these have not yet been experimentally pursued.

Study approval

All animal experiments were in accordance with the German regulations for Laboratory Animal Science (GVSOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocols for animal studies were approved by the *Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen* (LANUV-NRW), Germany (AZ: 84-02.2014.A092, 84-02-2017.A157 and 84-02.04.2016.A261).

Animals

Male wildtype C57BL/6JRj mice (Janvier Labs, France) aged 3 months (young) and 20 months (old) were used for experiments on thyroid dysfunction in isolated mouse hearts.

For experiments on the acute effect of T3 in isolated hearts male wildtype C57BL/6JRj mice (Janvier Labs, France) aged 3-6 months were used.

Generation of TRαGS knockin mice

Male $TR\alpha^{GS}$ mice were generated as previously described (Hones et al. 2017). Briefly, GS point mutations were introduced into the Thra (NM_178060.3) gene locus of C57BL/6J mice. This was done by using custom made zinc finger nucleases (ZFNs) and a donor plasmid, which contains the required sequence (Carbery et al. 2010). To change the amino acid sequence in the P-box from glutamic acid and glycine (EG) to glycine and serine (GS) a specified donor vector ($pTR\alpha^{GS}$-donor) was used. Consequently, the codons 71 (GAG) and 72 (GGC) of exon 3 were mutated to GGG and AGC. Next, oocytes of C57BL/6JCrl mice were injected with microinjection solution, containing the ZFN mRNA and the $pTR\alpha^{GS}$-donor. Transplantation of manipulated oocytes was done into oviducts of 0.5 days p.c. pseudopregnant mice. Ultimately, PCR and sequencing were used for offspring screening (Hones et al. 2017).

TRα knockout mice

Male TRα knockout ($TR\alpha^0$) mice were received from the European Mouse Mutant Archive and all mouse strains were backcrossed on C57BL/6JCrl for at least five generations. Homozygous mice and wildtype littermates were used for experiments.

Animal housing

Animals were housed in temperature- (23 ± 1° C) and light-controlled (inverse 12:12 hour light-dark cycle) conditions. Food and water were provided *ad libitum*.

Induction of thyroid dysfunction in mice

For experiments on thyroid dysfunction in 3 and 20 months old mice, chronic hyperthyroidism was induced by adding 1 µg/ml T4 to the drinking water (T4 was dissolved in 40 mM NaOH and 0.1% bovine serum albumin (BSA)) (Engels et al. 2016). For induction of chronic hypothyroidism, animals were fed a low-iodine diet and received drinking water supplemented with 0.02% methimazole, 0.5% perchlorate and 0.3% saccharine as sweetener (LoI/MMI/ClO4-) (Rakov et al. 2016). Control and hyperthyroid animals were fed a control diet (low iodine diet with added potassium iodide). The treatment period was 3 weeks.

Chronic hyperthyroidism in $TR\alpha^0$ mice was induced by adding 400 ng/ml T3 to the drinking water (T3 was dissolved in 40 mM NaOH and 0.1% BSA) (Hones et al. 2017). The treatment period was 3 weeks.

Isolated mouse hearts

After cervical dislocation, 200 IU of heparin were injected intraperitoneally to prevent coagulation, and hearts were rapidly excised through bilateral thoracotomy. Within two minutes hearts were cannulated under a stereomicroscope through the aorta in ice-cold 0.9% NaCl. Hearts were perfused in Langendorff mode (Fig. 7) (Botker et al. 2018; Langendorff 1895) with modified KHB (Table 6). The perfusion pressure was continuously monitored above the aortic cannula using a transducer and was held constant at 80 mmHg. The perfusate temperature was held constant by a heat exchanger located next to the aortic cannula. CF was measured by an in-line ultrasonic flow probe connected to the aortic cannula. A fluid-filled cling film balloon was inserted through the mitral valve into the left ventricular cavity and connected to a pressure transducer to allow continuous monitoring of left ventricular pressure (LVP). The left ventricular end-diastolic pressure was set at 5 to 15 mmHg. After cannulation and instrumentation, isolated hearts were subjected to a stabilization phase (baseline) of in total 30 minutes. Spontaneous heart rate was determined

within the first ten minutes of baseline, as hearts usually stabilized after this time. Hearts were then paced to 500 beats/min by right atrial electrical stimulation for another 20 minutes. CF, end-diastolic and peak LVP were continuously recorded. LVDP was calculated as the difference between maximal and minimal LVP (Hildebrandt et al. 2016). Preparations of untreated wildtype mouse hearts (controls) with a CF below 1 ml/min and a LVDP below 50 mmHg were excluded (Hildebrandt et al. 2016), as it is assumed that the supply of a mouse heart is not guaranteed under these conditions. For example, this can happen if the knot is set incorrectly and the supplying coronary arteries are pinched off. Preparations with a CF above 5 ml/min, a LVDP above 120 mmHg, or hearts with an arrhythmia lasting longer than 3 min were also excluded (Bell, Mocanu, and Yellon 2011). In these cases the aorta could be injured during the preparation, which might result in a leak and thus in a false positive high CF. LVDP and CF were calculated as mean values during the last minute of the baseline (30th min), at the beginning of ischaemia (5th min), at the end of ischaemia (25th min), and at 10, 20, 30, 40, 50 and 60 min of reperfusion. The temperature of the humidified organ chamber was continuously kept between 37.0 and 37.5° C to avoid hypothermia during ischaemia (Lindsey et al. 2018).

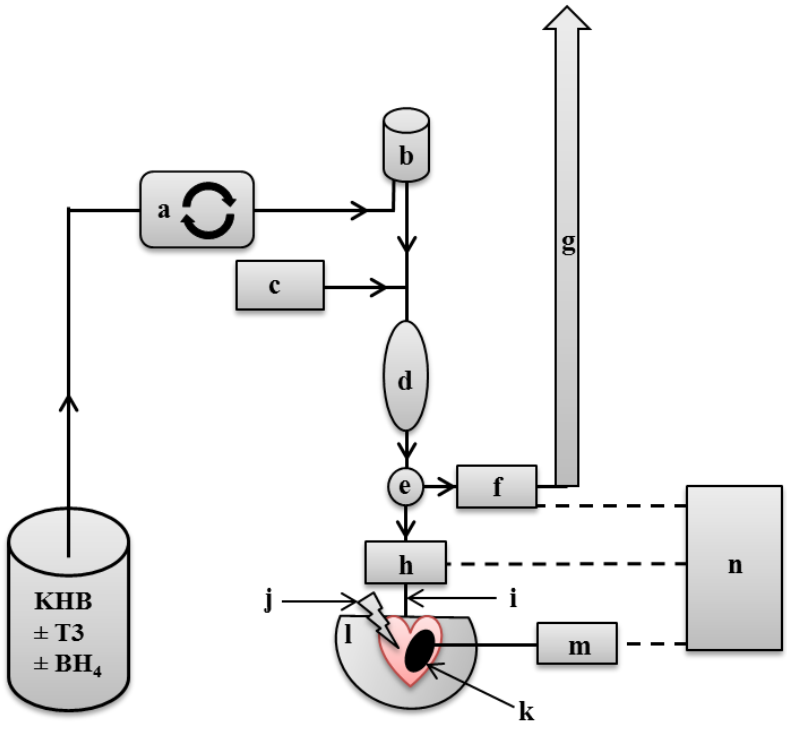

Figure 7: Schematic illustration of a Langendorff machine. **a** roller pump; **b** bubble trap; **c** ± medication, e.g. epinephrine via syringe pump; **d** double-walled and heated glas spiral, **e** three-way tap; **f** recording of aortic pressure (AP); **g** pipe for perfusion pressure; **h** recording of coronary flow (CF); **i** aortic cannula; **j** electrode for atrial electrical stimulation; **k** left ventricular balloon; **l** double-walled and heated organ chamber; **m** recording of left ventricular pressure (LVP); **n** computer; **KHB** indicates Krebs-Henseleit-buffer; **T3**: triiodothyronine; **BH_4**: tetrahydrobiopterin. AP, CF and LVP are recorded on software in the computer for continuous monitoring.

Protocols

After a stabilization period of 30 min hearts were subjected to 30 min global no-flow ischaemia by closing the three-way tap in heart direction and thus stopping heart perfusion for this time. This was followed by 120 min reperfusion (Fig. 8 and Fig. 9) (Lindsey et al. 2018). Time controls (TCs) were perfused for a duration period equal to the experimental protocol, i.e. 180 min but without ischaemia (Fig. 8 and Fig. 9).

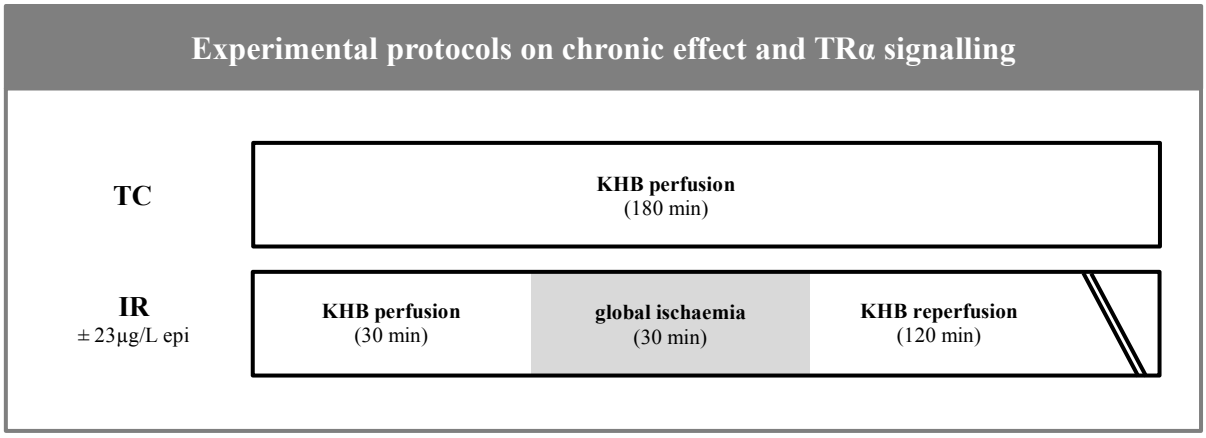

Figure 8: Experimental protocols for isolated pressure constant perfused mouse hearts with thyroid dysfunction and for transgenic mice. TC: time control; **IR**: global ischaemia/reperfusion; **epi**: epinephrine and **KHB**: Krebs-Henseleit-buffer.

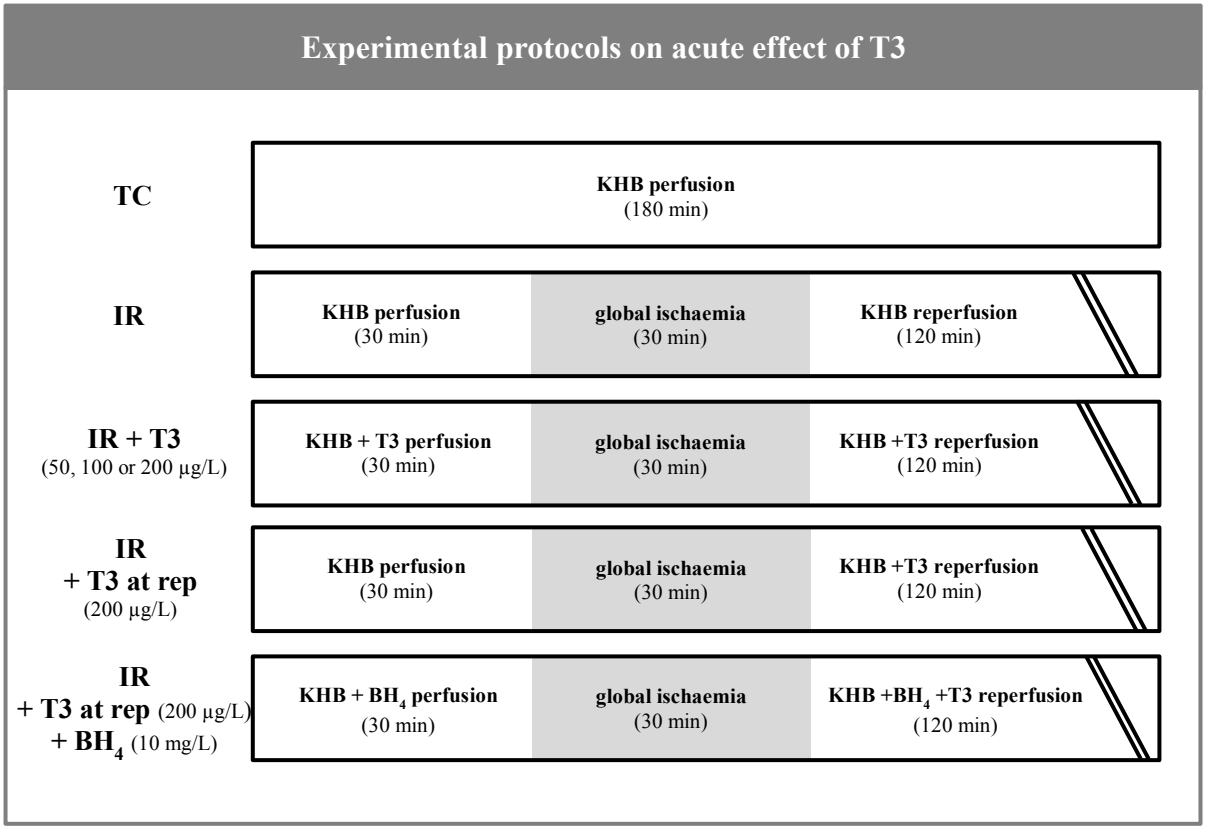

Figure 9: Experimental protocols for isolated pressure constant perfused mouse hearts with acute T3 delivery. TC: time control; **IR**: global ischaemia/reperfusion; **epi:** epinephrine; **T3**: triiodothyronine; **BH_4**: tetrahydrobiopterin; **KHB**: Krebs-Henseleit-buffer and **rep**: reperfusion.

Infarct staining

After 120 min reperfusion which is a sufficient duration for washing out reductive equivalents (Lindsey et al. 2018), hearts were frozen at -20° C overnight and cut into 4-6 transverse 1 mm thick slices. The slices were immersed in 2,3,5-triphenyltetrazolium chloride (TTC) solution (1% (w/V)) dissolved in phosphate buffer, consisting of 77.4% (V/V) 0.1 mol/L Na_2HPO_4 and 22.6% (V/V) 0.1 mol/L NaH_2PO_4, and incubated in a water bath at 37° C for 5 min. TTC dyes viable myocardium red because of a formazan reaction with NADH and NADPH that are washed out from irreversibly injured myocardium (Fishbein et al. 1981). After photographing the slices, they were quickly frozen in liquid nitrogen and stored at -80° C for later analysis. Total slice area and areas of viable tissue (red) and necrotic tissue (white) were measured by computerized planimetry. Infarct size was calculated as percent of the sum of left and right ventricles (% of LV+RV or % of ventricular mass).

Blood sample collection

After heart excision, blood samples from the abdominal caval vein were harvested from sacrificed animals in Microvette® tubes for capillary blood collection and stored on ice for at least 30 min to enable coagulation. After centrifugation at 4° C with 13,000 g for 20 min, serum was aliquoted in 500 µl reaction tubes and stored at -80° C.

Determination of thyroid hormone concentrations in serum

Total T4 (TT4), free T4 (FT4) and free T3 (FT3) serum concentrations were measured using commercial ELISA kits according to the manufacturer's instructions (minimum detectable TH concentrations: 0.5 µg/dl for TT4, 0.05 ng/dl for FT4 and 0.05 pg/ml for FT3). Serum samples with known TH concentrations were used as controls (Rakov et al. 2016; Engels et al. 2016).

Analysis of protein expression by immunoblot

Snap-frozen ventricular samples from the middle heart slice were homogenized in 100 mmol/L tris(hydroxymethyl)aminomethane (TRIS) with 2% sodium dodecyl sulfate (SDS; w/v), heated to 70° C for 5 min and centrifuged at 14,000 g for 10 min. The protein lysate containing supernatant was stored at -80° C in aliquots to prevent freeze- and thaw-cycles. Proteins were separated by electrophoresis on precasted SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were stained with Ponceau S as loading/transfer control. After blocking with fat-free milk, membranes were incubated with antibodies directed against the phosphorylated forms of proteinkinase B (AKT_{ser473}),

extracellular-signal regulated kinases ($ERK_{thr202/tyr204}$), signal transducer and activator of transcription 3 ($STAT3_{tyr705}$), p38-mitogen-activated protein kinase ($p38_{thr180/tyr182}$) and endothelial nitric oxide synthase ($eNOS_{ser1177\ and\ thr495}$). After incubation with the respective secondary antibody, immunoreactive signals were detected by chemiluminescence and quantified with ChemCam/LabImage1D software. Membranes were stripped and re-probed for the detection of the respective total form of AKT protein, ERK protein, STAT3 protein, p38 protein and eNOS protein. The immunoreactivity of phosphorylated proteins was normalized to the immunoreactivity of the respective total protein which, in turn, was normalized to Ponceau S staining.

Quantitative real-time PCR

Total RNA from mouse hearts was isolated using RNeasy Kit with some modifications (Hones et al. 2017). Briefly, 30 mg of ventricular tissue was homogenized in 300 µl of RLT-buffer (containing β-mercaptoethanol) using a rotor-stator homogenizer (Ultra-Turrax). Homogenates were loaded on *QIAshredder*™ columns for further cell disruption and were centrifuged for 1 min at 3,000 rpm. Lysates were subsequently digested with 10 µl proteinase K and 590 µl H_2O for 30 min at 37° C. Washing and isolating steps were performed according to manufactures' instructions. Finally, RNA concentration was measured using the nanodrop. Two µg of total RNA was reverse transcribed into cDNA with *SuperScript® III* and random hexamer primers. Using random-hexamer primer generates cDNA from the total RNA pool, including mRNA, miRNA and rRNA. A mix of 2 µg RNA, 2 µl random hexamer primer, 1 µl dNTP was generated, filled up to 10 µl with nuclease-free water and incubated for 5 min at 65° C. Ten µl of the cDNA-synthesis Master Mix, which contains 2 µl of 10x *RT Buffer*, 4 µl of 25 mM MgCl2, 0.1 M DTT, 1 µl of each *RNaseOUT*™ and *SuperScript® III* were added and incubated for 10 min at 25° C followed by 50 min at 50° C. The reaction was stopped by incubation for 5 min at 85° C. This was followed by the addition of 1 µl of RNase H to the 20 µl reaction volume. To remove template RNA, it was incubated for 20 min at 37° C. Quantitative real-time PCR was performed using *Roche SYBR Green I Master Mix* on a light cycler LC480® (Roche, Switzerland). A mix of 2.5 µl of cDNA, 5 µl of *SYBR Green I Master Mix*, 2.4 µl PCR-grade water (Roche) and 0.05 µl of forward and reverse primer were generated in a 96 well plate. The PCR programme started with melting at 95° C for 5 min, followed by 40 cycles at 95° C for 15 sec, 60° C for 10 sec and 72° C for 20 sec, respectively. The primer sequences are provided in Table 7. *Polr2a* (polymerase RNA II) was used as reference gene, analysis and calculation of the fold

change in gene expression were done on Ct-values ≤ 35 using the efficiency corrected method (Pfaffl 2001).

Table 7: List of qRT-PCR primers for gene expression analysis.

Gene	Forward	Reverse	Accession No.
Atp2a2	AAC TAC CTG GAA CAA CCC GC	TCA TGC AGA GGG CTG GTA GA	NM_009722.3
Hcn2	CCA GTC CCT GGA TTC GTC AC	TCA CAA TCT CCT CAC GCA GT	NM_008226.2
Hcn4	CAG CGT CAG AGC GGA TAC TT	CTT CTT GCC TAT GCG GTC CA	NM_001081192.1
Myh6	CAG ACA GAG ATT TCT CCA ACC CA	GCC TCT AGG CGT TCC TTC TC	NM_010856.4
Myh7	CAC GTT TGA GAA TCC AAG GCT C	CTC CTT CTC AGA CTT CCG CA	NM_080728.2
Polr2a	CTT TGA GGA AAC GGT GGA TGT C	TCC CTT CAT CGG GTC ACT CT	NM_009089

Statistical analysis

Investigators analysing infarct size and protein expression were blinded for the protocols. Data are presented as means ± standard deviations. One-way ANOVA (comparison of three or more groups) and unpaired t-test (comparison of two groups) were used to analyse data on infarct size, CF and LVDP at baseline in isolated mouse hearts, Western Blot and gene expression data. Two-way ANOVA for repeated measures was used for data on CF and LVDP in isolated mouse hearts with 120 min reperfusion (time point and treatment). For gene expression data, statistical significance was calculated on log-transformed data (to obtain normal distribution) as recommended by the MIQE guidelines (Bustin et al. 2009). When a significant difference was detected, individual mean values were compared by Bonferroni's post hoc tests (GraphPad Prism 6 (GraphPad, San Diego, USA)). Differences were considered significant at the level of $P < 0.05$.

Software for graphical design

Microsoft PowerPoint and *smart.servier medical art (www.smart.servier.com)* were used for graphical design.

Results

Effects of chronic hypo- and hyperthyroidism on myocardial ischaemia/reperfusion injury in mice

In order to investigate effects of chronic hypo- and hyperthyroidism on myocardial IR injury the isolated heart method according to Langendorff (Langendorff 1895) was used. For that purpose, experimental hypo- and hyperthyroidism were induced over 3 weeks in 3 months old male mice and were compared to untreated controls. Before examining myocardial infarct size – as the most robust end parameter in preclinical studies on cardioprotection (Lindsey et al. 2018; Botker et al. 2018) – it was verified whether induction of thyroid dysfunction was successful. Hence, serum concentrations of TT4, FT3 and FT4 were analysed (Engels et al. 2016; Rakov et al. 2016).

Serum TH concentrations in mice with manipulation of thyroid function

Treatment of mice for hypothyroidism with LoI/MMI/ClO_4^- over 3 weeks (hypo) decreased TT4 concentrations below the detection limit (Fig. 10 A), whereas FT4 and FT3 levels did not differ significantly from the controls (Fig. 10 B, C). In contrast, treatment for hyperthyroidism with T4 over 3 weeks (hyper) increased serum concentrations of TT4 (Fig. 10 A), FT4 (Fig. 10 B) as well as FT3 (Fig. 10 C). In summary, serum concentrations of TT4, FT4 and FT3 confirmed experimental successful induction of thyroid dysfunction in mice.

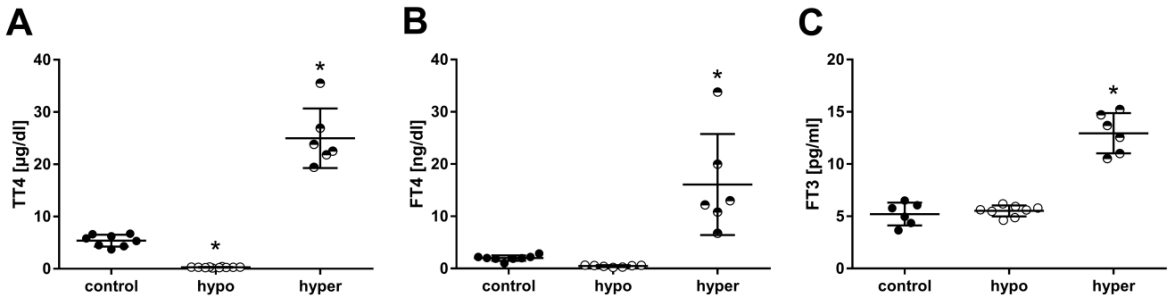

Figure 10: TH serum concentrations of male mice at 3 months of age. Serum concentration of total thyroxine (TT4) (A), free thyroxine (FT4) (B) and free thyronine (FT3) (C) in untreated (control), LoI/MMI/ClO_4^- treated (hypo) and T4 treated (hyper) mice, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Heart rate in mice with thyroid dysfunction

Similarly to hypo- and hyperthyroid patients in which brady- and tachycardia can manifest as a symptom (Chaker et al. 2017; De Leo, Lee, and Braverman 2016) heart rate is also a reliable baseline parameter that reflects thyroid dysfunction in mice (Rakov et al. 2016). Thus, after 10 minutes of stabilization, heart rate was measured in isolated mouse hearts. Assessment of the *ex vivo* heart rate in beats per minute [bpm] revealed a significantly lower heart rate in hypothyroid mouse hearts and an elevated heart rate in hyperthyroid mouse hearts compared to controls (Fig. 11). Thus, altered heart rate in isolated mouse hearts also confirmed successful induction of thyroid dysfunction in mice.

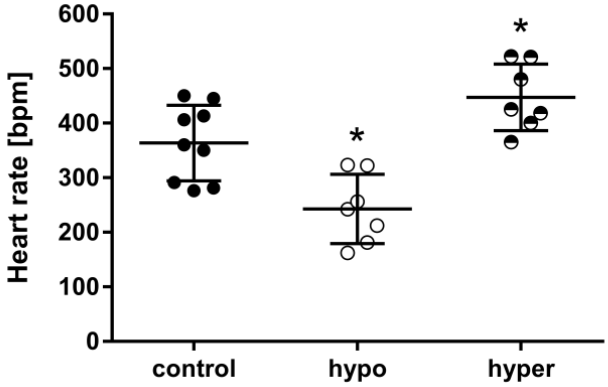

Figure 11: Heart rate of isolated, pressure constant perfused 3 months old male mouse hearts. *Ex vivo* heart rate in beats per minute [bpm] under control, hypothyroid (hypo) and hyperthyroid (hyper) conditions, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Impact of thyroid dysfunction on baseline left ventricular function in mice

After measuring *ex vivo* heart rate at baseline, all hearts were paced equally to 500 bpm to reach comparable experimental conditions between the groups. After 20 more minutes of pacing (30th min of baseline) CF and LVDP were recorded to evaluate myocardial functional performance before IR. Under heart rate matched conditions, baseline CF and LVDP were decreased in hypo and increased in hyper compared to control mouse hearts (Fig. 12 A, B).

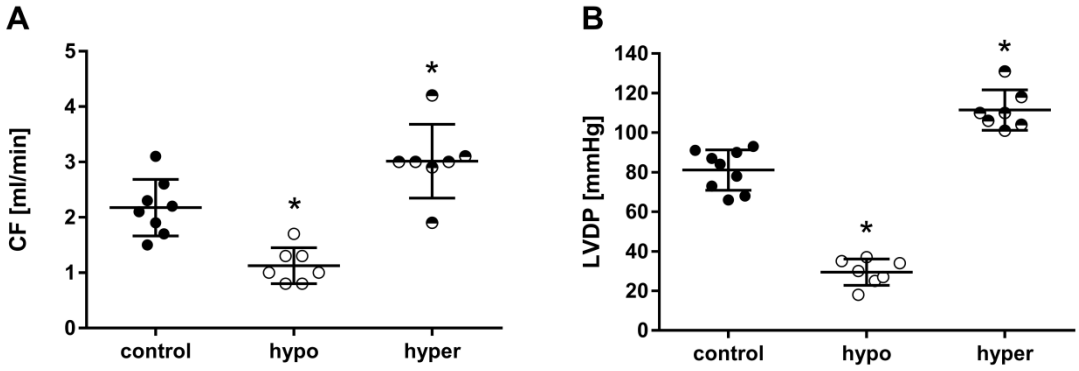

Figure 12: Baseline coronary flow (CF) (A) and left ventricular developed pressure (LVDP) (B) of isolated, pressure constant perfused 3 months old mouse hearts. CF in [ml/min] (A) and LVDP [mmHg] (B) in hearts of control, hypothyroid (hypo) and hyperthyroid (hyper) male mice, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Langendorff perfusion to determine extent of IR injury in mice

After IR, mouse hearts underwent TTC staining to differentiate between red (viable) and white (nonviable) tissue for infarct size determination. To verify whether Langendorff experiments are stable and that nonviable tissue only occurs after global IR, infarct sizes of control hearts that underwent global IR were compared with time controls (TCs) that were perfused for a duration period equal to the experimental protocol, i.e. 180 min but without ischaemia (Fig. 8). Thirty minutes of global ischaemia and 120 minutes of reperfusion (30/120 global IR) resulted in an infarct size of 51 ± 14% of ventricular mass in control mice. In TCs that did not undergo IR only infarction of 13 ± 1% of ventricular mass was detected (Fig. 13). TCs were regularly performed as stability control throughout all experiments.

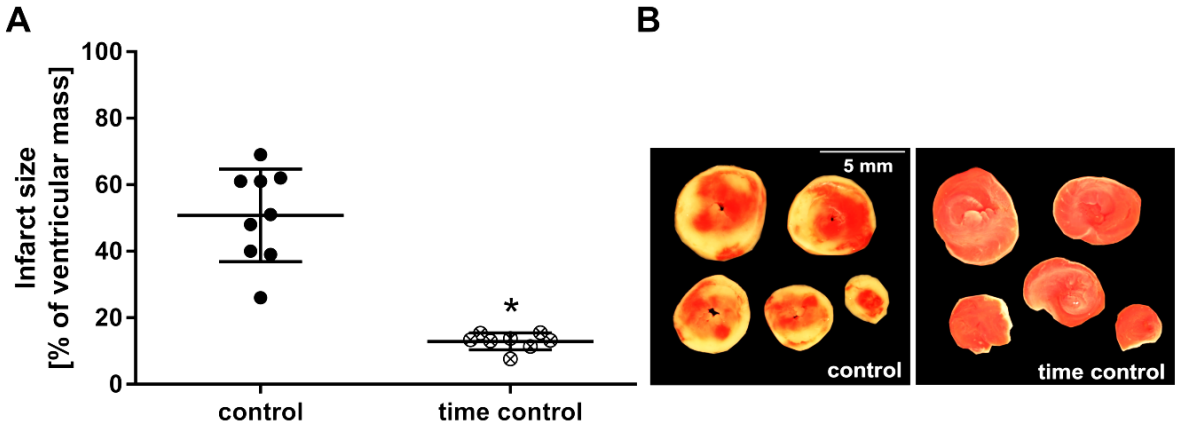

Figure 13: Infarct size of isolated pressure constant perfused 3 months old mouse hearts. Infarct size in [% of ventricular mass] of male mouse hearts that underwent global IR (control) and time controls that did not undergo global IR (time control) (A) and representative heart slices after TTC staining, respectively (B); Data are means ± standard deviations; Unpaired t-test; * $P < 0.05$ vs. control.

Impact of thyroid dysfunction in mice on myocardial infarct size

In comparison to controls, hypo- and hyperthyroid isolated mouse hearts were Langendorff perfused and infarct size was determined after 30/120 global IR. Hypothyroidism reduced infarct size to 15 ± 4% of ventricular mass, while hyperthyroidism enlarged infarct size to 75 ± 11% of ventricular mass compared to control mouse hearts with 51 ± 14% of ventricular mass, respectively (Fig. 14).

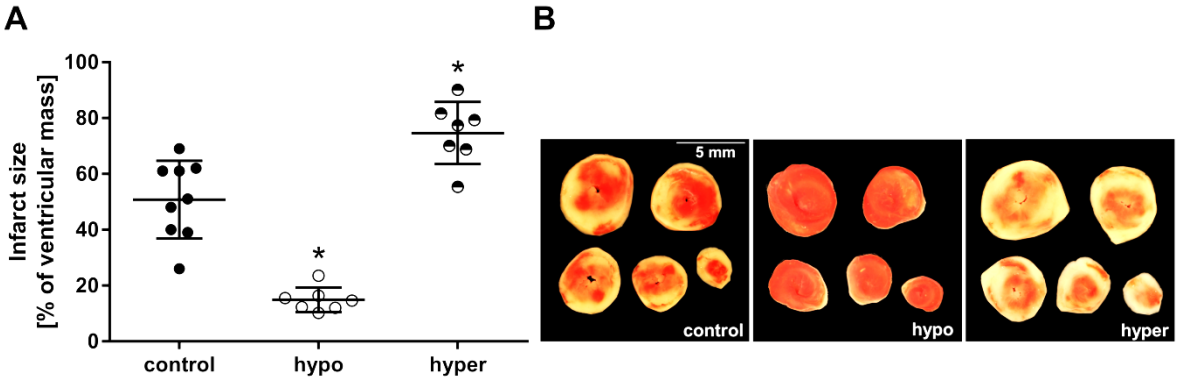

Figure 14: Infarct size of isolated pressure constant perfused 3 months old mouse hearts with thyroid dysfunction. Infarct size in [% of ventricular mass] of control, hypothyroid (hypo) and hyperthyroid (hyper) male mice (A) and representative heart slices after TTC staining, respectively (B); Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Recovery of left ventricular function in isolated mouse hearts

Recovery of CF and LVDP provides information about the functional myocardial performance after ischaemia. Control mouse hearts not only showed viable but also nonviable tissue after IR protocol (51 ± 14% of ventricular mass) (Fig. 13 and Fig. 14) and recovery of LVDP was decreased during reperfusion compared to baseline, while CF remained unaltered (Table 8). In contrast, CF and LVDP were stable over time in TCs (Table 8).

Table 8: Functional recovery of coronary flow and left ventricular developed pressure in 3 months old male mouse hearts that underwent global IR (control) and in time controls that did not undergo global IR. Mean coronary flow (CF) and mean left ventricular developed pressure (LVDP) of control and time control mouse hearts were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion, respectively. Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; # $P < 0.05$ vs. control; * $P < 0.05$ vs. baseline.

Protocol	Time	CF [ml/min]	LVDP [mmHg]
control	baseline	2.1 ± 0.5	81 ± 10
n = 9	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.7 ± 0.7	4 ± 4 *
	rep20	1.7 ± 0.8	17 ± 19 *
	rep30	1.7 ± 0.8	20 ± 20 *
	rep40	1.7 ± 0.8	23 ± 19 *
	rep50	1.7 ± 0.8	25 ± 18 *
	rep60	1.7 ± 0.8	25 ± 18 *
time control	baseline	2.6 ± 0.4	89 ± 21
n = 8	*isch5*	2.6 ± 0.4 #	84 ± 18 #
	isch25	2.6 ± 0.4 #	80 ± 10 #
	rep10	2.6 ± 0.4	86 ± 15 #
	rep20	2.6 ± 0.4	82 ± 11 #
	rep30	2.5 ± 0.3	80 ± 15 #
	rep40	2.5 ± 0.4	78 ± 13 #
	rep50	2.5 ± 0.4	81 ± 16 #
	rep60	2.5 ± 0.4	79 ± 16 #

Hypothyroid hearts (hypo) preserved CF and LVDP from the beginning until the end of reperfusion, wherein LVDP was increased at time point rep10 compared to controls (Table 9). On the other hand, hyperthyroid mouse hearts showed a decreased LVDP during reperfusion in comparison to baseline, in line with enlarged infarct size in this group (Table 9). CF in reperfusion of hyper did not differ compared to control mouse hearts or baseline values (Table 9).

Table 9: Functional recovery of coronary flow and left ventricular developed pressure in control, hypo- and hyperthyroid 3 months old male mouse hearts. Mean coronary flow (CF) and mean left ventricular developed pressure (LVDP) of control, hypothyroid (hypo) and hyperthyroid (hyper) mice were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion, respectively. Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; # $P < 0.05$ vs. control; * $P < 0.05$ vs. baseline.

Protocol	Time	CF [ml/min]	LVDP [mmHg]
control	baseline	2.1 ± 0.5	81 ± 10
n = 9	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.7 ± 0.7	4 ± 4 *
	rep20	1.7 ± 0.8	17 ± 19 *
	rep30	1.7 ± 0.8	20 ± 20 *
	rep40	1.7 ± 0.8	23 ± 19 *
	rep50	1.7 ± 0.8	25 ± 18 *
	rep60	1.7 ± 0.8	25 ± 18 *
hypo	baseline	1.1 ± 0.3 #	28 ± 6 #
n = 7	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.9 ± 0.5	21 ± 8 #
	rep20	1.4 ± 0.5	23 ± 7
	rep30	1.3 ± 0.5	25 ± 10
	rep40	1.5 ± 0.5	24 ± 6
	rep50	1.4 ± 0.6	25 ± 10
	rep60	1.4 ± 0.5	26 ± 10
hyper	baseline	3.0 ± 0.7 #	111 ± 10 #
n = 8	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.4 ± 0.4	1 ± 2 *
	rep20	2.4 ± 0.4	3 ± 5 *
	rep30	2.3 ± 0.4	5 ± 10 *
	rep40	2.3 ± 0.5	12 ± 24 *
	rep50	2.2 ± 0.5	15 ± 30 *
	rep60	2.2 ± 0.5	16 ± 34 *

Phosphorylation of classical cardioprotective proteins in mice with thyroid dysfunction

To investigate the impact of thyroid dysfunction on classical cardioprotective pathways (Heusch 2015), protein expression and phosphorylation of the reperfusion injury salvage kinase pathway (*RISK*) with its key enzymes including protein kinase B (AKT) and extracellular signal-regulated kinases (ERK1/2), the survival activating factor enhancement pathway (*SAFE*) with its key signal transducer and activator of transcription (STAT3), the endothelial nitric oxide synthase (eNOS) and p38-mitogen-activated protein kinase (p38) were analysed after IR protocol and hypo and hyper groups were compared to controls (Fig. 15). Phosphorylation of AKT (p-AKT/total-AKT) (Fig. 15 A), ERK (p-ERK/total-ERK) (Fig. 15 B), STAT3 (p-STAT3/total-STAT3) (Fig. 15 C), p38 (p-p38/total-p38) (Fig. 15 D) and eNOS (p-eNOS(ser)/total-eNOS) (Fig. 15 E) did not differ significantly between hypo or hyper mice compared to controls. However, there was a small, yet not significant decrease in p-ERK/total-ERK and p-STAT3/total-STAT3 in hypo compared to control group (Fig. 15 C).

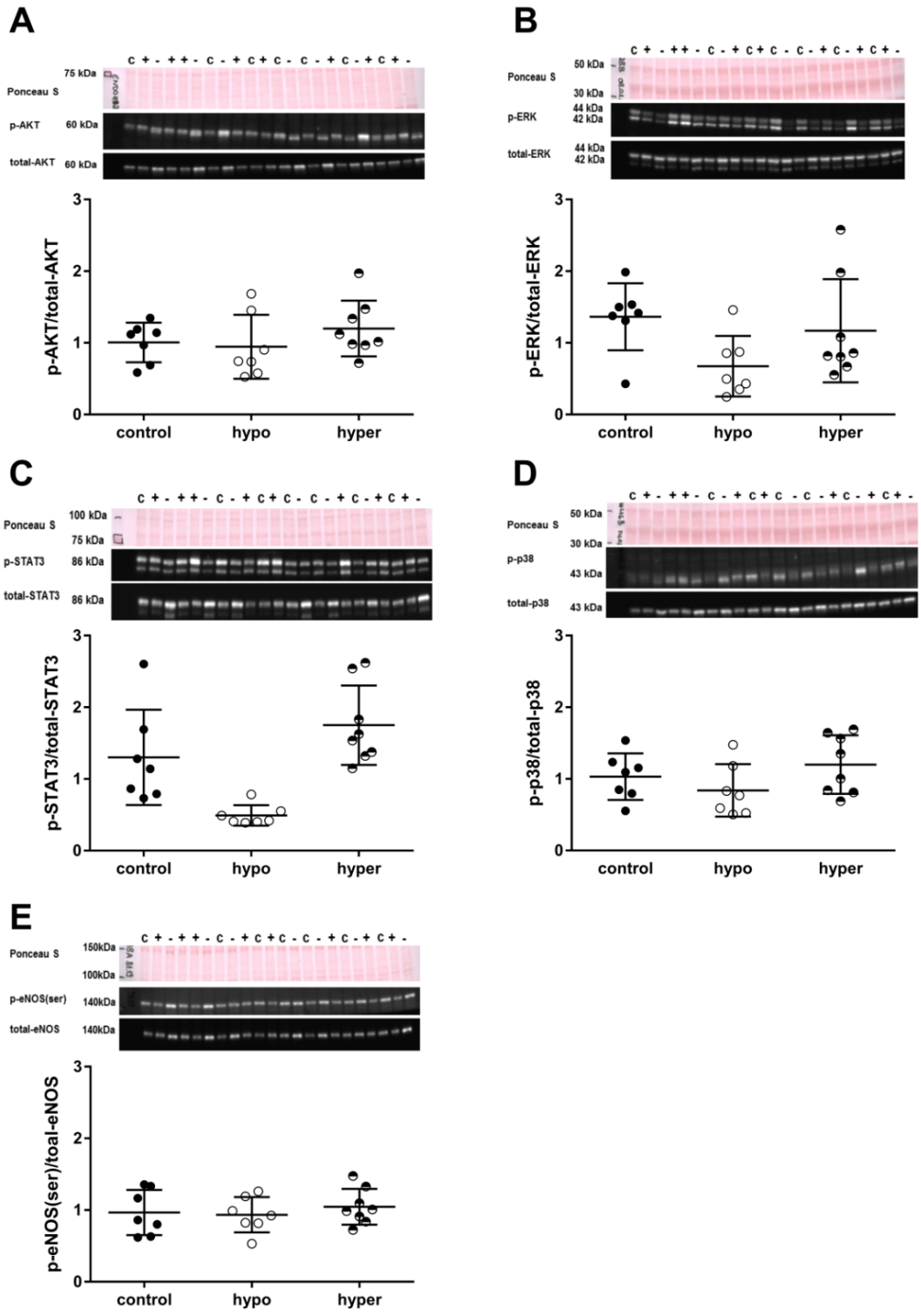

Figure 15: Phosphorylation of proteinkinase B (p-AKT/total-AKT) (A), extracellular-signal regulated kinases (p-ERK/total-ERK) (B), signal transducer and activator of transcription 3 (p-STAT3/total-STAT3) (C), p38-mitogen-activated proteinkinase (p-p38/total-p38) (D) and endothelial nitric oxide synthase (p-eNOS(ser)/total-eNOS) (E) after myocardial ischaemia/reperfusion in left ventricular tissue of 3 months old mouse hearts. Top, middle, bottom: Membranes stained with Ponceau S, immunoreactivity signals for phosphorylated and total protein (**c**: control; **-** : hypo; and **+** : hyper). The phosphorylation of proteins in control, hypothyroid (hypo) and hyperthyroid (hyper) mouse hearts was normalized to the respective total protein expression. Data are means ± standard deviations; One-Way ANOVA with Bonferroni's correction.

Impact of epinephrine addition to hypothyroid mouse hearts on functional performance and infarct size

During experiments in hypothyroid isolated mouse hearts, we noticed that LVDP broke down as soon as the pacer had been switched on after determination of basal heart rate (Fig. 16 A). Usually, control hearts maintained their LVDP when pacing at 500 bpm. However, it is known that hypothyroid hearts have decreased expression of *Hcn4* and *Myh6* (Rakov et al. 2016) in line with reduced heart rate and decreased LVDP at baseline (Vetter et al. 2011). Thus, hypothyroid hearts might not be able to beat 500 times per minute. However, in order to maintain comparable experimental conditions, pacing remained essential. Additionally, we had to exclude that the reduced LVDP in hypothyroid hearts, which resembled the "phenomenon of cardioplegia" that defines the resting and thus saving mode of a heart (Nishina and Chambers 2018; Chambers and Fallouh 2010; Das et al. 1987), was not causally involved in infarct size reduction (Fig. 14) and improved functional recovery (Table 9). Therefore, in a control experiment, LVDP was matched to the level of control hearts by adding 23 µg/L epinephrine (epi) to the hypothyroid hearts. Subsequently, infarct size and functional performance were measured. Since epinephrine is known to act lusitropic, chronotropic and inotropic (Broadley 1970), a rapid increase in LVP and heart rate was observed in isolated hypothyroid mouse hearts (Fig. 16 B).

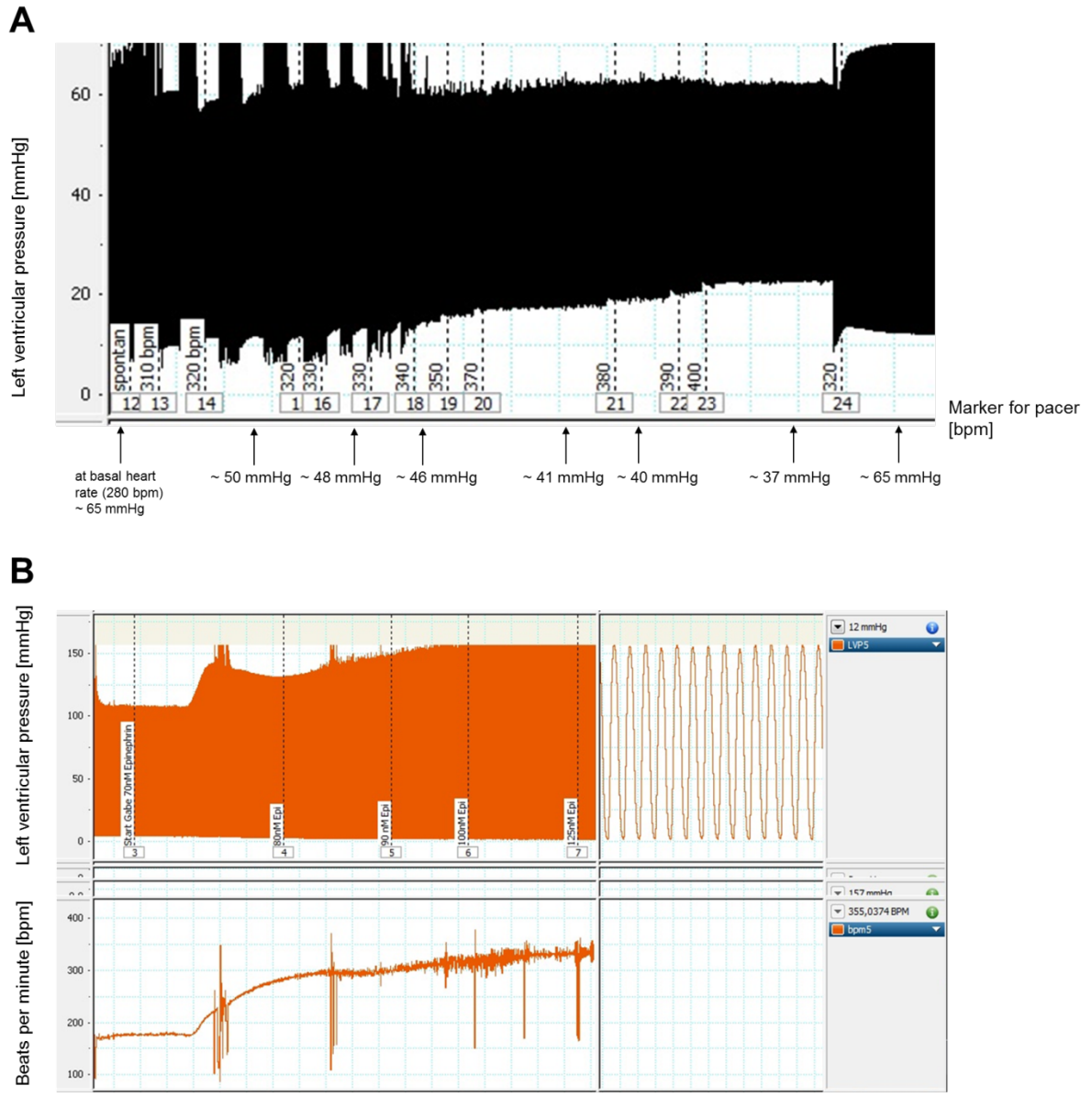

Figure 16: Original registrations of left ventricular pressure and heart rate in hypothyroid isolated mouse hearts in absence (A) and in presence of epinephrine (B). In black: Original registration of left ventricular pressure (LVP) in [mmHg] of an isolated hypothyroid mouse heart with increasing electrical atrial stimulation in [bpm] (A). LVP decreases with increasing electrical stimulation. In orange: Original registration of LVP in [mmHg] (above) and heart rate in [bpm] (below) of an isolated hypothyroid mouse heart with increasing epinephrine (epi) concentration without electrical atrial stimulation (B). LVP and heart rate increase with increasing epinephrine concentration. End concentration of epinephrine was 23 µg/L.

Under heart rate matched conditions, the addition of epinephrine increased baseline CF (Fig. 17 A) and LVDP (Fig. 17 B) of hypo hearts to the CF and LVDP levels of control hearts, which were 2.1 ± 0.5 ml/min and 81 ± 10 mmHg, respectively.

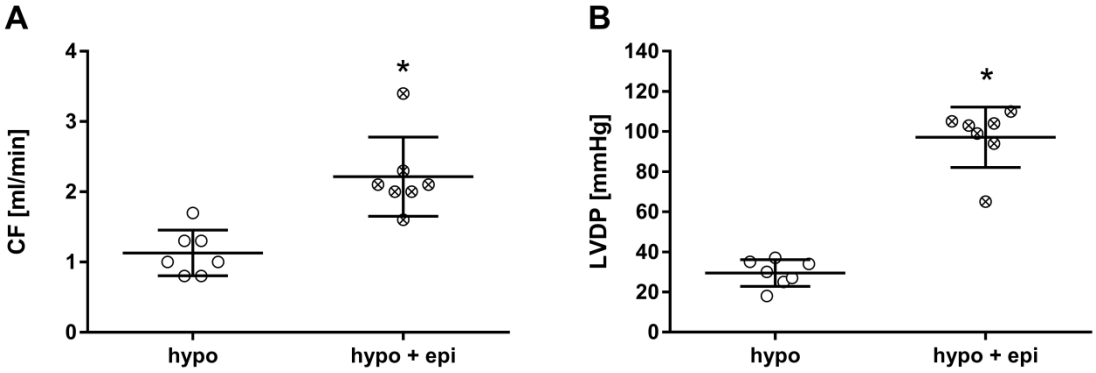

Figure 17: Baseline coronary flow (CF) (A) and left ventricular developed pressure (LVDP) (B) of isolated, pressure constant perfused hypothyroid mouse hearts in absence (hypo) or in presence of epinephrine (hypo + epi). CF in [ml/min] (A) and LVDP in [mmHg] (B). Data are means ± standard deviations; Unpaired t-test; * $P < 0.05$ vs. hypo.

Infarct size of hypo hearts in presence of epinephrine (hypo + epi) was 20 ± 7% of ventricular mass and was comparable to hypo hearts in absence of epinephrine (hypo) (Fig. 18). Thus, epinephrine increased functional performance at baseline but this had no impact on infarct size.

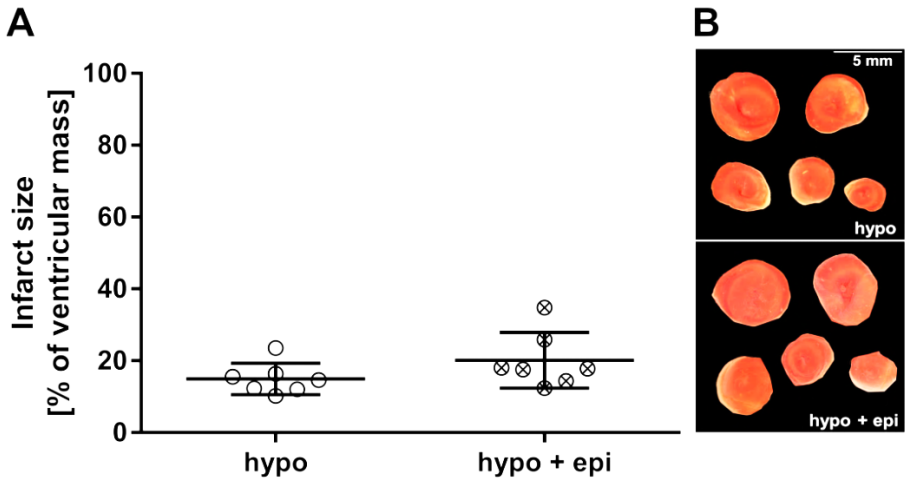

Figure 18: Infarct size of isolated pressure constant perfused hypothyroid mouse hearts in absence (hypo) or in presence of epinephrine (hypo + epi). Infarct size in [% of ventricular mass]. Data are means ± standard deviations; Unpaired t-test.

CF during reperfusion was increased in hypo + epi hearts compared to hypo hearts and it was also increased compared to baseline (Fig. 19 A). LVDP of hypo + epi hearts was increased during reperfusion in comparison to hypo group, while LVDP during reperfusion decreased in comparison to baseline (Fig. 19 B).

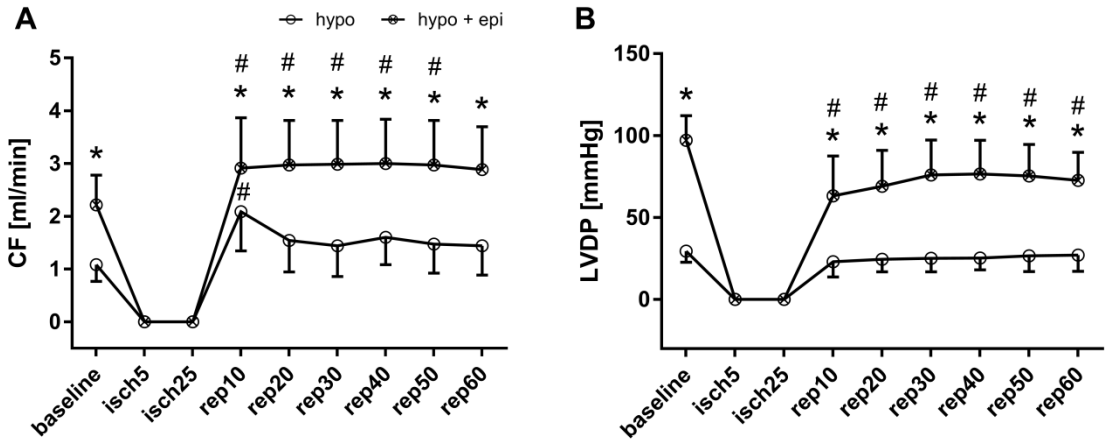

Figure 19: Functional recovery of coronary flow (A) and left ventricular developed pressure (B) in hypothyroid mouse hearts in absence (hypo) and in presence of epinephrine (hypo + epi). Mean coronary flow (CF) (A) and mean left ventricular developed pressure (LVDP) (B) were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion, respectively. Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; * $P < 0.05$ vs. hypo; # $P < 0.05$ vs. baseline.

In summary, hypothyroidism decreased heart rate and baseline CF and LVDP in 3 months old male mice. It also reduced myocardial infarct size and preserved left ventricular function after 30/120 global IR. Infarct size reduction under hypothyroidism was irrespective of reduced CF and LVDP at baseline, as CF and LVDP were matched to the level of control hearts by adding epinephrine to hypothyroid hearts. On the other hand, hyperthyroidism increased heart rate and baseline CF and LVDP in 3 months old male mice. Infarct size was enlarged in hyperthyroid hearts compared to controls and functional recovery after IR was strongly reduced compared to baseline situation. There were no significant changes in the phosphorylation of classical cardioprotective proteins in left ventricular tissue of isolated mouse hearts with altered thyroid status. However, small decreases of p-STAT3/total-STAT3 and p-ERK/total-ERK were noted.

Thyroid hormone effects on myocardial ischaemia/reperfusion injury in aged mice

The risk for both, thyroid and heart diseases increases with age (Selmer et al. 2014; Diab et al. 2019), however it is still unclear whether ageing affects the outcome of IR injury under thyroid dysfunction (Jabbar et al. 2017). We aimed to clarify whether 20 months old mice suffer more from IR injury under thyroid dysfunction than 3 months old mice. Therefore myocardial infarct size of 20 months old male mice after inducing experimental thyroid dysfunction over 3 weeks was investigated. Since experiments in 3 months old hypothyroid mice had shown that reduced baseline LVP is not causally involved in infarct size reduction (Fig. 18), old hypothyroid hearts were not tested in presence of epinephrine.

Serum TH concentrations in aged mice with manipulation of thyroid function

Treatment with T4 over 3 weeks increased concentrations of TT4 (Fig. 20 A), FT4 (Fig. 20 B) and FT3 (Fig. 20 C) in sera of 20 months old mice compared to controls, while there were only small, but not significant, decreases in TT4 (72% decrease) (Fig. 20 A) and FT4 (Fig. 20 B) in hypo compared to control hearts. In 3 months old mice, LoI/MMI/ClO_4^- treatment decreased TT4 serum concentrations significantly by 98%. Thus, manipulation of thyroid function was less pronounced in aged compared to younger mice.

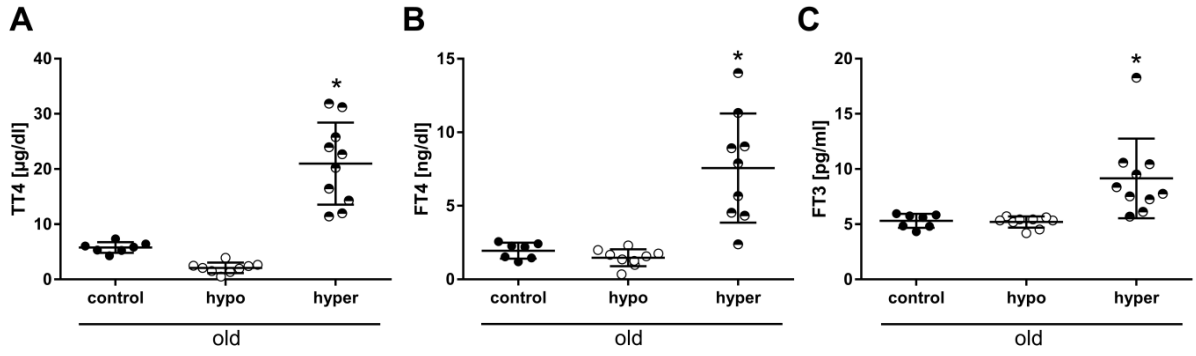

Figure 20: TH serum concentrations of male mice at 20 months of age. Serum concentrations of total thyroxine (TT4) (A), free thyroxine (FT4) (B) and free thyronine (FT3) (C) of control, hypothyroid (hypo) and hyperthyroid (hyper) male mice, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Heart rate in aged mice with thyroid dysfunction

Hypothyroidism resulted in a decreased heart rate and hyperthyroidism in increased heart rate compared to controls in 20 months old mice (Fig. 21). The assessment of basal heart rate yielded the same degree of changes in 3 and 20 months old mice.

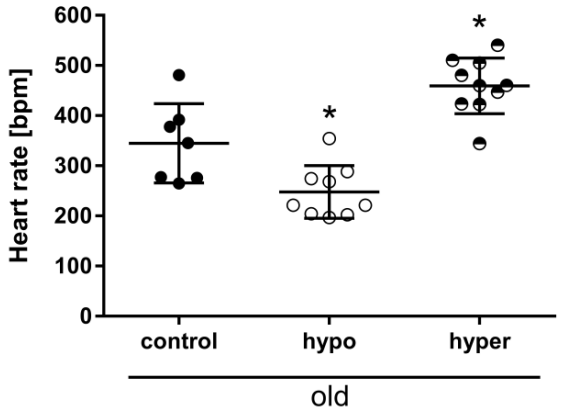

Figure 21: Heart rate of isolated, pressure constant perfused 20 months old mouse hearts. *Ex vivo* heart rate in beats per minute [bpm] of control, hypothyroid (hypo) and hyperthyroid (hyper) mouse hearts, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Impact of thyroid dysfunction on baseline left ventricular function in aged mice

CF showed a small, but not significant, decrease (by 25%) (Fig. 22 A) and LVDP decreased significantly in aged hypo mouse hearts (Fig. 22 B). Under hyperthyroidism, CF increased (Fig. 22 A), while LVDP remained unaltered in 20 months old mouse hearts compared to controls (Fig. 22 B). In 3 months old mice, hypothyroidism decreased CF significantly by 50%, while LVDP increased significantly by 37% by hyperthyroidism. Thus, the effects of TH status on CF and LVDP were less pronounced in 20 months old compared to 3 months old mouse hearts.

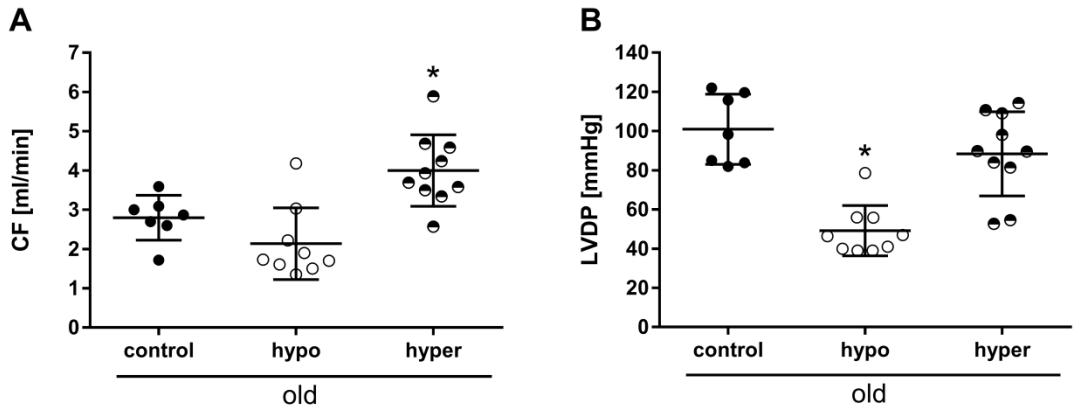

Figure 22: Baseline coronary flow (CF) (A) and left ventricular developed pressure (LVDP) (B) of isolated, pressure constant perfused 20 months old mouse hearts. CF in [ml/min] (A) and LVDP [mmHg] (B) of control, hypothyroid (hypo) and hyperthyroid (hyper) mouse hearts, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Impact of thyroid dysfunction on myocardial infarct size in aged mice

While hypothyroidism decreased infarct size by 51%, hyperthyroidism resulted in a small, but not significant increase of 21% in aged mouse hearts (Fig. 23). In 3 months old mice, hypothyroidism decreased infarct size by 71%, whereas hyperthyroidism increased infarct size by 47%. Taken together, in 20 months old mice, the pattern of infarct size distribution remained similar to 3 months old mouse cohort, but the differences were less pronounced.

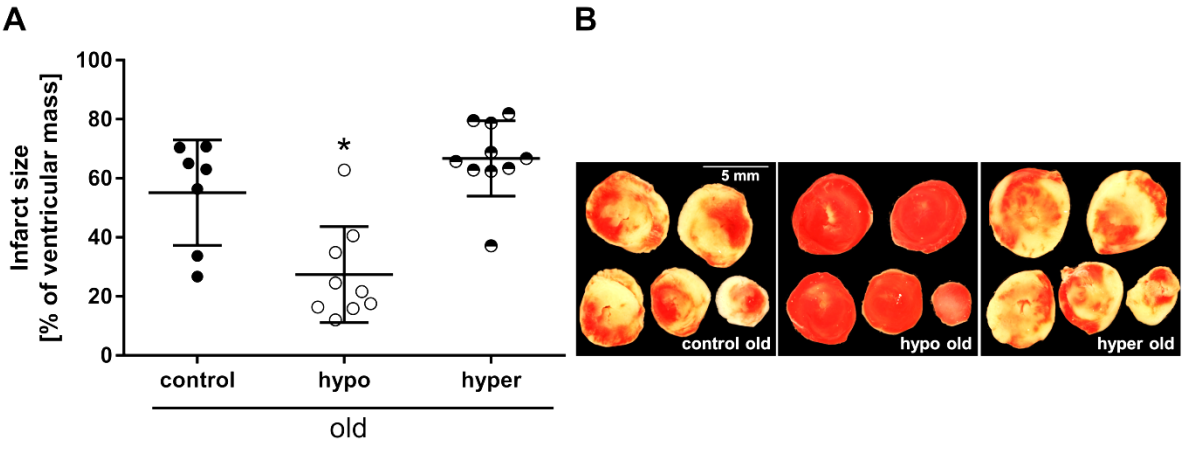

Figure 23: Infarct size of isolated pressure constant perfused 20 months old mouse hearts. Infarct size in [% of ventricular mass] of control, hypothyroid (hypo) and hyperthyroid (hyper) male mice (A) and representative heart slices after TTC staining, respectively (B); Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Recovery of left ventricular function in aged mice with thyroid dysfunction

Hearts of control and hyperthyroid 20 months old mice had a decreased LVDP during reperfusion in comparison to baseline (Table 10), reflecting the non-viable tissue of these hearts after IR injury (Fig. 23). In aged hypothyroid hearts, LVDP during reperfusion was increased compared to baseline value (Table 10). In 3 months old mouse hearts, hypothyroidism did not increase LVDP after ischaemia. Taken together, effects of hypothyroidism on functional recovery were more pronounced in aged mouse hearts.

Table 10: Functional recovery of coronary flow and left ventricular developed pressure in control, hypo- and hyperthyroid 20 months old mouse hearts. Mean coronary flow (CF) and mean left ventricular developed pressure (LVDP) were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion, respectively. Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; # $P < 0.05$ vs. control; * $P < 0.05$ vs. baseline.

Protocol	Time	CF [ml/min]	LVDP [mmHg]
control	baseline	2.8 ± 0.6	104 ± 18
old	isch5	0.0 ± 0.0 *	0 ± 0 *
n = 7	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.5 ± 1.2	21 ± 4 *
	rep20	2.7 ± 1.4	17 ± 34 *
	rep30	2.6 ± 1.4	49 ± 43 *
	rep40	2.5 ± 1.4	55 ± 41 *
	rep50	2.4 ± 1.3	55 ± 38 *
	rep60	2.3 ± 1.3	55 ± 36 *
hypo	baseline	2.1 ± 0.9	49 ± 13 #
old	isch5	0.0 ± 0.0 *	0 ± 0 *
n = 9	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.7 ± 1.0	36 ± 23
	rep20	2.9 ± 1.0	64 ± 27
	rep30	2.9 ± 1.0	71 ± 26
	rep40	2.9 ± 1.1	75 ± 25 *
	rep50	2.8 ± 1.0	74 ± 22 *
	rep60	2.8 ± 1.0	73 ± 21 *
hyper	baseline	3.8 ± 0.7 #	86 ± 21
old	isch5	0.0 ± 0.0 *	0 ± 0 *
n = 10	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.5 ± 1.0	1 ± 2 *
	rep20	2.5 ± 1.1	6 ± 10 *
	rep30	2.6 ± 1.2	11 ± 15 *
	rep40	2.6 ± 1.2	17 ± 16 *
	rep50	2.5 ± 1.1	18 ± 17 *
	rep60	2.5 ± 1.1	20 ± 19 *

Phosphorylation of classical cardioprotective proteins in aged mice with thyroid dysfunction

There was no significantly increased protein phosphorylation in AKT, ERK, STAT3, eNOS and p38 detectable (Fig. 24 A-E). However, phosphorylation of STAT3 (p-STAT3/total-STAT3) was decreased in hypo hearts compared to control hearts of 20 months old mice (Fig. 24 C). Additionally, under hypothyroidism a small, but not significant, decrease was noted in the phosphorylation of AKT (Fig. 24 A), ERK (Fig. 24 B) and p38 (Fig. 24 D), whereas hyperthyroidism led to a small, yet not significant, increase in phosphorylation of p38 compared to controls (Fig. 24 D).

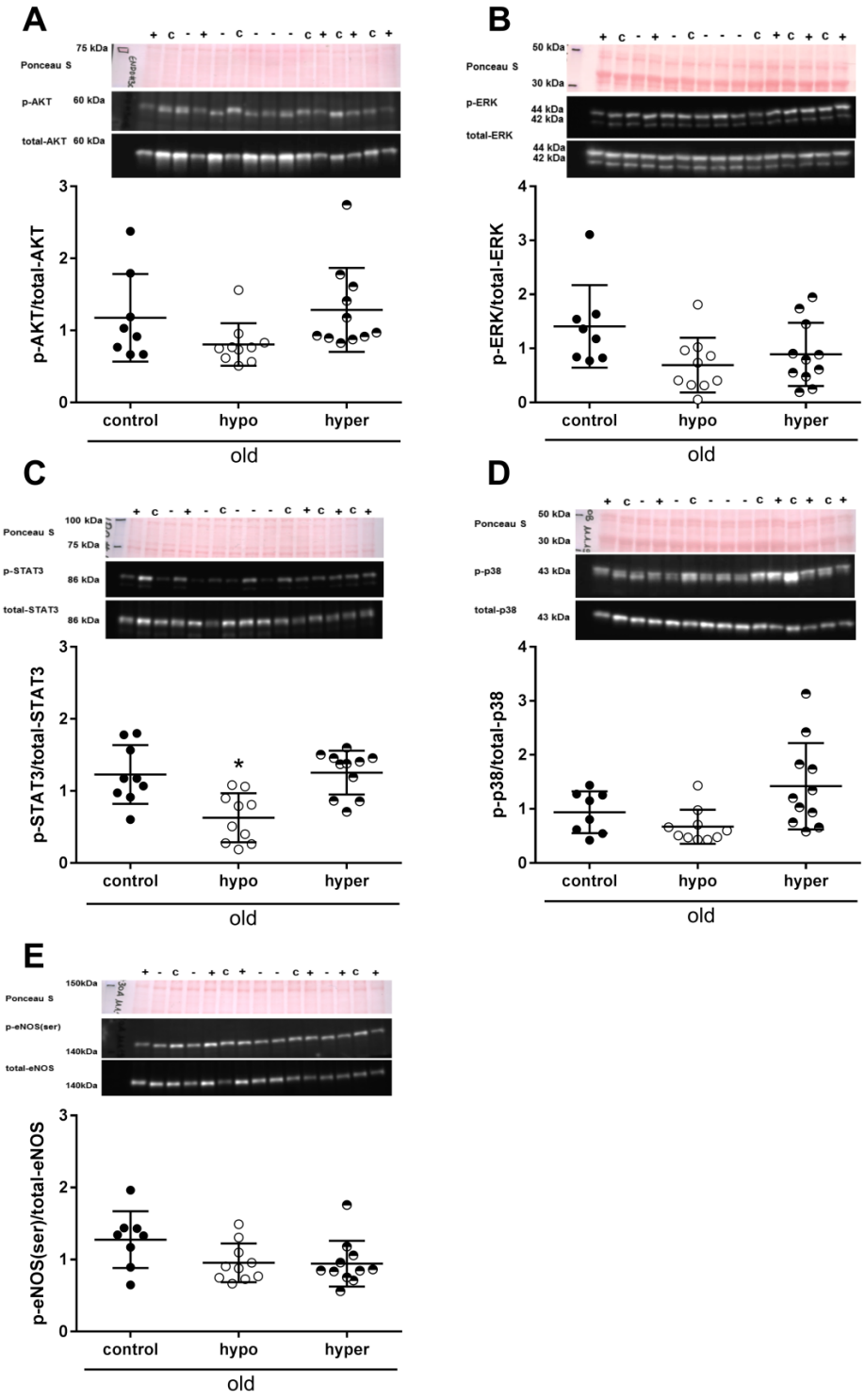

Figure 24: Phosphorylation of proteinkinase B (p-AKT/total-AKT) (A), extracellular-signal regulated kinases (p-ERK/total-ERK) (B), signal transducer and activator of transcription 3 (p-STAT3/total-STAT3) (C), p38-mitogen-activated proteinkinase (p-p38/total-p38) (D) and endothelial nitric oxide synthase (p-eNOS(ser)/total-eNOS) (E) after myocardial ischaemia/reperfusion in left ventricular tissue of 20 months old mouse hearts. Top, middle, bottom: Membranes stained with Ponceau S, immunoreactivity signals for phosphorylated and total protein (**c**: control; **-** : hypo; and **+** : hyper). The phosphorylation of proteins in control, hypothyroid (hypo) and hyperthyroid (hyper) mouse hearts was normalized to the respective total protein expression. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

In summary, hypothyroidism decreased heart rate and baseline LVDP in 20 months old mice. It also reduced myocardial infarct size and improved recovery of left ventricular function after ischaemia in comparison to baseline. On the other hand, hyperthyroidism increased heart rate and baseline CF in 20 months old male mice. Infarct size was marginally, but not significantly, enlarged compared to controls, whereas functional recovery was markedly reduced compared to baseline situation in aged mouse hearts. There was no significant increase in the phosphorylation of cardioprotective proteins in left ventricular tissue of 20 months old mouse hearts with altered thyroid status. However, a decrease was noted in p-STAT3/total-STAT3 under hypothyroidism in old mouse hearts. Taken together, in comparison to 3 months old mice, most effects of thyroid dysfunction on myocardial IR injury were less pronounced in 20 months old male mice.

Role of TRα signalling on myocardial ischaemia/reperfusion injury

TRα is the predominantly expressed receptor in the myocardium and therefore primarily mediates cardiac TH action (Brent 2012). In previous studies it was shown that noncanonical action of TRα determines physiological regulation of heart rate (Hones et al. 2017) and that treatment of rats with a selective TRα inhibitor impaired functional performance after *in vivo* MI in mice (Mourouzis et al. 2013). However it remained unclear whether canonical or noncanonical TRα signalling affects myocardial functional parameters and infarct size after global IR. To clarify the role of TRα signalling during IR injury, isolated hearts of wildtype mice (control), mice lacking TRα ($TR\alpha^0$) and mice expressing a mutant TRα ($TR\alpha^{GS}$) that is incapable of binding to the DNA were used. Consequently the comparison of control, $TR\alpha^0$ and $TR\alpha^{GS}$ mice allowed differentiation between canonical and noncanonical TRα effects (Hones et al. 2017; Flamant and Gauthier 2013; Flamant et al. 2017). As in previous experiments, infarct size and left ventricular function were determined as readout parameters. In addition, to distinguish between indirect TH effects in the organ and direct TRα dependent effects, a group with T3 hyperthyroid $TR\alpha^0$ mice ($TR\alpha^0$hyper) was generated. To confirm successful induction of hyperthyroidism in $TR\alpha^0$hyper mice, TH serum concentrations of transgenic mice were measured.

Serum TH concentrations in mice with altered TRα signalling in absence and in presence of systemic hyperthyroidism

Treatment with T3 over 3 weeks revealed no significant changes in TT4 serum concentration between the groups, although there was a small decrease in TT4 levels in hyperthyroid $TR\alpha^0$ ($TR\alpha^0$hyper) compared to control mice (Fig. 25 A). FT4 decreased significantly in $TR\alpha^0$hyper compared to controls (Fig. 25 B), while FT3 concentration strongly increased in hyperthyroid $TR\alpha^0$ ($TR\alpha^0$hyper) compared to control mice. TT4, FT4 and FT3 serum concentrations were comparable in untreated controls, $TR\alpha^0$ and $TR\alpha^{GS}$ mice (Fig. 25).

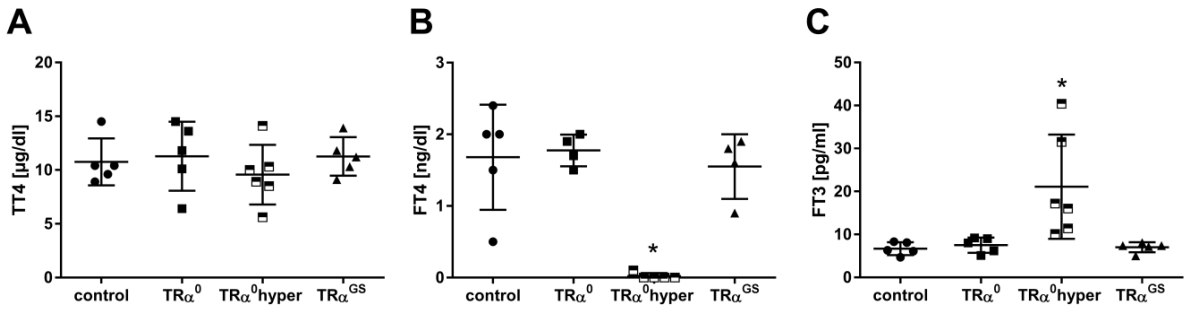

Figure 25: TH serum concentrations of transgenic mice. Serum concentrations of total thyroxine (TT4) (A), free thyroxine (FT4) (B) and free thyronine (FT3) (C) of control, $TR\alpha^0$, $TR\alpha^0$hyper and $TR\alpha^{GS}$ mice, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Heart rate in mice with altered TRα signalling in absence and in presence of systemic hyperthyroidism

Very similar to the hypothyroid state, where heart rate decreased by 33% compared to controls, lack of canonical TRα signalling, which is absent in $TR\alpha^0$ and $TR\alpha^{GS}$ mice, decreased heart rate by 32% and 29%, respectively (Fig. 26). Interestingly, there was no significant difference between control and hyperthyroid $TR\alpha^0$ ($TR\alpha^0$hyper) isolated mouse hearts (Fig. 26). Thus, *ex vivo* heart rate decreased in mice lacking canonical TRα signalling, while it remained comparable between controls and $TR\alpha^0$ mice with systemic hyperthyroidism.

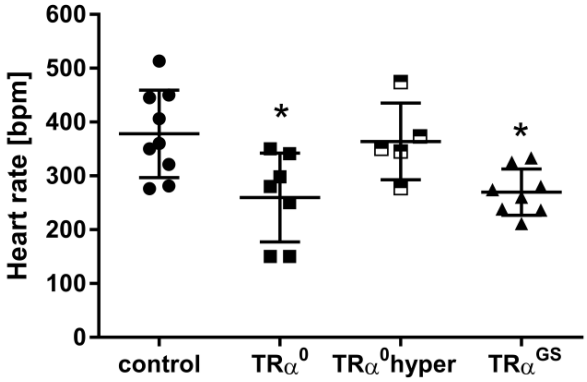

Figure 26: Heart rate of isolated, pressure constant perfused transgenic male mouse hearts. *Ex vivo* heart rate in beats per minute [bpm] of wildtype (control), $TR\alpha^0$, hyperthyroid $TR\alpha^0$ ($TR\alpha^0$hyper) and $TR\alpha^{GS}$ mice, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Impact of TRα signalling on baseline left ventricular function in mice

To evaluate myocardial functional performance before IR injury, LVDP and CF of transgenic mice were compared to controls. CF was comparable in control, TRα^0, TRα^{0}hyper and TRα^{GS} mouse hearts (Fig. 27 A). LVDP was decreased in TRα^0 mouse hearts compared to controls, while LVDP of TRα^{0}hyper and TRα^{GS} mouse hearts did not differ in comparison to controls (Fig. 27 B).

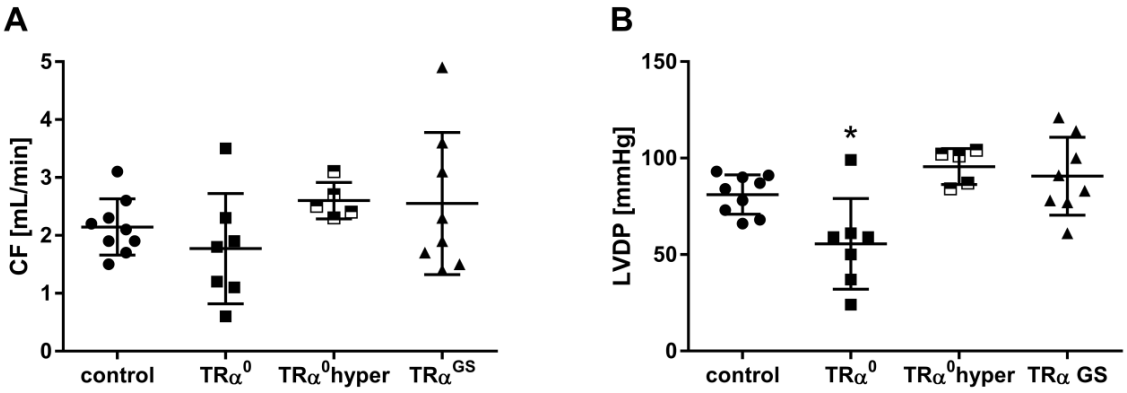

Figure 27: Baseline coronary flow (CF) (A) and left ventricular developed pressure (LVDP) (B) of isolated, pressure constant perfused transgenic mouse hearts. CF in [ml/min] (A) and LVDP [mmHg] (B) of wildtype (control), TRα^0, hyperthyroid TRα^0 (TRα^{0}hyper) and TRα^{GS} mouse hearts, respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Impact of TRα signalling on myocardial infarct size in mice

Infarct size was determined after 30/120 global IR. Compared to controls, infarct sizes were markedly reduced in TRα^0 (decrease by 65%), TRα^{0}hyper (decrease by 59%) and TRα^{GS} mice (decrease by 65%) (Fig. 28). This is almost in line with infarct size reduction in hypothyroid mouse hearts of the same age with a decrease by 71% compared to controls (Fig. 13).

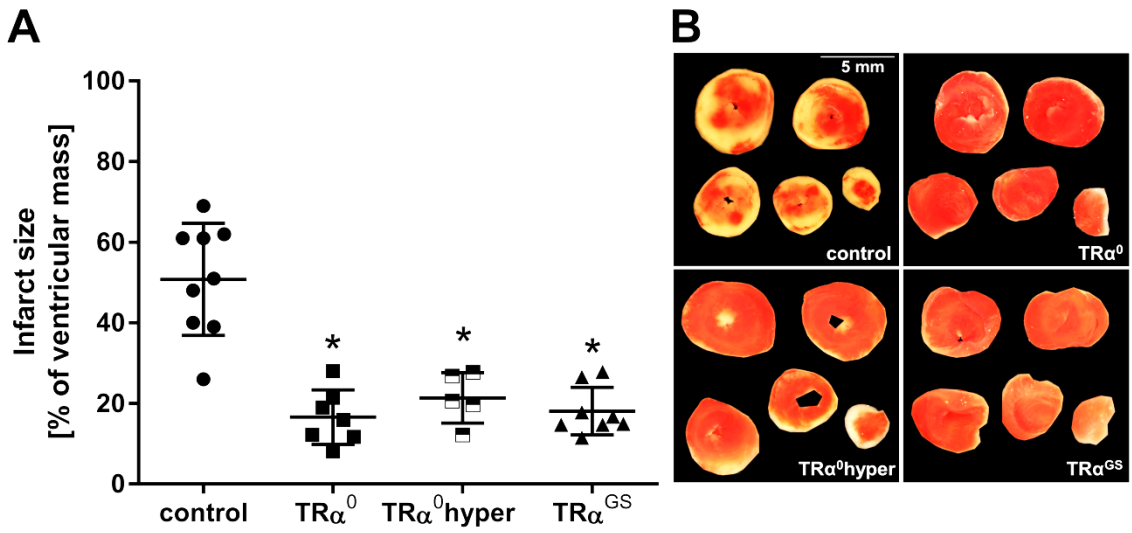

Figure 28: Infarct size of isolated pressure constant perfused transgenic mouse hearts. Infarct size in [% of ventricular mass] of wildtype (control), TRα^0, hyperthyroid TRα^0 (TRα^{0}hyper) and TRα^{GS} mice, respectively (A) and representative heart slices after TTC staining, respectively (B); Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Recovery of left ventricular function in mice with altered TRα signalling

To verify whether the reduced myocardial infarcts (Fig. 28) are also reflected in the improved functional recovery, CF and LVDP were assessed every ten minutes after ischaemia. During reperfusion the recovery of LVDP was improved in TRα^0, TRα^{0}hyper and TRα^{GS} mice compared to controls (Table 11), in line with reduced infarct sizes of all three groups (Fig. 28). Compared to baseline, LVDP at reperfusion decreased in control and also in TRα^{0}hyper mouse hearts. CF was comparable in all groups (Table 11).

Table 11: Functional recovery of coronary flow and left ventricular developed pressure in wildtype (control), TRα^0, hyperthyroid TRα^0 (TRα^0 hyper) and TRα^{GS} mouse hearts. Mean coronary flow (CF) and mean left ventricular developed pressure (LVDP) of mouse hearts were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion; n=5-9; Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; # P < 0.05 vs. control, * P < 0.05 vs. baseline.

Protocol	Time	CF [ml/min]	LVDP [mmHg]
control	baseline	2.1 ± 0.5	81 ± 10
n = 9	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.7 ± 0.7	4 ± 4 *
	rep20	1.7 ± 0.8	17 ± 19 *
	rep30	1.7 ± 0.8	20 ± 20 *
	rep40	1.7 ± 0.8	23 ± 19 *
	rep50	1.7 ± 0.8	25 ± 18 *
	rep60	1.7 ± 0.8	25 ± 18 *
TRα^0	baseline	1.8 ± 1.0	56 ± 23 #
n = 7	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.7 ± 0.8	40 ± 19 #
	rep20	1.7 ± 0.8	47 ± 17 #
	rep30	1.7 ± 0.8	51 ± 18 #
	rep40	1.7 ± 0.8	52 ± 16 #
	rep50	1.7 ± 0.7	54 ± 16 #
	rep60	1.8 ± 0.7	53 ± 13 #
TRα^{0}hyper	baseline	2.4 ± 0.4	96 ± 9
n = 5	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.9 ± 0.6	18 ± 20 *
	rep20	2.8 ± 0.5	53 ± 23 * #
	rep30	2.8 ± 0.4	61 ± 21 * #
	rep40	2.7 ± 0.4	63 ± 14 * #
	rep50	2.5 ± 0.4	64 ± 10 * #
	rep60	2.4 ± 0.4	64 ± 7 * #
TRα^{GS}	baseline	2.5 ± 1.5	90 ± 20
n = 8	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.9 ± 1.4	73 ± 25 #
	rep20	2.8 ± 1.4	82 ± 19 #
	rep30	2.7 ± 1.4	81 ± 19 #
	rep40	2.7 ± 1.4	80 ± 15 #
	rep50	2.6 ± 1.4	79 ± 14 #
	rep60	2.6 ± 1.3	76 ± 14 #

Phosphorylation of classical cardioprotective proteins in mice with altered TRα signalling

Classical cardioprotective proteins were investigated in TRα^0, TRα^{0}hyper and TRα^{GS} mouse hearts after IR injury (Fig. 29). Comparable to the protein phosphorylation pattern in hypothyroid hearts, no increase in expression was found for any of the investigated key cardioprotective proteins in TRα^0, TRα^{0}hyper and TRα^{GS} mouse hearts compared to controls (Fig. 29 A-E). In fact, phosphorylation of STAT3 (p-STAT3/total-STAT3) was decreased in TRα^0, TRα^{0}hyper and TRα^{GS} mouse hearts compared to controls (Fig. 29 C), similarly reflecting the STAT3 situation in hypothyroid mouse hearts. Additionally, there was a small, but not significant, decrease in the phosphorylation of AKT (Fig. 29 A), ERK (Fig. 29 B) and p38 (Fig. 29 D) in all transgenic groups compared to control mouse hearts.

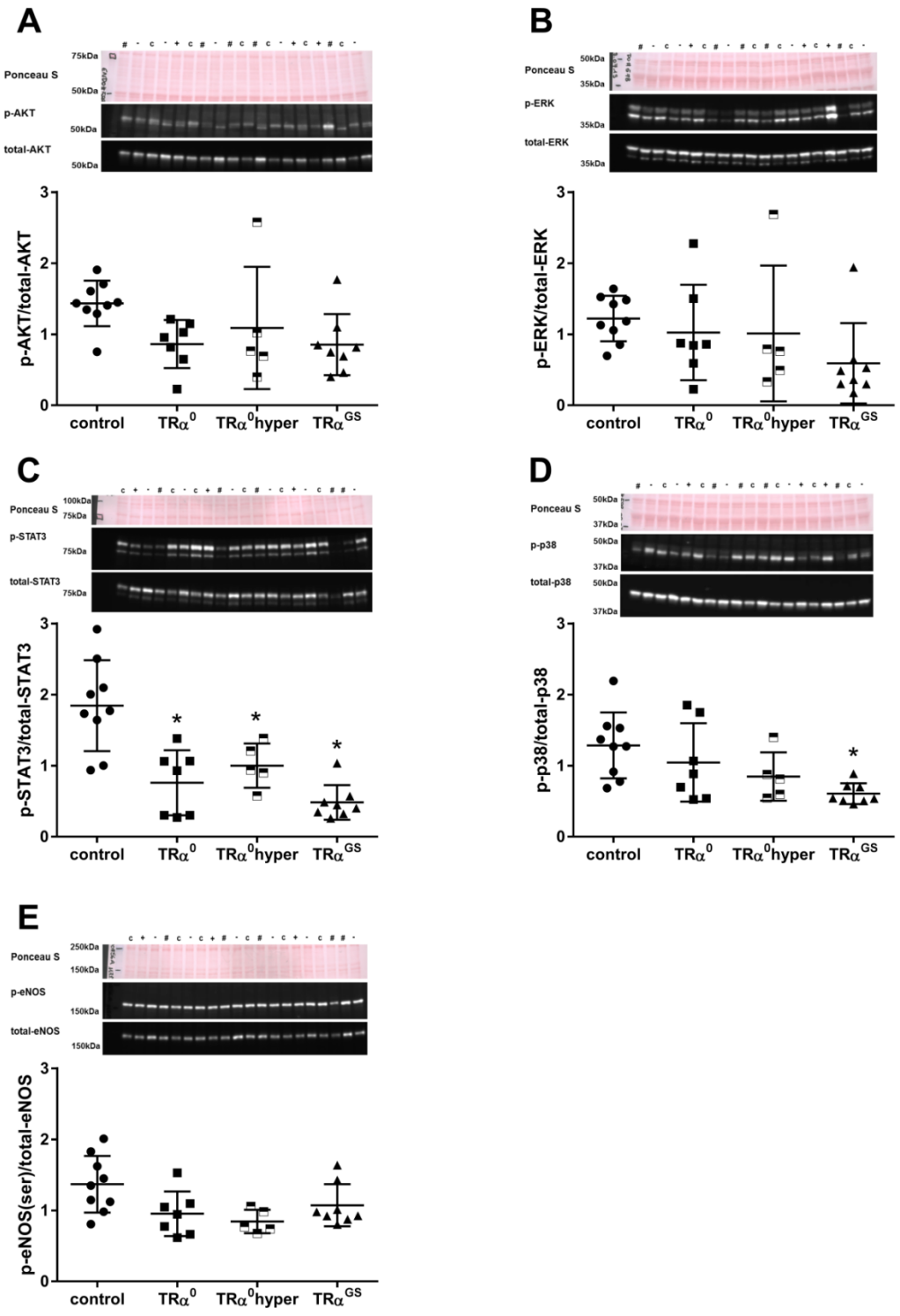

Figure 29: Phosphorylation of proteinkinase B (p-AKT/total-AKT) (A), extracellular-signal regulated kinases (p-ERK/total-ERK) (B), signal transducer and activator of transcription 3 (p-STAT3/total-STAT3) (C), p38-mitogen-activated proteinkinase (p-p38/total-p38) (D) and endothelial nitric oxide synthase (p-eNOS(ser)/total-eNOS) (E) after myocardial ischaemia/reperfusion in left ventricular tissue of transgenic mouse hearts. Top, middle, bottom: Membranes stained with Ponceau S, immunoreactivity signals for phosphorylated and total protein (**c**: control; **-** : TRα^0; + : TRα^{0}hyper; and # : TRα^{GS}). The phosphorylation of proteins in wildtype (control), TRα^0, TRα^{0}hyper and TRα^{GS} mouse hearts was normalized to the respective total protein. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

Impact of TRα signalling on cardiac gene expression

Since there was no increase in the phosphorylation of any of the investigated proteins that could explain infarct size reduction in mouse hearts lacking canonical TRα signalling (Fig. 29), transcriptome was investigated by measuring known TH responsive genes (Portman 2008; Kahaly and Dillmann 2005) in isolated transgenic mouse hearts after IR injury (Fig. 30). Previously, it has already been shown that $TR\alpha^0$ and $TR\alpha^{GS}$ mice display hypothyroid like gene expression patterns with a decrease in e.g. hyperpolarization activated cyclic nucleotide gated potassium channel 2 (*Hcn2)* (Hones et al. 2017), however cardiac gene expression has not yet been investigated in hyperthyroid $TR\alpha^0$ isolated mouse hearts. While myosin heavy chain 6 (*Myh6)* expression which has a function in cardiac muscle contraction showed a small, but not significant, increase in hearts of $TR\alpha^0$hyper compared to control mice, $TR\alpha^0$ and $TR\alpha^{GS}$ mouse hearts had comparable *Myh6* levels to controls (Fig. 30 A). *Myh7* transcript increased in $TR\alpha^0$ and $TR\alpha^{GS}$ mouse hearts compared to controls, whereas $TR\alpha^0$hyper remained unaltered in comparison to control group (Fig. 30 B). In $TR\alpha^0$hyper mouse hearts, expression of *Hcn2* increased in comparison to controls, while expression of *Hcn2* was marginally, but not significantly, decreased in $TR\alpha^0$ and $TR\alpha^{GS}$ compared to controls (Fig. 30 C). No differences were found for *Hcn4* in any of these groups (Fig. 30 D). Expression of sarcoplasmic reticulum calcium ATPase *(Atp2a2)* was increased in $TR\alpha^0$hyper isolated mouse hearts compared to control group (Fig. 30 E).

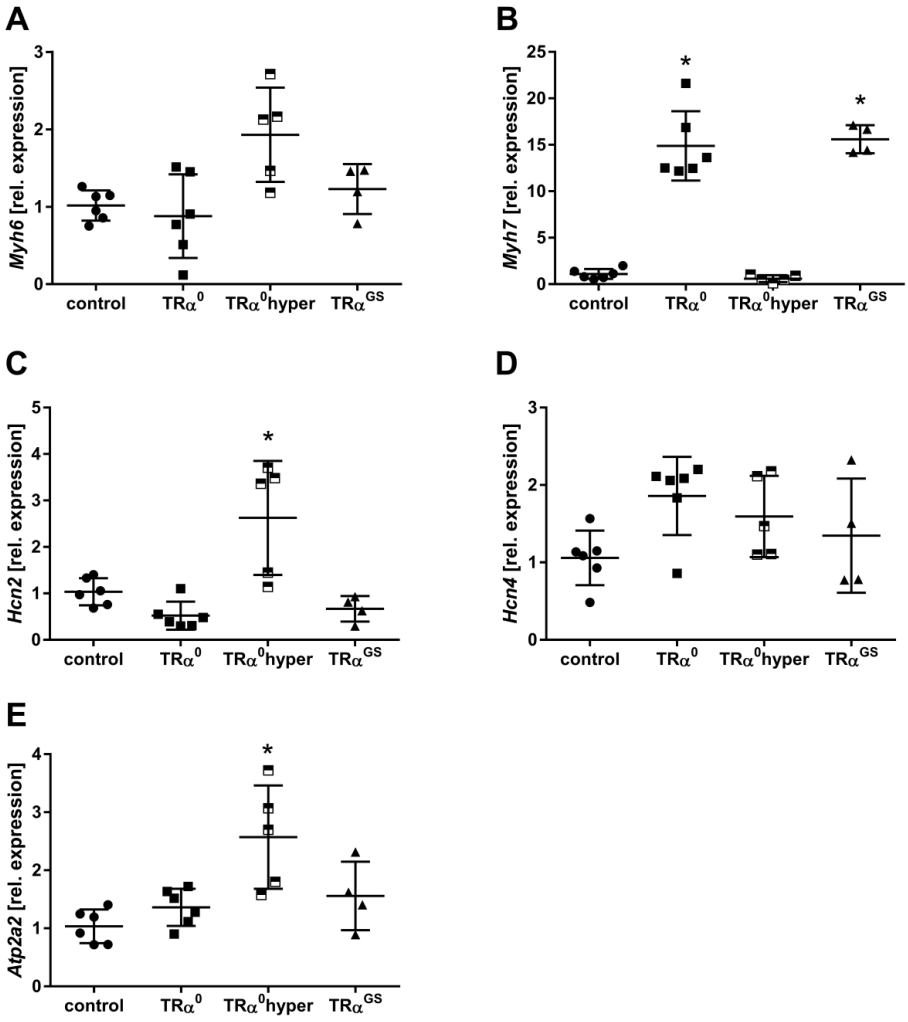

Figure 30: Impact of TRα signalling on TH target gene expression in left ventricular tissue of isolated mouse hearts after ischaemia/reperfusion injury. Relative (rel.) expression of myosin heavy chain 6 (*Myh6)* (A)*, Myh7* (B)*,* hyperpolarization activated cyclic nucleotide gated potassium channel 2 *(Hcn2)* (C), *Hcn4* (D) and sarcoplasmic reticulum calcium-ATPase 2 (SERCA2) *(Atp2a2)* (E) in wildtype (control), $TR\alpha^0$, hyperthyroid $TR\alpha^0$ ($TR\alpha^0$hyper) and $TR\alpha^{GS}$ isolated mouse hearts. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

In summary, the lack of canonical TRα signalling, which is absent in $TR\alpha^0$ and $TR\alpha^{GS}$ mice, resulted in a decreased heart rate, comparable to the hypothyroid situation. Baseline LVDP was decreased in $TR\alpha^0$ mouse hearts in line with hypothyroid state, while LVDP was comparable between $TR\alpha^0$hyper, $TR\alpha^{GS}$ and control hearts. Infarct size was reduced in $TR\alpha^0$, $TR\alpha^0$hyper and $TR\alpha^{GS}$ mouse hearts. Thus, lack of canonical TRα signalling reduced infarct size similar to the observed reduction under hypothyroidism. Recovery of LVDP was also improved in these hearts. While phosphorylation of STAT3 decreased in $TR\alpha^0$, $TR\alpha^0$hyper and $TR\alpha^{GS}$ mouse hearts, cardiac expression of *Myh7* increased in $TR\alpha^0$ and $TR\alpha^{GS}$ mice and *Hcn2* and *Atp2a2* increased in $TR\alpha^0$hyper group.

Effects of T3 pre- and postconditioning on myocardial ischaemia/reperfusion injury in isolated mouse hearts

In experimental AMI a low TH tissue state caused by upregulation of cardiac DIO3 resulting in reduced T3 availability in the myocardium has been reported (Janssen et al. 2016). This has long been viewed as an adaptive, energy-preserving effect in the heart (Galli, Pingitore, and Iervasi 2010). However, the contrary may be true, with some clinical and experimental data suggesting that TH intervention in AMI may in fact improve outcome (Gerdes and Iervasi 2010; Jabbar et al. 2017; Pingitore et al. 2019). A number of studies, mostly in rats, suggested that T3 administration may reduce IR injury (Nicolini et al. 2016; Zeng et al. 2019; Forini et al. 2018; Sabatino et al. 2016; da Silva et al. 2018; Bi et al. 2019) and could improve functional recovery post MI (Pantos et al. 2007; Pantos et al. 2009; Bi et al. 2019). However, the precise role of acute TH intervention on myocardial infarct size, the most robust endpoint of cardioprotection in preclinical studies (Botker et al. 2018), still requires clarification, in particular regarding the causally involved molecular pathways and cellular origin (Jabbar et al. 2017; Cappola et al. 2019). Therefore, the role of T3 when given in pre- (before ischaemia) and postconditioning (after ischaemia) mode on myocardial infarct size was addressed.

Impact of acute T3 delivery on baseline left ventricular function

We asked, whether acute T3 administration influences baseline myocardial functional performance of isolated mouse hearts *per se*. For this, baseline CF and LVDP were compared between controls and T3 perfused groups with increasing T3 dosages. There were no differences in CF (Fig. 31 A) and LVDP (Fig. 31 B) between control and T3 perfused isolated mouse hearts at baseline. Taken together, acute T3 administration had no impact on baseline left ventricular function.

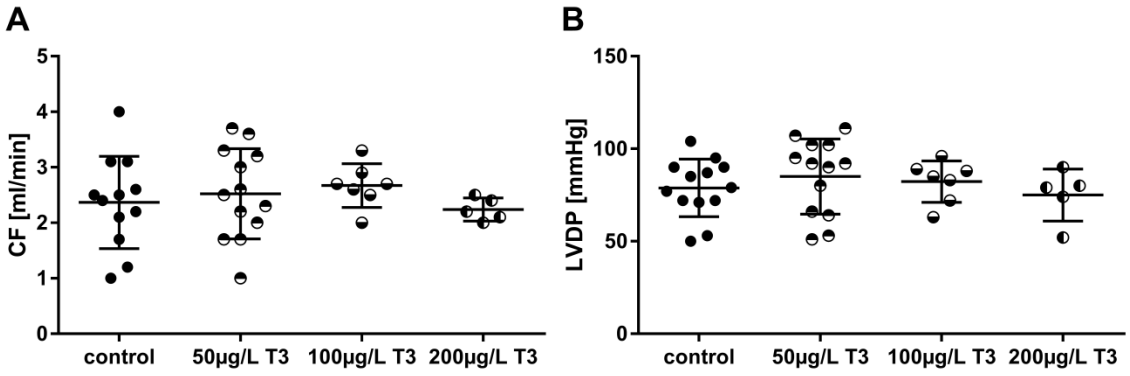

Figure 31: Baseline coronary flow (CF) (A) and left ventricular developed pressure (LVDP) (B) of isolated, pressure constant perfused mouse hearts with acute T3 delivery. CF in [ml/min] (A) and LVDP [mmHg] (B) of wildtype mice in absence (control) and in presence of T3 in different concentrations (50 µg/L T3, 100 µg/L T3, 200 µg/L T3), respectively. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction.

Impact of acute T3 delivery on myocardial infarct size

After 30/120 global IR injury, infarct size of KHB perfused control hearts was 52 ± 7% of ventricular mass (Fig. 32). Administration of 50 µg/L T3 from baseline until end of reperfusion decreased infarct size to 42 ± 9% of ventricular mass, while addition of 100 µg/L and 200 µg/L T3 led to a further decrease in infarct size to 31 ± 11 and 24 ± 3% of ventricular mass, respectively (Fig. 32). Thus, there was a dose-dependent infarct size reduction under acute T3 administration.

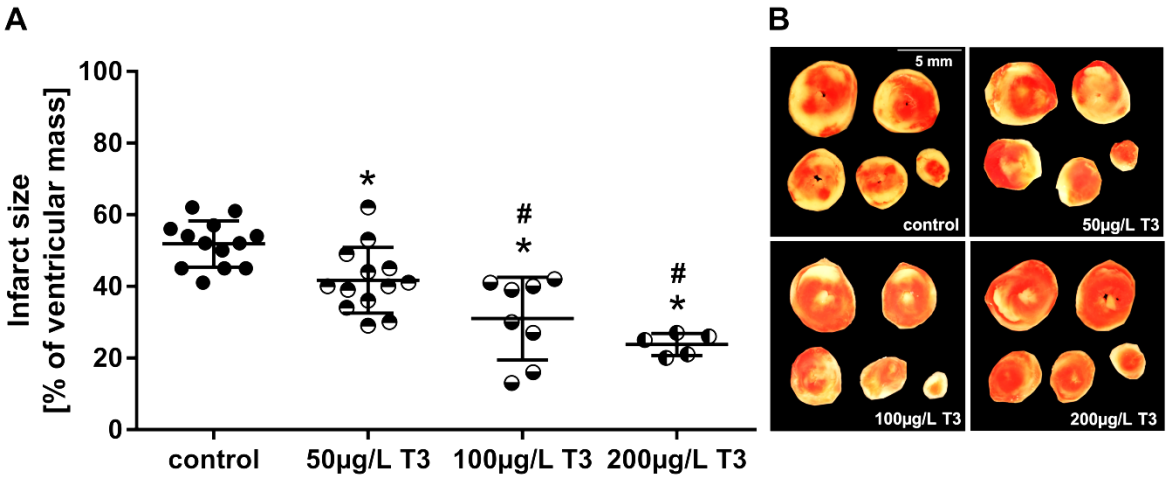

Figure 32: Infarct size of isolated pressure constant perfused mouse hearts with acute T3 delivery. Infarct size in [% of ventricular mass] of wildtype mice in absence (control) and in presence of T3 in different concentrations (50 µg/L T3, 100 µg/L T3, 200 µg/L T3) (A) and representative heart slices after TTC staining, respectively (B); Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control; # $P < 0.05$ vs. 50 µg/L T3.

Recovery of left ventricular function in mouse hearts with acute T3 administration

CF during reperfusion was consistently increased in hearts perfused with 100 µg/L T3 compared to controls (Table 12). LVDP during reperfusion showed a small increase in all T3 perfused hearts compared to controls, while the increase was significant at time point rep30 and rep40 in 100 µg/L T3 group only (Table 12). CF during reperfusion decreased compared to baseline in controls, while it increased in hearts perfused with 100 µg/L T3 and 200 µg/L T3 (Table 12). Taken together, T3 improved recovery of CF and LVDP.

Table 12: Functional recovery of coronary flow and left ventricular developed pressure in isolated wildtype mouse hearts in absence (control) or in presence of T3 in different concentrations from baseline (50 µg/L T3, 100 µg/L T3, 200 µg/L T3) and at reperfusion (200 µg/L T3 at rep). Mean coronary flow (CF) and mean left ventricular developed pressure (LVDP) of mouse hearts CF_{mean} and $LVDP_{mean}$ were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion; n=5-13; Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; # $P < 0.05$ vs. control; * $P < 0.05$ vs. baseline.

Protocol	Time	CF [ml/min]	LVDP [mmHg]
control	baseline	2.1 ± 1.0	81 ± 16
n = 13	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.9 ± 1.0	10 ± 16 *
	rep20	1.9 ± 1.0 *	24 ± 24 *
	rep30	1.8 ± 1.0 *	30 ± 23 *
	rep40	1.8 ± 1.0 *	34 ± 22 *
	rep50	1.7 ± 1.0 *	36 ± 20 *
	rep60	1.7 ± 1.0 *	36 ± 19 *
50 µg/L T3	baseline	2.5 ± 0.8	85 ± 20
n = 13	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.5 ± 1.0	18 ± 23 *
	rep20	2.5 ± 1.0	37 ± 30 *
	rep30	2.5 ± 1.0	48 ± 27 *
	rep40	2.4 ± 1.0	50 ± 20 *
	rep50	2.3 ± 1.0	51 ± 18 *
	rep60	2.4 ± 1.0	47 ± 16 *
100 µg/L T3	baseline	2.6 ± 0.4	82 ± 11
n = 8	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	3.6 ± 1.1 * #	22 ± 31 *
	rep20	3.4 ± 0.8 * #	46 ± 20 *
	rep30	3.4 ± 0.8 * #	57 ± 17 * #
	rep40	3.2 ± 0.7 * #	58 ± 15 * #
	rep50	3.1 ± 0.7 * #	57 ± 15 *
	rep60	3.0 ± 0.6 * #	56 ± 14 *
200 µg/L T3	baseline	2.2 ± 0.2	81 ± 6
n = 5	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	3.0 ± 0.2 *	10 ± 7 *
	rep20	2.9 ± 0.1 *	31 ± 10 *
	rep30	2.9 ± 0.1 *	48 ± 5 *
	rep40	2.8 ± 0.2 *	54 ± 6 *
	rep50	2.6 ± 0.2	56 ± 9 *
	rep60	2.5 ± 0.2	55 ± 9 *
200 µg/LT3 at rep	baseline	2.7 ± 0.6	88 ± 14
n = 9	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	3.6 ± 1.2 *	20 ± 24 *
	rep20	3.5 ± 1.3 *	46 ± 27 *
	rep30	3.5 ± 1.3 *	55 ± 25 *
	rep40	3.4 ± 1.2 *	61 ± 21 *
	rep50	3.3 ± 1.2 *	63 ±18 *
	rep60	3.3 ± 1.2 *	63 ± 16 *

Impact of T3 pre- and postconditioning mode on IR injury

Next, we asked whether administration of T3 before (T3 preconditioning) or after ischaemia (T3 postconditioning) has an effect on infarct size. For this purpose, the most efficient T3 concentration from the dose response curve where T3 was given from baseline was used (200 µg/L T3) (Fig. 32). There was no significant difference in infarct size detectable, whether T3 was given in pre- or post-conditioning mode (Fig. 33). The recovery of CF and LVDP was also comparable between pre- and post-conditioning mode (Table 12).

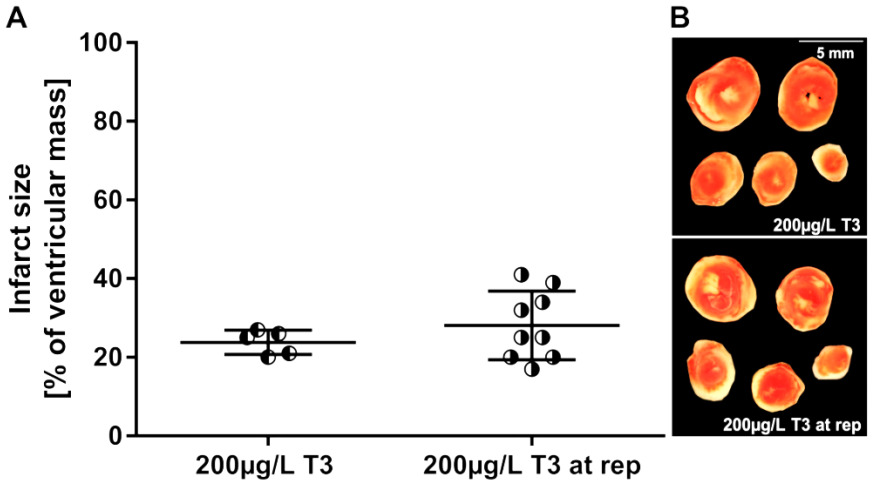

Figure 33: Infarct size of isolated pressure constant perfused mouse hearts with acute T3 delivery at baseline or at reperfusion. Infarct size in [% of ventricular mass] of wildtype mice in presence of 200 µg/L T3 at baseline (200 µg/L T3) and in presence of 200 µg/L T3 at reperfusion (200 µg/L T3 at rep) (A) and representative heart slices after TTC staining, respectively (B); Data are means ± standard deviations; Unpaired t-test.

Phosphorylation of classical cardioprotective proteins in mouse hearts with acute T3 delivery

To investigate the involvement of classical cardioprotective pathways on infarct size reduction by acute T3 administration, expression and phosphorylation of key proteins was compared between the groups (Fig. 34). Protein phosphorylation of AKT, ERK, STAT3, $eNOS_{ser1177}$ and p38 did not differ significantly between controls and T3 perfused groups, however a consistent, but not significant, decrease of these proteins in groups treated with increasing concentrations of T3 compared to controls was noted (Fig. 34 A-E). In contrast, $eNOS_{thr495}$ phosphorylation, which is proposed to inactivate eNOS (Forstermann and Sessa 2012), was strongly increased in hearts treated with 100 µg/L and 200 µg/L T3 compared to controls (Fig. 34 F).

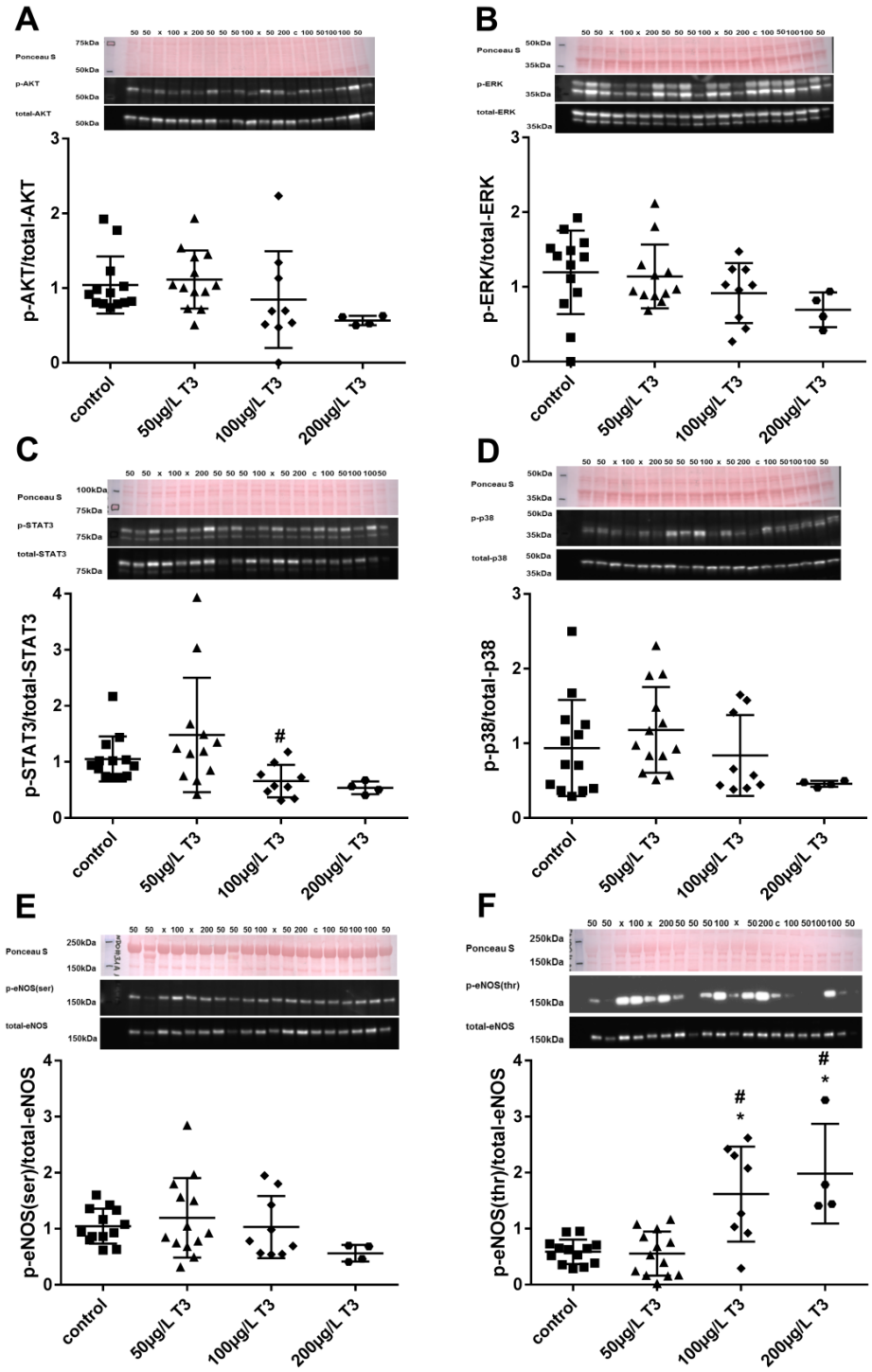

Figure 34: Phosphorylation of protein kinase B (p-AKT/total-AKT) (A), extracellular-signal regulated kinases (p-ERK/total-ERK) (B), signal transducer and activator of transcription 3 (p-STAT3/total-STAT3) (C), p38-mitogen-activated protein kinase (p-p38/total-p38) (D) and endothelial nitric oxide synthase (p-eNOS(ser)/total-eNOS) (E) and p-eNOS(thr)/total-eNOS) (F) after myocardial ischaemia/reperfusion in left ventricular tissue of isolated mouse hearts in absence or in presence of T3 in different concentrations (50 µg/L T3, 100 µg/L T3, 200 µg/L T3). Top, middle, bottom: Membranes stained with Ponceau S, immunoreactivity signals for phosphorylated and total protein (**c**: control; **50**: 50 µg/L T3; **100**: 100 µg/L T3; **200**: 200 µg/L T3; traces marked with an **x** are not part of the present data set). The phosphorylation of proteins was normalized to the respective total protein expression. Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control; # $P < 0.05$ vs. 50 µg/L T3.

Impact of tetrahydrobiopterin on ischaemia/reperfusion injury and protein phosphorylation

Based on the finding of an increased $eNOS_{thr495}$ phosphorylation (Fig. 34 F), we hypothesized that acute T3 administration in IR injury might increase eNOS uncoupling (Forstermann and Sessa 2012). To test this hypothesis, we added 10 mg/L tetrahydrobiopterin (BH_4), an essential cofactor of eNOS-synthesis, to the perfusion buffer (Forstermann and Sessa 2012). As shown in Fig. 33, 200 µg/L T3 at reperfusion decreased infarct size compared to controls (Fig. 35). The sole administration of BH_4 in perfusion buffer resulted in a marginal, but not significant, increase in infarct size compared to KHB perfused controls (Fig. 35). The combination of 200 µg/L T3 at rep and BH_4 (200 µg/L T3 at rep + BH_4) increased infarct size back to the level of controls (Fig. 35), suggesting that BH_4 antagonized infarct size reduction by acute T3.

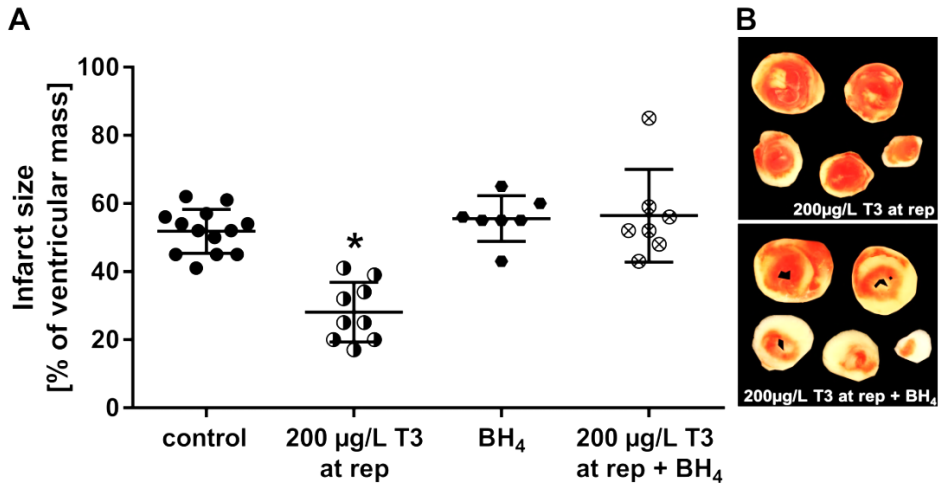

Figure 35: Infarct size of isolated pressure constant perfused mouse hearts after acute T3 delivery at reperfusion and in presence of tetrahydrobiopterin. Infarct size in [% of ventricular mass] of wildtype mouse hearts in absence (control) and in presence of 200 µg/L T3 at reperfusion (200 µg/L T3 at rep), in presence of 10 mg/L tetrahydrobiopterin (BH_4) and in presence of 200 µg/L T3 at reperfusion + 10 mg/L BH_4 (200 µg/L T3 at rep + BH_4) (A), and representative heart slices after TTC staining of 200 µg/L T3 at rep and 200 µg/L T3 at rep + BH_4, respectively (B); Data are means ± standard deviations; One-way ANOVA with Bonferroni's correction; * $P < 0.05$ vs. control.

CF at baseline in mouse hearts perfused with BH_4 and T3 + BH_4 was increased in comparison to hearts perfused with T3 only, while LVDP was comparable (Table 13). The recovery of LVDP after ischaemia decreased in hearts perfused with BH_4 and T3 + BH_4 compared to hearts perfused with 200 µg/L T3 at rep only (Table 13), suitably reflecting enlarged infarct size under BH_4 addition (Fig. 35). The recovery of CF after ischaemia did not differ between the groups (Table 13). LVDP during reperfusion was consistently decreased in all groups

compared to the baseline situation (Table 13). Taken together, BH_4 addition decreased functional recovery of LVDP after ischaemia.

Table 13: Functional recovery of coronary flow and left ventricular developed pressure in isolated wildtype mouse hearts in absence vs. in presence of 200 µg/L T3 at reperfusion (200 µg/L T3 at rep), tetrahydrobiopterin (BH_4) and T3 + BH_4 (200 µg/L T3 at rep + BH_4). Mean coronary flow (CF) and mean left ventricular developed pressure (LVDP) of mouse hearts CF_{mean} and $LVDP_{mean}$ were analysed at different time points: at baseline, at 5/25 ischaemia and at 10/20/30/40/50/60 reperfusion; n=5-9; Data are means ± standard deviations; Two-way ANOVA for repeated measures with Bonferroni's correction; # $P < 0.05$ vs. 200 µg/L; * $P < 0.05$ vs. baseline.

Protocol	Time	CF [ml/min]	LVDP [mmHg]
control	baseline	2.1 ± 1.0	81 ± 16
n = 13	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	1.9 ± 1.0	10 ± 16 *
	rep20	1.9 ± 1.0 *	24 ± 24 *
	rep30	1.8 ± 1.0 *	30 ± 23 *
	rep40	1.8 ± 1.0 *	34 ± 22 *
	rep50	1.7 ± 1.0 *	36 ± 20 *
	rep60	1.7 ± 1.0 *	36 ± 19 *
200 µg/L T3 at rep	baseline	2.7 ± 0.6	88 ± 14
n = 9	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	3.6 ± 1.2 *	20 ± 24 *
	rep20	3.5 ± 1.3 *	46 ± 27 *
	rep30	3.5 ± 1.3 *	55 ± 25 *
	rep40	3.4 ± 1.2 *	61 ± 21 *
	rep50	3.3 ± 1.2 *	63 ±18 *
	rep60	3.3 ± 1.2 *	63 ± 16 *
BH_4	baseline	3.5 ± 0.7 #	92 ± 17
n = 7	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	2.8 ± 0.9	1 ± 1 *
	rep20	2.7 ± 0.7	3 ± 3 * #
	rep30	2.8 ± 0.7	10 ± 9 * #
	rep40	2.9 ± 0.7	15 ± 11 * #
	rep50	2.9 ± 0.7	18 ± 12 * #
	rep60	2.9 ± 0.7	20 ± 12 * #
200 µg/L T3 at rep + BH_4	baseline	3.5 ± 0.6 #	93 ± 9
n = 7	isch5	0.0 ± 0.0 *	0 ± 0 *
	isch25	0.0 ± 0.0 *	0 ± 0 *
	rep10	3.0 ± 0.9	1 ± 1 *
	rep20	3.0 ± 0.9	11 ± 14 * #
	rep30	3.1 ± 0.9	19 ± 19 * #
	rep40	3.2 ± 0.9	22 ± 20 * #
	rep50	3.2 ± 0.9	25 ± 21 * #
	rep60	3.2 ± 0.9	28 ± 22 * #

On the protein level, phosphorylation of $eNOS_{thr}$ decreased in T3 + BH_4 perfused mouse hearts in comparison to mouse hearts perfused with 200 µg/L T3 at rep only (Fig. 36).

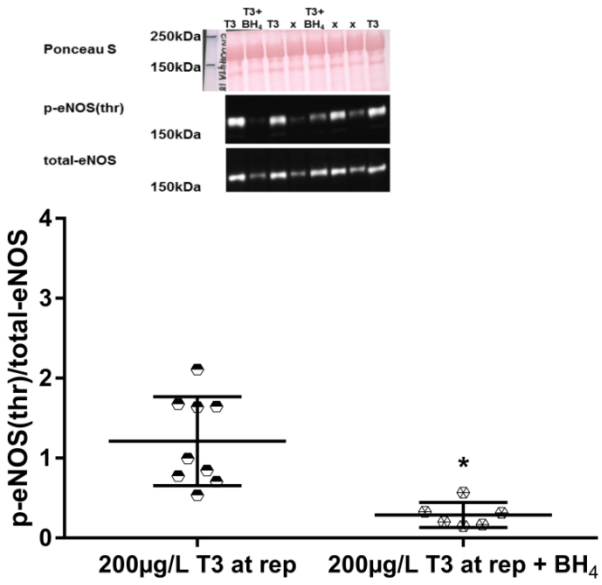

Figure 36: Threonine$_{495}$-phosphorylation normalized to total expression of endothelial nitric oxide synthase (p-eNOS(thr)/total-eNOS) of isolated hearts after ischaemia/reperfusion injury in presence of 200 µg/L T3 at reperfusion (200 µg/L T3 at rep) vs. T3 + tetrahydrobiopterin (BH_4) perfusion (200 µg/L T3 at rep + BH_4). Top, middle, bottom: Membrane stained with Ponceau S, immunoreactivity signals for phosphorylated and total protein (**T3**: 200 µg/L T3 at rep; **T3 + BH_4**: 200 µg/L T3 at rep + BH_4; traces marked with an **x** are not part of the present data set). Data are means ± standard deviations; Unpaired t-test; * $P < 0.05$ vs. 200 µg/L T3 at rep.

In summary, acute T3 administration did not affect baseline left ventricular function. However, in acute ischaemia T3 reduced infarct size dose-dependently and improved recovery of LVDP after IR injury. Importantly, infarct size was also reduced when T3 was given after ischaemia. Therefore, infarct size was reduced by T3 in a pre- and postconditioning mode. Furthermore, T3 administration increased phosphorylation of $eNOS_{thr}$ (p-eNOS(thr)/total-eNOS). Thus, BH_4 addition to T3 postconditioned hearts reversed infarct size reduction, functional performance and also $eNOS_{thr}$ phosphorylation.

Discussion

In order to clarify the impact of chronic TH action on myocardial infarct size in a young as well as ageing organism, male C57BL/6 mice aged 3 and 20 months were rendered hypo- and hyperthyroid. To address the role of TRα signalling during IR injury, mouse models with altered TRα signalling were used and compared to wildtype littermates. Hearts of mice were isolated and pressure constant perfused according to Langendorff (Langendorff 1895) in order to determine the impact of circulating TH concentrations, age and TRα signalling on cardiac functional parameters such as heart rate, CF, LVP as well as infarct size after global IR. Since the isolated heart method works well to investigate direct pharmacological effects on the heart (Watanabe and Okada 2018), hearts of C57BL/6 mice were isolated and examined during IR protocol under administration of T3 in different doses and time points.
Thus, this study allowed investigations about the impact of chronic vs. acute TH action on myocardial infarct size in mice including hemodynamic parameters and key cardioprotective signal transduction cascades.

Thyroid dysfunction inversely affects myocardial infarct size

Under thyroid dysfunction TH action in the heart is changed at the transcriptome but also at the functional level (Dillmann 1990). However, the impact of thyroid dysfunction on the outcome after cardiovascular events such as MI is still not fully understood (Jabbar et al. 2017; Razvi et al. 2018). Previous data from animal models presented ambivalent findings on whether THs are protective or detrimental in myocardial IR injury (Seara et al. 2018; Pantos et al. 2006; Venditti, Agnisola, and Di Meo 2002). In addition, underlying mechanisms remained elusive (Cappola et al. 2019). Thus, in the first part of this thesis effects of chronic hypo- and hyperthyroidism on myocardial infarct size were investigated in 3 months old isolated pressure constant perfused mouse hearts with special attention to generate comparable experimental and hemodynamic conditions, since it is known that they may influence IR injury outcome *per se* (Botker et al. 2018; Heusch 2017). Molecularly, classical cardioprotective signalling pathways (Heusch 2015) were investigated.

Brady- and tachycardia induced by thyroid dysfunction are heart intrinsic effects

Ex vivo heart rate was assessed prior to IR and resulted in bradycardia due to hypothyroidism and tachycardia due to hyperthyroidism in the isolated heart in our study, which is in line with decreased and increased heart rate in hypo- and hyperthyroid patients, respectively (Chaker et al. 2017; De Leo, Lee, and Braverman 2016). Non-invasive electrocardiography measurements of our group also displayed increased and decreased heart rate in 5 months old

male and female mice under hyperthyroidism and hypothyroidism, respectively (Rakov et al. 2016). However, these *in vivo* measurements did not allow a statement about intrinsic effects of the heart because extrinsic factors such as the autonomous nervous system may also play a role and can only be excluded in isolated heart measurements (Lindsey et al. 2018). Decreased *ex vivo* heart rate was reported in myosin heavy chain (MHC) mutant mice displaying a hypothyroid like cardiac phenotype with increased *Myh7* transcript expression (Pazos-Moura et al. 2000) and was confirmed in our study in mice with hypothyroidism. This suggests that brady- and tachycardia induced by thyroid dysfunction are heart-intrinsic. To exclude heart rate dependency on myocardial infarct size, all hearts were paced equally to the same heart rate of 500 bpm after 10 min of baseline to generate comparable experimental situations.

Serum TH status determines myocardial hemodynamic performance

We could show that baseline LVDP and CF decreased in hypothyroid isolated mouse hearts compared to controls. Hypothyroidism also resulted in reduced left ventricular contractility in hypothyroid rats compared to controls (Shao et al. 2016). In contrast, increased CF and LVDP at baseline was found in our hyperthyroid mouse hearts. This is consistent with an increase in cardiac contractility (Seara et al. 2018) and cardiac hypertrophy (Lino, Demasi, and Barreto-Chaves 2019) after TH treatment. Taken together, serum TH status was correlated with CF and LVDP at baseline in isolated perfused mouse hearts.

Chronic hypothyroidism is cardioprotective in absence of favorable hemodynamics

In the Langendorff model of global IR injury, myocardial infarct size is regarded as a robust endpoint in preclinical studies on cardioprotection (Botker et al. 2018; Lindsey et al. 2018). In our study, chronic hypothyroidism drastically reduced heart infarct size. Recently, infarct size reduction has also been shown in hypothyroid isolated rat hearts, however in this study heart rate was not matched by atrial electrical stimulation (Seara et al. 2018). Since heart rate was matched during IR injury in our study, we suggest that cardioprotection by hypothyroidism occurs irrespective of heart rate during IR. To demonstrate that the “phenomenon of cardioplegia” (describing the resting and thus saving mode of a heart) (Santer et al. 2019; Heusch 2017), expressed in reduced baseline LVDP in hypothyroid hearts, is not causally involved in reduced infarct size and improved functional recovery, baseline LVP of hypothyroid mouse hearts was matched to level of controls by adding catecholamines, in form of epinephrine, and infarct size and functional performance were measured under these conditions. In 1998 it has already been shown, that catecholamine levels are decreased in

hypothyroid myocardium (Mano et al. 1998), suggesting that decreased catecholamine levels may contribute to decreased LVP in hypothyroid hearts. Since catecholamines are known to increase cardiac contractility (Furnival, Linden, and Snow 1971), addition of epinephrine resulted in an increased baseline CF and LVDP in our hypothyroid hearts but remarkably this had no impact on myocardial infarct size. Thus, we could demonstrate for the first time that cardioprotection by hypothyroidism is not a result of a favorable hemodynamic situation during IR *per se*.

Hyperthyroidism confers additional damage in IR injury

In contrast, preceding hyperthyroidism increased heart infarct size in our study. Using a rat heart model, Seara and colleagues did not observe a difference in infarct size between hyperthyroid and control rats (Seara et al. 2018) and other studies even reported a cardioprotective phenotype under hyperthyroidism with an improved recovery of LVDP (da Silva et al. 2018; Pantos et al. 2006). However, da Silva and colleagues did not observe a significant increase of serum T3 in their "hyperthyroid" mice, although they were intraperitoneally injected with T3 for 14 days (da Silva et al. 2018) and infarct size was not determined. Taken together, the results of our study reflect the increased risk for cardiovascular events and cardiac mortality under hyperthyroidism in epidemiological studies (Jabbar et al. 2017; Selmer et al. 2014).

Functional recovery of LVDP is correlated with myocardial infarct size

In our study and in line with previous reports in rat heart (Seara et al. 2018; Pantos, Malliopoulou, Mourouzis, et al. 2003), hypothyroidism preserved LVDP after ischaemia in mice. In contrast, hyperthyroidism strongly decreased functional recovery of LVDP compared to baseline, which is consistent with an impaired cardiac functional recovery under hyperthyroidism previously reported in rats (Venditti, Agnisola, and Di Meo 2002).

Possible underlying mechanisms for cardioprotection under hypothyroidism

To address signalling pathways that could be involved in the observed effects of chronic thyroid dysfunction on IR, key proteins of the *RISK*, *SAFE* and *NO/PKG* pathways, that were previously identified in association with cardioprotection (Heusch 2015), were investigated. The *RISK* pathway does not seem to be involved, as there were no differences between the two treatment groups (hypo and hyper) and the control group in the phosphorylation of AKT and ERK after IR. Interestingly, phosphorylation of STAT3 was downregulated in hypothyroid hearts and this was a consistent finding throughout all experiments on TH action

in this thesis. However, according to previous findings, an increased phosphorylation of STAT3 is associated with cardioprotection after IR injury (Kleinbongard et al. 2018; Zhang et al. 2017; Lieder et al. 2018; Boengler et al. 2008). To verify whether decreased STAT3 phosphorylation is causally involved in cardioprotection, the addition of a STAT3 activator would be the method of choice to see if infarct size reverses. However, it is unlikely that decrease in STAT3 activation is causally involved in infarct size reduction by hypothyroidism, since in a cardiomyocyte-specific STAT3 knockout mouse model a comparable infarct size with wildtype littermates was reported (Boengler et al. 2010). It is more likely that the decrease in STAT3 phosphorylation is associated with the chronic situation of TH deprivation, since physiological levels of THs have been shown to promote phosphorylation of STAT3 *in vitro* (Lin et al. 1999).

However, very recent data explained the higher tolerance to IR injury under hypothyroidism by an increased stoichiometric ratio of two mitochondrial uniplex subunits and the threshold to cytosolic Ca^{2+}, suggesting a reduced Ca^{2+} content in mitochondria which could result in more tolerance to Ca^{2+} overload and thus delaying mPTP opening and mitochondrial dysfunction (Chapoy-Villanueva et al. 2019). This is in line with rodent studies of hepatic mitochondria, where hypothyroidism decreased the respiratory rate compared to controls (Silvestri et al. 2018). Irrespective of respiratory substrates, both, the exogenous H_2O_2 release and removal rate decreased in hypothyroid rat hearts compared to controls. These findings suggest a lower susceptibility to oxidants (Venditti et al. 2019). Therefore, there is evidence for a cardiomyocyte effect and that mitochondria are the target organelles in cardioprotection by hypothyroidism. Ultimately, to prove this, measurement of myocardial mitochondrial respiration would be necessary to show that mitochondrial ADP stimulated respiration and consequent ATP production is still preserved after IR injury in hypothyroid mouse hearts. In fact, this has been indicated in our hypothyroid mice whose cardiac mitochondria have been isolated after global IR and measured in preliminary experiments at the Institute for Pathophysiology (medical doctoral thesis of Merlin Stroetges).

Thyroid hormone effects are preserved through ageing

The risk for both, thyroid disorders and heart disease increases with age (De Leo, Lee, and Braverman 2016; Sawin et al. 1994; Chaker et al. 2017). However, experimental set-ups often lack the aspect of ageing. Previous studies of our group have addressed this and suggest a reduced activity of the aged thyroid gland that may be responsible for the systemic low TH state in old mice and that TH tissue response is altered in an aged organism (Rakov et al. 2019). Thus, the age-dependent influence of experimental hypo- and hyperthyroidism on myocardial infarct size was investigated in 20 months old isolated pressure constant perfused mouse hearts with a focus on infarct size, assessment of functional parameters and cardioprotective pathways.

Brady- and tachycardia induced by thyroid dysfunction persist in aged mice

Although TH serum concentrations decreased to a smaller degree in LoI/MMI/ClO_4^- treated 20 months old mice compared to 3 months old mice, which might be due to higher iodine content in older thyroids, bradycardia was equally pronounced in 3 and 20 months old hypothyroid isolated mouse hearts. Thus, serum TH might not exactly reflect organ specific TH action or indicate an age-dependent TH demand of the heart (Kerp, Gassen, and Fuhrer 2020). T4 treatment also increased TH concentrations to a smaller degree in old compared to young mice, which might have been a result of age-dependent changes in DIO activity (Barbesino 2018; Visser et al. 2016). Nevertheless, tachycardia was found in young and aged mice. These findings confirm that brady- and tachycardia induced by thyroid dysfunction are not only heart-intrinsic (Klein and Danzi 2007) but also age-independent effects.

Serum TH status determines myocardial hemodynamic performance in aged mice

The decrease in baseline LVDP and CF under hypothyroidism was less pronounced in aged compared to young isolated mouse hearts. Similarly, increase of CF and LVDP under hyperthyroidism was also less pronounced in the aged compared to the young organism. This reflects the age variation in TH serum concentrations detected in 20 months old compared to 3 months old mice.

The cardioprotective effect of hypothyroidism is age-independent

Experimental hypothyroidism reduced myocardial infarct size after IR in 3 months old as well as in 20 months old isolated mouse hearts to a similar extent, suggesting that cardioprotection under hypothyroidism is an age-independent effect. Under hyperthyroidism infarct size increased in younger mice only. However, this is inconsistent to clinical data reporting an

increased cardiovascular mortality in older people (De Leo, Lee, and Braverman 2016; Sawin et al. 1994). Possibly, investigated mice with 20 months of age were not old enough to represent the pathophysiological situation in old hyperthyroid human hearts. Furthermore, while risk factors such as atherosclerosis rise in older patients (Warren et al. 2017), the C57BL/6J mouse model is not susceptible to develop atherosclerosis and hence lacks the human equivalent of coronary heart disease (Mancini et al. 1965; Paigen et al. 1985).

Recovery of LVDP is pronounced in hypothyroid hearts of aged mice

Interestingly, LVDP recovered age-dependently after IR, as hypothyroidism preserved LVDP after ischaemia in young mice, but even better in aged mice, suggesting that hypothyroidism is not detrimental in the elderly, also in view of the increased cardiovascular risk in old age *per se* (Selmer et al. 2014). In contrast, hyperthyroidism decreased functional recovery of LVDP in young and aged mice, which is consistent with an impaired recovery of LVDP in hyperthyroid rats (Venditti, Agnisola, and Di Meo 2002) and the clinical scenario that hyperthyroidism increases cardiovascular morbidity (Selmer et al. 2014).

In summary, it could be shown that although ageing in mice that were rendered hypothyroid was associated with less pronounced decrease in TH serum concentrations, this resulted in a similar degree of bradycardia, decrease of myocardial infarct size and increase in functional recovery of LVDP after ischaemia compared to 3 months old mouse hearts. Hyperthyroidism resulted in a smaller increase of TH serum concentrations with a similar degree in tachycardia, increased infarct size in younger hearts only and decreased functional recovery in both ages. Congruent to protein data in young mouse hearts, no increased activation of cardioprotective proteins, but a significant decrease in the phosphorylation of STAT3 was noted in aged hypothyroid mice. Additionally, p38 showed a small, but not significant, decrease in aged hypothyroid mouse hearts and a small increase in aged hyperthyroid mouse hearts after global IR. This correlates with infarct size and is in line with a concept that p38 aggravates cardiovascular pathologies (Martin, Bassi, and Marber 2015). Since p38 was not altered in 3 months old hypo- and hyperthyroid mouse hearts, an age effect could be suggested in p38 regulation after IR injury. On the other hand, group size in 3 months old mice was smaller compared to 20 months old mice which could be a reason for not having detected a more pronounced effect in p38 regulation.

TRα signalling influences baseline cardiac performance and outcome after IR injury

It still remains unclear whether canonical or noncanonical TRα signalling affects not only infarct size but also functional parameters such as LVDP. Therefore, isolated pressure constant perfused mouse hearts of control, TRα^0 and TRα^{GS} mice were used. To analyse and separate indirect TH effects in the organ and direct TRα dependent effects, an additional group of T3 treated TRα^0 mice (TRα^{0}hyper) was included in the study. To address rapid noncanonical and canonical TRα effects on function and infarct size, protein phosphorylation of classical cardioprotective proteins and gene expression were analysed.

Lack of canonical TRα signalling determines bradycardia

The lack of canonical TRα signalling decreased *ex vivo* heart rate in our study, while T3 treated TRα^0 mouse hearts had *ex vivo* heart rates comparable to controls. Previously, *in vivo* bradycardia was noted in TRα1 deficient mice, which increased by T3 treatment again, but did not reach heart rate of controls (Wikstrom et al. 1998), suggesting an influence of the autonomous nervous system and not only heart intrinsic effects.

We could show that bradycardia in hypothyroid myocardium is a heart intrinsic effect, strongly suggesting TRα^0 and TRα^{GS} mice to have a hypothyroid like myocardium as well, since TRα is known to be the dominant receptor for TH action in the heart (Yen 2001). Consistently, TRα has been shown to be a target for action potential repolarization and pacemaker channels such as *Hcn2* and *Hcn4* (Gloss et al. 2001). Moreover, it was already shown that hearts of TRα^0 and TRα^{GS} mice display hypothyroid like gene expression patterns (Hones et al. 2017). Ultimately, in a euthyroid state the lack of canonical TRα signalling is causal for inducing heart intrinsic bradycardia or, *vice versa*, canonical TRα signalling is necessary to maintain intrinsic heart rate (Fig. 37). Surprisingly, hyperthyroidism increased *ex vivo* heart rate in TRα^0 to the level of controls again, suggesting an involvement of TRβ signalling.

Noncanonical TRα signalling determines baseline left ventricular pressure

In addition, LVDP decreased in TRα^0 isolated mouse hearts at baseline, which was similar to the hypothyroid condition observed in C57BL/6J mouse hearts. An altered cardiac structure as well as a decrease in cardiac contractility has previously been reported in mice with a deletion of TRα (Liu et al. 2016). Moreover, contractile function and levels of mRNA coding for contractile proteins, i.e. *Myh6*, were decreased in TRα knockout mice (Gloss et al. 2001). Strikingly, hearts of TRα^{GS} mice developed LVP which was similar to C57BL/6J controls in this study. Therefore, it could be shown that the lack of noncanonical TRα signalling leads to

a decrease in LVDP. Consequently, noncanonical TRα signalling contributes to a normal LVDP under euthyroidism (Fig. 37).

Comparable to the hyperthyroid condition in C57BL/6J mice, TRα^{0}hyper mice were able to improve LVDP compared to TRα^0 mouse hearts, which was also reflected in an increased *Atp2a2* expression, suggesting a possible involvement of TRβ signalling on cardiac contractility (Chang et al. 1997). This is supported by a contractile deficit found in mice with a non-functional TRβ with increased T4 serum concentrations (do Imperio et al. 2015).

Lack of canonical TRα signalling is cardioprotective

The lack of canonical TRα signalling confered cardioprotection, because hearts from mice expressing a mutant TRα (TRα^{GS}) that is incapable of canonical but still capable of noncanonical TRα signalling, also exhibited a very small infarct size and improved recovery of LVDP similar to hypo, TRα^0 and TRα^{0}hyper groups. In a previous study, the treatment of rats with a selective TRα inhibitor impaired functional performance after ischaemia of isolated rat hearts to ischaemic stress (Mourouzis et al. 2013), which already led to the assumption about an involvement of TRα in IR injury. However, it remained unclear how the permanent absence of canonical or noncanonical TRα signalling affects myocardial infarct size in a specifically modified mouse model. This has been addressed for the first time in our study and underlines a fundamental relevance of TRα signalling for cardioprotection.

TH serum status contributes to loss of ventricular function after ischaemia

Recovery of LVDP was improved in TRα^{0}hyper and TRα^{GS} mouse hearts compared to controls while LVDP in reperfusion was also preserved in TRα^0 mouse hearts compared to LVDP at baseline. Moreover, inconsistent to hyperthyroid mouse hearts, functional recovery of LVDP in TRα^{0}hyper hearts did not decrease compared to controls, however LVDP at reperfusion decreased in comparison to baseline. Taken together, TH serum status does not reflect cardioprotective phenotype, as TRα^{0}hyper hearts were also protected against IR injury. TH action improves LVP at baseline, however this has also an adverse effect on the functional recovery after IR injury. Remarkably, noncanonical TRα signalling in TRα^{GS} mouse hearts also improves LVP at baseline to level of controls without inversely affecting the outcome, as cardioprotection arises as soon as canonical TRα signalling is absent (Fig. 37).

Finally, lack of canonical TH action via TRα, and no cytoplasmic signal transduction, seems to be responsible for cardioprotection under hypothyroidism, which is supported by the present protein data showing no increased activation in any of these cardioprotective key proteins. In contrast, as already seen under hypothyroidism in 3 and 20 months old mice,

phosphorylation of STAT3 consistently decreased in mouse hearts lacking canonical TRα signalling, again correlating with infarct size. Thus, THs may contribute to maintenance of myocardial STAT3 signal via TRα signalling in IR injury. A decrease in p-p38 in $TR\alpha^0$, $TR\alpha^0$hyper and $TR\alpha^{GS}$ mouse hearts that was significant in $TR\alpha^{GS}$ mouse hearts only, suggests a possible involvement of downregulation of p-p38 in cardioprotection not only by hypothyroidism in 20 months old mouse hearts but also by lack of canonical TRα signalling. Larger groups of $TR\alpha^0$ and $TR\alpha^0$hyper mice might also have had a significant effect in the downregulation of p38 after myocardial IR injury.

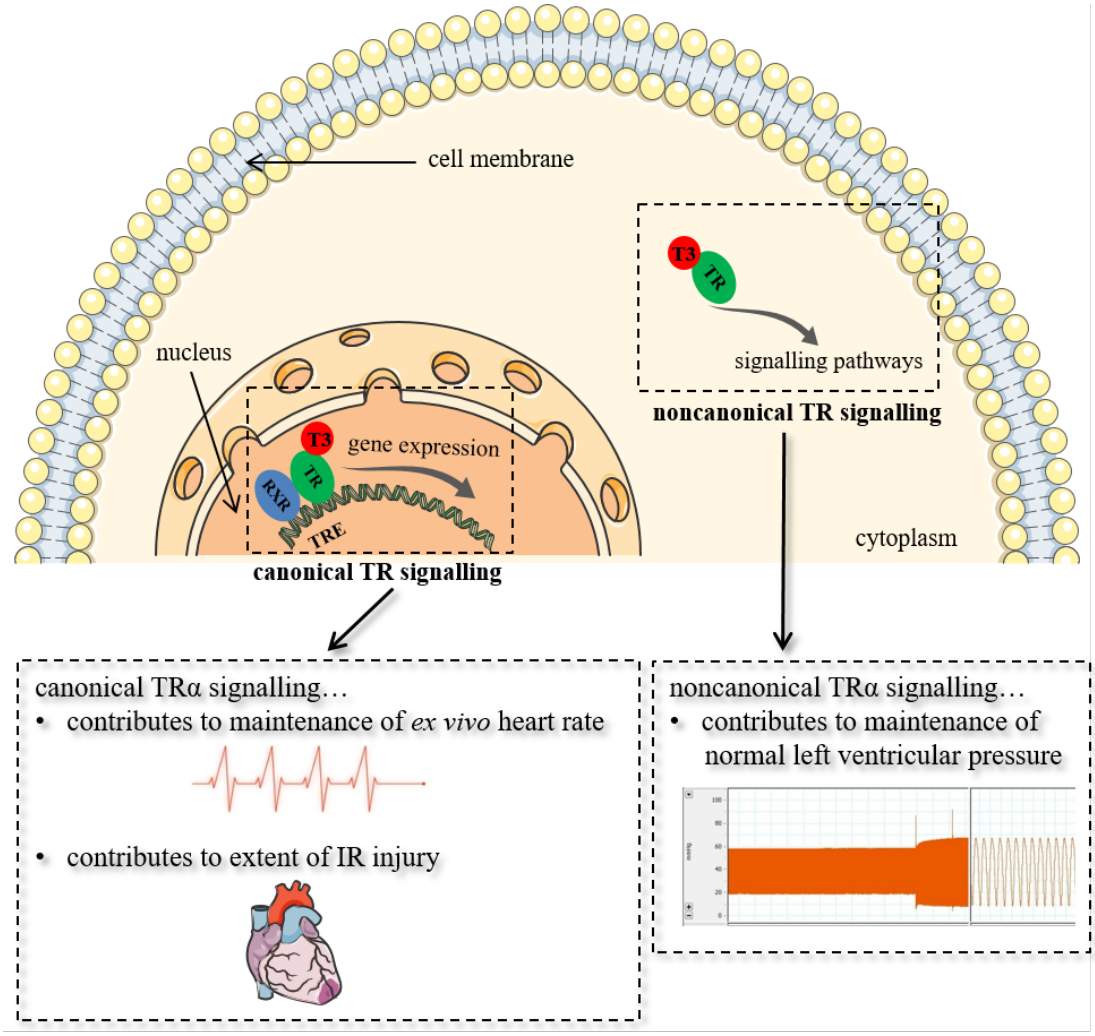

Figure 37: Impact of TR signalling on cardiac parameters. TRs are constitutively bound to thyroid hormone responsive elements (TREs) and form either homo- or heterodimers with retinoic acid receptors (RXR). Via binding of T3 to TR, gene expression of different target genes is stimulated or repressed (canonical TR signalling or type 1 signalling). Noncanonical TR action (type 3 signalling) happens rapidly and is independent from DNA binding and protein synthesis. Within this study, contribution to maintenance of *ex vivo* heart rate and extent of ischaemia/reperfusion (IR) injury could be attributed to canonical TRα signalling. Contribution to maintenance of normal left ventricular pressure could be attributed to noncanonical TRα signalling.

Acute T3 administration reduces myocardial infarct size

Before mice were treated with THs for several weeks to investigate effects of chronic thyroid dysfunction on myocardial infarct size, the effects of THs have been tested when it was given exogenously to the heart during IR protocol. Within these Langendorff test run series, it already emerged that administration of T3 can acutely modify infarct size. In fact treatment with THs after MI may have great therapeutic potential (Gerdes and Iervasi 2010; Jabbar et al. 2017; Pingitore et al. 2019). Thus, a number of studies, mostly in rats, showed that T3 administration not only reduces hypoxia/reoxygenation injury in cardiomyocytes (Nicolini et al. 2016; Zeng et al. 2019; Forini et al. 2018; Sabatino et al. 2016; da Silva et al. 2018; Bi et al. 2019) but also improves post-ischaemic functional recovery in isolated rat hearts (Pantos et al. 2007; Pantos et al. 2009; Bi et al. 2019). To investigate the acute effect of T3 on IR injury further, the role of different doses of exogenously administered T3 on myocardial infarct size was addressed. It was also examined whether there is a difference when T3 was given in pre- (before ischaemia) or postconditioning (after ischaemia) mode, as this may have influences and could be relevant for translation to the human clinical situation.

Cardioprotection by acute T3 administration is a rapid dose-dependent effect and targets reperfusion damage

In our study acute T3 administration was cardioprotective in global IR injury in male mice, as infarct size was decreased. This effect was dose-dependent, but independent from pre- or postconditioning mode, suggesting that this rapid effect is a noncanonical mode of T3 action targeting reperfusion damage. These data are consistent with very recent reports showing a dose-dependent beneficial effect of T3 in IR injury in isolated rat hearts (Bi et al. 2019). However, Bi and colleagues did not measure infarct size but only determined left ventricular function which also depends on reversible injury (stunning) and the function of remote myocardium (Kloner and Jennings 2001; Gelpi et al. 2002). Pantos and colleagues had previously shown cardioprotective effects of T3 at reperfusion at a lower dose in isolated perfused rat hearts, but again only with respect to functional recovery of the left ventricle (Mourouzis et al. 2012; Pantos et al. 2009; Pantos et al. 2011; Pantos, Malliopoulou, Paizis, et al. 2003). In an *in vivo* model of MI in rat, T3 infusion decreased myocardial infarct size (Forini et al. 2014). However, T3 infusion was continued for several days after ligation of the left descending coronary artery, precluding an assessment of the rapid T3 effect on myocardial infarct size. Treatment with a TH analog (DITPA) also decreased infarct size and macrophage accumulation in a mouse model of MI after 3 days (Abohashem-Aly et al. 2011), but again this was not reflecting an acute setting. Similar to previous studies reporting an

improved functional recovery of LVDP with T3 addition during reperfusion in isolated rat hearts (Pantos et al. 2009; Bi et al. 2019; Pantos, Malliopoulou, Paizis, et al. 2003), LVDP and CF improved in T3 treated isolated mouse hearts in our study, however the increase was not significant at most time points. LVDP's recovery during reperfusion reflects a decrease in infarct size (Botker et al. 2018), but infarct size is still the most robust endpoint of cardioprotection in preclinical studies (Botker et al. 2018; Lindsey et al. 2018) and was studied the for first time in our experiments in mouse hearts.

Cardioprotection by acute T3 administration indicates an involvement of eNOS uncoupling

Very recently, in rat heart it has been suggested that cardioprotection by acute T3 targets mitochondria (Forini, Nicolini, and Iervasi 2015; Bi et al. 2019), leading to improved mitophagy (Bi et al. 2019) and decreased apoptosis (Forini et al. 2014). Activation of the eNOS pathway is also known to protect myocardium against IR injury (Schulz, Kelm, and Heusch 2004; Heusch, Boengler, and Schulz 2008) and it may also target mitochondria, e.g. mitochondrial ROS formation and mPTP opening (Heusch 2015; Rassaf et al. 2014). Yet, the link between eNOS signalling and acute TH induced postconditioning has not been investigated in great detail. Selective overexpression of TRα in endothelial cells attenuated infarct size, suggesting a possible role of TRα in cardioprotection and TH related signalling pathways in the endothelium (Pantos et al. 2011; Suarez et al. 2014; Pantos and Mourouzis 2015). Contrary to our expectations, phosphorylation of $eNOS_{ser1177}$ did not differ between T3 and control groups in our study, suggesting that the classical activation of eNOS is not responsible for cardioprotection by acute T3 administration. In contrast, we observed $eNOS_{thr}$ phosphorylation which was increased in T3 treated hearts with smaller infarct size. Rescue experiments with BH_4 reversed $eNOS_{thr}$ on phosphorylation in T3 treated mouse hearts and this was associated with increased infarct size. The vasodilatory effect of BH_4 was reflected in a higher CF at baseline, in line with previous studies investigating CF under BH_4 in isolated hearts (Dumitrescu et al. 2007; Yamashiro et al. 2002). Classically, the addition of BH_4 as an essential cofactor of eNOS-synthesis is associated with functional eNOS or eNOS coupling (Forstermann and Sessa 2012), and in consequence of NO availability for cardioprotection (Xie et al. 2019; Xie et al. 2015; Siu et al. 2015). However, reactive nitrogen species, especially NO, may exert distinct effects during IR injury (Schulz, Kelm, and Heusch 2004). Thus, while protective effects of low NO concentrations on left ventricular function have been found in humans (Rassaf et al. 2006), high NO concentrations can have detrimental effects on cardiac contractility (Heinzel et al. 2008). In a study by Farah and colleagues eNOS uncoupling driven by an increase in NO metabolites was required for exercise-induced

cardioprotection in rats since the beneficial effect was reversed with BH_4 addition to perfusion buffer (Farah et al. 2013). In our acute setting, it is unlikely that excessive NO amounts are driven by an increase in NO metabolites. However, excessive NO levels can also be NOS mediated (Totzeck, Hendgen-Cotta, and Rassaf 2017). In fact, under T3 administration an increase in iNOS expression has been reported in mouse heart tissue (da Silva et al. 2018) and in a model of IR liver injury upregulation of iNOS expression was protective, suggesting a role of transient and reversible oxidative stress (Fernandez et al. 2009). To test whether this assumption is correct for our heart models further studies are mandatory. In summary, cardioprotection by acute TH postconditioning is suggested to be mediated by eNOS uncoupling that may protect the myocardium from excessive NO levels during reperfusion. Although there are hints for T3 mediated cardioprotective pathways in the endothelium (Suarez et al. 2014), uncoupling of eNOS may also occur in cardiomyocytes. Ultimately, it is unclear in which cellular compartment cardioprotection by acute T3 takes place. Thus, this observation emphasizes the importance of TH action in cardiovascular pathophysiology and necessitates further analysis/clarification of cells-specific requirements for T3 postconditioning to make it an attractive avenue for advanced cardioprotective therapy.

Conclusion and future perspectives

The results of the present study indicate that hypothyroidism is cardioprotective in IR injury irrespective of favorable hemodynamics such as decreased heart rate and LVDP. Determination of the exact underlying mechanisms could not be achieved in this study. However, by comparison of control, hypo, TRα^0, TRα^{0}hyper and TRα^{GS} mouse hearts, cardioprotection could be attributed to the lack of canonical TRα signalling (Fig. 37 and Fig. 38). While hypothyroidism was beneficial after IR injury, hyperthyroidism was detrimental, an effect which is age-independent (Fig. 38). This reflects the increased cardiovascular morbidity and mortality in patients with excess THs.

However, before recommendations concerning TH status for cardiovascular high-risk patients should be adjusted, *in vivo* and clinical studies need to verify these observations. Usually, cardiovascular risk patients have a diseased heart. Hearts of our mice were not morbidly changed before IR injury and thus experiments on prediseased hearts, e.g. diabetic hearts could be suggested. However in the future, patients with chronic heart disease receiving TH treatment may not be over-supplied with THs irrespective of age or it would even be recommended to carefully consider TH substitution in elderly patients with hypothyroidism and cardiovascular risk.

In addition, there is evidence that noncanonical TRα signalling maintains cardiac contractility under euthyroidism (Fig. 37) and that heart rate might not only be influenced by TRα signalling. Thus, studies of TRβ^0 and TRβ^{GS} mouse hearts would be necessary to confirm possible influence of TRβ on different cardiac readout parameters. In particular, organ-specific transgenic variants would be the mouse models of choice to exclude systemic side effects of respective global knockout/knockin mice, which in turn impede investigations on local TH action in the heart.

We showed that acute T3 administration reduces myocardial infarct size dose-dependently. Cardioprotective effect of acute T3 seems to be a rapid noncanonical effect, which is irrespective of pre- or postconditioning mode, underscoring the promising therapeutic potential in emergency rooms (Fig. 38). Although it still remains unclear in which cellular compartment of the heart cardioprotection by acute T3 takes place, this study suggests that uncoupling of eNOS may be relevant, emphasizing adverse effects of excessive NO levels during IR. Recently, the usefulness of T3 “replacement” therapy in patients with MI accompanied by low T3 levels has been investigated in the THIRST study (Pingitore et al. 2019). This was the first pilot experience in which T3 therapy was safe and able to improve

myocardial dysfunction, however no effect on infarct size could be observed yet. Probably it still needs to be clarified in which time frame which dose promises optimal success in humans and whether only patients with low T3 after MI should be included. In order to further improve outcome in MI patients by acute T3 treatment, it would be necessary to clarify how (via which TR signalling pathway), where exactly (in which cell type) and when (in which timeframe) cardioprotection takes place. Therefore cardiomyocyte- and endothelium-specific transgenic mouse strains would be the method of choice aiming for a more targeted and efficient T3 treatment after MI with translational potential.

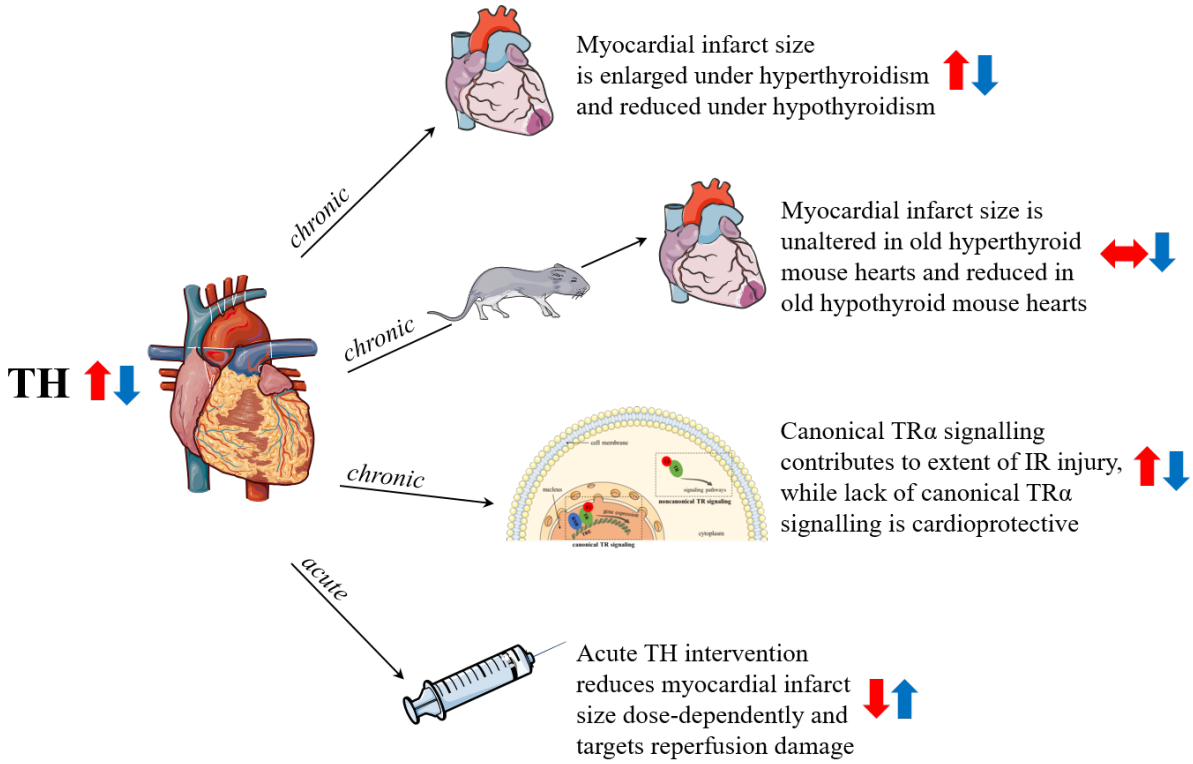

Figure 38: Influence of TH on myocardial ischaemia/reperfusion injury in the chronic and acute setting.

In conclusion, our *ex vivo* Langendorff data provide evidence that THs impact myocardial infarct size differently, depending on whether they are administered chronically (before global IR) or acutely (in/after global IR) and acting via different modes of TR signalling. The studies suggest a promising therapeutic potential of tissue TH status for cardioprotection and intervention in AMI which should to be further explored in basic, translational and clinical studies.

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Abbreviations and acronyms

AMI	acute myocardial infarction
ATP	adenosine triphosphate
BL	baseline
bpm	beats per minute
CF	coronary flow
CNS	central nervous system
DIO	deiodinase
epi	epinephrine
FT4	free 3,3´,5,5´-tetraiodothyronine; free thyroxine
FT3	free 3,3´,5-triiodothyronine; free thyronine
HPT axis	hypothalamic-pituitary-thyroid axis
hyper	hyperthyroid
hypo	hypothyroid
isch	ischaemia
IR	ischaemia/reperfusion
KHB	Krebs-Henseleit-buffer
LoI	low iodine diet
LV	left ventricle
LVDP	left ventricular developed pressure
LVP	left ventricular pressure
MCT	mono carboxylate transporter
MI	myocardial infarction
MMI	methimazole
mPTP	mitochondrial permeability transition pore
NIS	sodium-iodide-symporter
NO	nitric oxide
rep	reperfusion

RISK	Reperfusion Injury Salvage Kinase
ROS	reactive oxygen species
rT3	reverse triiodothyronine; reverseT3
RV	right ventricle
SERCA	sarcoplasmic reticulum calcium-ATPase
SAFE	Survival Activating Factor Enhancement
T2	3,3´-diiodothyronine
T3	3,3´,5-triiodothyronine
T4	3,3´,5,5´-tetraiodothyronine, thyroxine
TBG	thyroxine binding globulin
TC	time control
Tg	thyroglobulin
TH	thyroid hormone
TPO	thyroid peroxidase
TR	thyroid hormone receptor
TRE	TH-responsive element
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone; thyrotropin
TTC	triphenyltetrazoliumchloride

List of figures

List of tables

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"Man kann die eigenen Grenzen nur feststellen, indem man sie gelegentlich überschreitet. Das gilt für jene, die man sich selbst setzt, ebenso wie für jene, die einem andere setzen."
Josef Broukal

Publications

1) Gassen Janina et al. **Timing of thyroid hormone status is crucial for cardioprotection**. *In preparation.*

2) Gassen Janina et al. **Thyroid dysfunction affects outcome of myocardial ischaemia/reperfusion injury in aged mice.** *In preparation.*

3) Gassen Janina et al. **Lack of canonical thyroid hormone receptor alpha signalling is cardioprotective.** *In preparation.*

4) Kerp Helena, Hönes Sebastian, Hönes-Wendland Judith, Gassen Janina, Möller Lars Christian, Lorenz Kristina & Führer Dagmar. **Thyroid hormone deprivation stops the progression of maladaptive cardiac hypertrophy and heart failure in mice.** *Submitted.*

5) Kerp Helena, Gassen Janina, Führer Dagmar. **Age and sex influence thyroid hormone effects in target tissues with organ-specific responses.** *Experimental and Clinical Endocrinology & Diabetes. Exp Clin Endocrinol Diabetes. 2020 Jan 13. DOI: 10.1055/a-1083-6272.*

6) Hönes G. Sebastian, Rakov Helena, Logan John, Liao Xiao-Hui, Werbenko Eugenie, Pollard Andrea S., Præstholm Stine M., Siersbæk Majken S., Rijntjes Eddy, Gassen Janina, Latteyer Sören, Engels Kathrin, Strucksberg Karl-Heinz, Zwanziger Denise, Rozman Jan, Klein-Hitpass Ludger, Köhrle Josef, Armstrong David L., Grøntved Lars, Bassett J. H. Duncan, Williams Graham R., Refetoff Samuel, Führer Dagmar, Moeller Lars C. **Non-canonical thyroid hormone signalling mediates cardiometabolic effects in vivo.** *Proc Natl Acad Sci U S A. 2017 Dec 26;114(52):E11323-E11332. DOI: 10.1073/pnas.1706801115.*

Congress contributions

Oral presentations:

10/2019 Gassen Janina, Lieder Helmut Raphael, Kerp Helena, Moeller Lars Christian, Pesch Marion, Heusch Gerd, Kleinbongard Petra, Führer Dagmar. **Chronic hypothyroidism protects from myocardial ischaemia/reperfusion injury in male mice.** *21st Annual YARE Meeting (Young Active Reserach in Endocrinology), Essen.*

12/2018 Gassen Janina, Lieder Helmut Raphael, Kerp Helena, Moeller Lars Christian, Pesch Marion, Heusch Gerd, Kleinbongard Petra, Führer Dagmar. **Protective and detrimental effects of experimental hypo- and hyperthyroidism on myocardial infarct size in mouse heart.** *Annual Retreat Graduate School of Biomedical Science, Münster.*

11/2018 Gassen Janina, Rakov Helena, Lieder Helmut Raphael, Gedik Nilgün, Stroetges Merlin, Heusch Gerd, Moeller Lars Christian, Kleinbongard Petra, Führer Dagmar. **Protective and detrimental effects of experimental hypo- and hyperthyroidism on myocardial infarct size and functional recovery in mouse heart.** *48th Annual Conference of Sektion Schilddrüse, Essen.*

09/2018 Gassen Janina, Rakov Helena, Lieder Helmut, Gedik Nilgün, Stroetges Merlin, Heusch Gerd, Möller Lars, Kleinbongard Petra, Führer-Sakel Dagmar. **Protective and detrimental effects of experimental hypo- and hyperthyroidism on myocardial infarct size and functional recovery in mouse heart.** *41st Annual Meeting of the European Thyroid Association, Newcastle, UK.*

06/2018 Gassen Janina, Rakov Helena, Führer-Sakel Dagmar. **Molecular mechanisms of age-dependent thyroid hormone action.** *SPP1629 6th Annual Meeting, Berlin.*

03/2018 Gassen Janina, Rakov Helena, Lieder Helmut, Gedik Nilgün, Stroetges Merlin, Heusch Gerd, Möller Lars, Kleinbongard Petra, Führer-Sakel Dagmar. **Influence of thyroid hormones on myocardial infarct size and functional recovery in mouse heart.** *61st Annual Meeting of the German Society for Endocrinology, Bonn.*

11/2017 Gassen Janina, Rakov Helena, Lieder Helmut, Gedik Nilgün, Stroetges Merlin, Heusch Gerd, Möller Lars, Kleinbongard Petra, Führer-Sakel Dagmar. **Influence of thyroid hormones on myocardial infarct size and functional recovery after global ischaemia/reperfusion (IR) in the isolated pressure constant perfused mouse heart.** 47th *Annual Conference of Sektion Schilddrüse, Bremen.*

06/2017 Gassen Janina, Rakov Helena, Führer-Sakel Dagmar. **Molecular mechanisms of age-dependent thyroid hormone action.** *SPP1629 5th Annual Meeting, Bremen*.

Poster presentations:

03/2020 Gassen Janina, Kerp Helena, Lieder Helmut, Geist Daniela, Hönes Sebastian, Heusch Gerd, Möller Lars, Kleinbongard Petra, Führer-Sakel Dagmar. **Lack of canonical thyroid hormone receptor alpha is cardioprotective.** *13th International Workshop on Resistance to Thyroid Hormone (IWRTH), Monterey, California, USA (accepted; cancelled due to Covid-19 pandemic).*

06/2019 Gassen Janina, Lieder Helmut, Kerp Helena, Möller Lars, Heusch Gerd, Kleinbongard Petra & Führer-Sakel Dagmar. **Influence of thyroid hormones on myocardial ischaemia/reperfusion injury**. *2nd International Meeting of the Priority Program SPP1629 in Berlin*.

12/2018 Gassen Janina, Rakov Helena, Lieder Helmut Raphael, Gedik Nilgün, Stroetges Merlin, Heusch Gerd, Moeller Lars Christian, Kleinbongard Petra, Führer Dagmar. **Protective and detrimental effects of experimental hypo- and hyperthyroidism on myocardial infarct size and functional recovery in mouse heart.** *18. Forschungstag der Medizinischen Fakultät, Essen.*

11/2017 Gassen Janina, Rakov Helena, Lieder Helmut, Zwanziger Denise, Heusch Gerd, Möller Lars, Kleinbongard Petra, Führer-Sakel Dagmar. **Influence of thyroid hormones on myocardial infarct size and functional recovery after global ischaemia/reperfusion in the isolated perfused mouse heart.** *Annual Retreat Graduate School of Biomedical Science, Bonn.*

Awards:

11/2017 "**Best poster presentation**", *Annual Retreat Graduate School of Biomedical Science, Bonn.*

Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Eidesstattliche Erklärungen

Erklärung I:

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Prof. Dr. Dr. Dagmar Führer-Sakel