Physiological characterization of β-cells in zebrafish larvae

FINAL REPORT

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Abstract

Various studies focusing on mature β-cells as found in adults have revealed detailed insight to molecular and physiological mechanisms underlying insulin secretion. However, how these properties are established during pancreas development and how defects in early pancreas development impact on later functions is currently not well understood. In my PhD project I am addressing these questions using a combination of genetic, live imaging and pharmacological approaches in zebrafish larva. In our laboratory, we recently established *in vivo* imaging models for addressing β-cell function under wild type conditions and in early larval lethal mutants for genes that are predicted to affect insulin secretion. The major aim of this 3 months' research stay was a detailed physiological analysis of these models concerning embryonic β-cell functionality. *Ex-vivo* studies were performed on islets from wild type larvae at different developmental stages as well as from $Ca_V1.2$ mutants to determine requirements of these channels in the glucose induced insulin secretion pathway. An ultimate aim is to enhance our understanding of β-cell function in health and disease. This study may open new perspectives in pancreatic cell physiology during embryonic development that are necessary for understanding an integral comprehension of the regulation of glucose homeostasis and the development of diabetes.

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List of Abbreviations

T2D	Type 2 Diabetes
GLUT2	glucose transporter type 2
SLC2A2	Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 2
Gcg	glucose transporter type 2
Ins	insulin
Sst	somatostatin
pp/PP	pancreatic polypeptide
DB	dorsal bud
VB	ventral bud
Hpf	hours post-fertilization
Dpf	days post fertilization
GK	glucokinase
K_{ATP} channels	ATP-sensitive potassium channels
ATP	adenosine triphosphate
ADP	adenosine diphosphate
PEPCK/c	phosphoenopyruvate carboxykinase
VDCCs	voltage gated Ca channels
$Ca_V1.2$	L-type calcium channel alpha1 subunit
isl	island beat
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
KCl	potassium chloride
FIU	Fluorescence Intensity Unit
Cx36	Connexin 36
O/N	overnight
PK-L	pyruvat kinase, liver
AMY	amylase

1. Introduction

1.1   Pancreas Architecture

In higher vertebrates, endocrine cells are arranged in small clusters termed islets (islet of Langerhans in mammals) that contain up to five cells types, each expressing a specific hormone: α-, β-, δ-, ε- and PP cells, expressing glucagon (gcg), insulin (ins), somatostatin (sst), ghrelin and pancreatic polypeptide (pp), respectively [1]. Importantly, the pancreas in mammal and zebrafish has a conserved physiological function, a very similar cellular architecture, and conserved expression and function of most developmental genes (Pan and Wright, 2011). Non-mammalian vertebrates like zebrafish (Danio rerio) have become very popular alternative organisms to study metabolic diseases like diabetes [2]. With its ease of genetic manipulation, high fecundity, and ready access for in vivo imaging, the zebrafish has been highly productive for studies of pancreas development[3–5] and regeneration [6,7]. The zebrafish pancreas develops from the endodermal germ layer comprising endocrine and exocrine tissue and is conserved from mammals to fish [8]. Exocrine cells - also called acinar cells - generate digestive enzymes, which are transported into a branched intra-pancreatic duct, while endocrine islet cells are responsible for maintenance of blood glucose homeostasis. Uniquely in zebrafish an early primary islet forms within the first day of development expressing all endocrine hormones, which expands [8,9] and shows duct-related secondary islets as development progresses [10,11]. The zebrafish pancreas arises from two progenitor domains, termed dorsal bud (DB) and ventral bud (VB). However, in contrast to the dorsal and ventral pancreatic buds in mammals, the DB and VB in zebrafish have distinct differentiation potentials [5,12]. The DB is formed before 24 hours post-fertilization (hpf) and consists of early endocrine cells clustered to form the principal islet. The VB arises from the gut tube after 34 hpf, and these cells migrate to engulf the principal islet [12]. In zebrafish, the VB derived cells contribute to the large primary islet and scattered smaller secondary islets[10,13,14]. It is proposed that DB derived β-cells may constitute a specialized cell-type that releases insulin to support growth during embryonic development. In contrast, VB derived β-cells may have the capacity to differentiate into fully functional β-cells [15]. However, it is still not clear whether the dorsal and ventral bud derived β-cells are also distinct in their function.

1.2   Glucosensing and metabolism in pancreatic β-cells

Sensors are located in different parts of the body to monitor the changes in blood glucose concentration. The molecular basis of glucodetection is relatively well understood in mammalian pancreatic β-cells [16,17], however, whether glucosensing generally, as well as insulin secretion mechanisms specifically, are conserved in and physiologically significant for lower vertebrates like zebrafish needs to be further studied.

In mammals, glucose is taken up by glucose transporter type 2 (GLUT2), phosphorylated by glucokinase (GK) and metabolized through glycolysis to increase the intracellular ATP/ADP ratio. This leads to the closure of K_{ATP} channels (composed by Kir6.2 and SUR1 subunits), membrane depolarization, and opening of the voltage-gated Ca^{2+} channels. The entry of Ca^{2+} triggers increased neuronal activity and neurotransmitter secretion in brain regions and insulin release in pancreatic β-cells [17]. Ca^{2+} influx to the β-cells occurs rhythmically, due to a rhythmic nature of the underlying electrical activity [18]. In isolated mouse islets, changes in membrane potential induced by various secretagogues trigger synchronous changes in $[Ca^{2+}]_i$ in all β-cells of the islets [19]. Also, oscillations in secreted insulin, driven by oscillations of $[Ca^{2+}]_i$ have been demonstrated, indicating a very good temporal correlation between electrical activity, Ca^{2+} signaling, and insulin secretion in isolated islets. Moreover, each burst of electrical activity or calcium oscillation is associated with a pulse of insulin secretion recorded *in vivo,* similarly to the ones described in isolated islets studied shortly after isolation [18–20].

A K_{ATP} channel-independent 'pathway' has also been described, in which glucose stimulates insulin secretion, even when the K_{ATP} channel is maintained open (by diazoxide treatment) and the β-cells are depolarized by extracellular K^+ [21]. Both the K_{ATP} channel-dependent and –independent 'pathways' require energy production from glucose. β-cells differ in their individual rates of glucose-induced insulin release. Heterogeneity in glucose sensitivity among pancreatic β-cells can be explained by intercellular differences in glucose phosphorylation or by variations in the number of expressed ion channels in the different β-cells rather than in glucose transport [22]. The glucokinase (GK) enzyme, the first enzyme to phosphorylate glucose in the pancreas and liver, has been studied a lot in mammals. In glucose homeostasis of the pancreatic tissue, GK is considered as the primary glucosensor, and 150 mutations in the GK gene have been found to alter the glucosensing capacity of the pancreatic cells. The major feature of the regulation of GK expression is the dual

control of this enzyme by insulin in the liver and by glucose in the pancreatic β-cells. Unlike mammals, glucose appears to be a minor energy fuel in fish. Fish species cover their energy needs by oxidation of protein and lipids. In addition, there are big differences for glucose tolerance tests between fish species. Most teleost fish show poor glycaemic control that was mostly explained by the lack of expression of GK in pancreatic β-cells. This hypothesis was contradicted by the discovering of GK in many fish species [23]. Moreover, it was also discovered that the first feeding induces the expression of GK even if the diets are without glucose. In addition, when rainbow trout larvae were fed with carbohydrates, there was significantly higher GK gene expression [24]. In larval zebrafish, exogenous glucose inhibited enzymes involved in gluconeogenesis (PEPCK, phosphoenopyruvate carboxykinase) and also preproinsulin expression. These findings highlight that the regulation of glucose homeostasis by levels of carbohydrates occurs very early in the life of fish and that fish species could be a key to better understanding of the evolution and function of enzymes that are associated with glucose metabolism [25]. However, all these studies regarding glucose homeostasis in fish were carried out using whole body samples. Still very little is known about how these genes are regulated in pancreatic β-cells during embryonic development and whether the transition between endogenous and exogenous feeding in larval fish, similarly to mouse beta cells at weaning, results in switch in the expression of metabolic enzymes of the β-cells. In mouse β-cells, the drastic nutritional shift (fat-enriched maternal milk is replaced by a carbohydrate-rich diet) requires metabolic adaptations to maintain energy homeostasis that is potentially affecting β-cells and mostly correlated with the acquisition of β-cell glucose responsiveness [26].

1.3   Pancreatic β-cell failure: hallmark of type 2 diabetes

The secretion of islet hormones is highly regulated and collectively maintains glucose homeostasis through actions on many peripheral tissues such as liver, muscle and adipose tissue. Insulin stimulates glucose uptake by peripheral tissues, whereas glucagon mobilizes glucose from the liver into the circulation. Somatostatin inhibits both α- and β-cell secretions. Pancreatic polypeptide (PP) may exert an inhibitory role in pancreatic exocrine secretion, and ghrelin inhibits insulin secretion. All islet endocrine cells therefore play a central role in maintaining appropriate levels of blood glucose [27]. However, β-cells are claimed to be the most significant player in diabetes mellitus that currently affects 415

million people worldwide and this number is projected to increase [28]. A heritability of 30-70% for Type 2 Diabetes indicates a strong genetic influence. Consistently with this notion, hundreds of variants affecting more than 75 genomic loci have been found to robustly associate with increased T2D risk [29]. Many of these genes affect β-cell mass and function. Reduction in β-cell mass increases the secretory demand to the remaining β-cells, thereby disturbing β-cell function. This may lead to hyperglycemia, which may again induce β-cell apoptosis, thereby aggravating the β-cell deficit. Along the same lines, the vicious circle may be initiated by a primary defect in β-cell function[30].

In the mammalian pancreatic β-cell, the main stimulus for insulin release is elevated blood glucose levels following a meal. The circulating blood glucose is taken up by the facilitative glucose transporter GLUT2 (SLC2A2). Once inside the cell, glucose undergoes glycolysis, thereby generating adenosine triphosphate (ATP), resulting in an increased ATP/ADP ratio. This altered ratio then leads to the closure of ATP-sensitive K+-channels (K_{ATP}-channels) leading to the depolarization of the membrane, and consequent opening of VDCCs (voltage gated Ca channels). Insulin is stored in large dense-core vesicles that are recruited to the proximity of the plasma membrane following stimulation such that insulin is readily available [31].The increase in intracellular calcium concentration eventually triggers the fusion of insulin-containing granules with the membrane and the subsequent release of their content. The whole secretory process is biphasic with the first phase peaking around 5 minutes after the glucose stimulus with the majority of insulin release occurring during this first phase. In the second, somewhat slower, phase, additional insulin is secreted.

Emfinger et al. recently demonstrated that zebrafish islet β-cells express functional K_{ATP} channels of similar subunit composition, structure and metabolic sensitivity to their mammalian counterparts showing that β-cell K_{ATP} channel expression and function are conserved between zebrafish and mammals. The evolutionary conservation of islet metabolic sensing from fish to humans lends relevance to the use of zebrafish to model islet glucose sensing and diseases of membrane excitability such as neonatal diabetes [32].

Besides the K_{ATP} channels, the voltage gated L-type Ca^{2+} channels plays also a crucial role in glucose-secretion coupling, and appropriatetrafficking and distribution of L-type Ca^{2+} channels are essential for insulin release. The K_{ATP} channel-dependent mechanisms trigger first-phase insulin secretion by opening $Ca_V1.2$ and $Ca_V1.3$ channels [33]. Both $Ca_V1.2$ and $Ca_V1.3$ have been found in human, rodent as well as in zebrafish islets. Studies in mutant

mice with $Ca_V1.2$ or $Ca_V1.3$ deficiencies have confirmed that L-type Ca^{2+} channels are crucial for β-cell physiology. Up-regulation of β-cells Ca^{2+} channel activity results in enhanced insulin exocytosis and more efficient glucose homeostasis. Moreover, downregulation of voltage gated Ca^{2+} channels activity causes less insulin secretion and glucose intolerance which is associated with a group of type 2 diabetic patients [34–36]. Thus, it is clear that mutation in the Cav1.2 or Cav1.3 channels will perturb the insulin secretion pathway of pancreatic β-cells. However, the exact mechanism of this perturbation in the islet function is poorly understood.

1.4   Research Problem and Hypothesis

Study of the Ca^{2+} fluxes induced by glucose stimulation of β-cells is ongoing in our laboratory as a part of my PhD study. Optogenetic tools developed for studying neurons [37] can be used to reveal physiology and interactivity of cells in the pancreatic islet, as these cell types share many features [38]. Among genetically encoded activity sensors, those detecting calcium (GCaMPs) are of particular interest, as calcium influx has been documented to correlate with several key function in endocrine cells, in addition to glucose-stimulated insulin secretion [39]. *In vivo* analyses of transgenic zebrafish embryos expressing a GCaMP-variant under control of the insulin promoter (Tg(ins:GCaMP6s ins:H2B-RFP)) revealed glucose-induced oscillating calcium fluxes in β-cells *(Figure 1.).* Experiments in 5 dpf (days post fertilization) larvae revealed rapid fluorescence signal alteration (peak within 10 sec) in response to intravenous injection of higher concentration of D-glucose (>10 mM final concentration). There was no response in 4 dpf old larvae, however, only lower concentration of glucose was injected (<10 mM final concentration). To confirm that the response is due to glucose metabolism, rather than osmotic stress, L-glucose was also injected at the same concentration, and showed no response *(Figure 2.)*
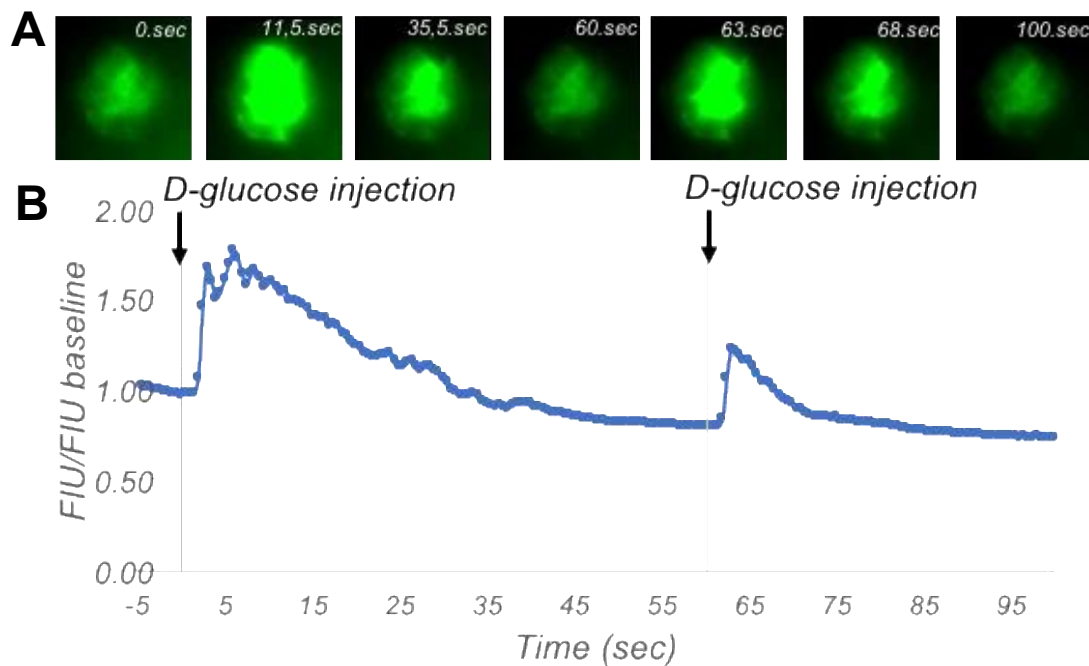
Figure 1: *Fluorescence image series of the islet showing beta cell-specific GCaMP6 signal at the indicated time-points after intravenous injection of 0,5 M D-glucose (˜1 nl) (>10 mM final concentration) in 5 days post fertilization (dpf) larva.* **(B)** *Quantitation GCaMP6 signal intensity in the time series shown in (A), showing calcium influx after sequential glucose injections. FIU intensities for the whole islet were determined using Image-J and normalized to the FIU baseline (intensities measured in the first 10 sec before injection). FIU= Fluorescence Intensity Units (Arbitrary Units).*

In vivo imaging of beta cell physiology in zebrafish embryos and larvae offers unique possibilities for addressing beta cell functions in the context of whole-organism physiology, in conjunction with defined genetic backgrounds and pharmacological manipulations [40]. However, *ex vivo* physiological measurements are essential to perform perfusion experiments in which the concentration of different components/nutrients delivered to the tissue (pancreatic islet) can be controlled, even sequentially. *Ex vivo* results can be correlated to the dataset that we get *in vivo* in the β-cells´ natural environment. Our hypothesis was that the glucose induced Ca^{2+} oscillations that we observed in the pancreatic β-cells in living zebrafish larvae (*in vivo*) reveals the same Ca^{2+} dynamics in the isolated pancreatic islet tissue (*ex vivo*). Furthermore, based on my preliminary *in vivo* results - 4 dpf old zebrafish larvae do not respond to intravenous injection of D-glucose *(Figure 2.)* - I predicted that the pancreatic β-cells differ in their glucose responsiveness during embryonic development and that the Ca^{2+} oscillations in the pancreatic β-cells of zebrafish, similarly to mammals, are glucose concentration dependent.
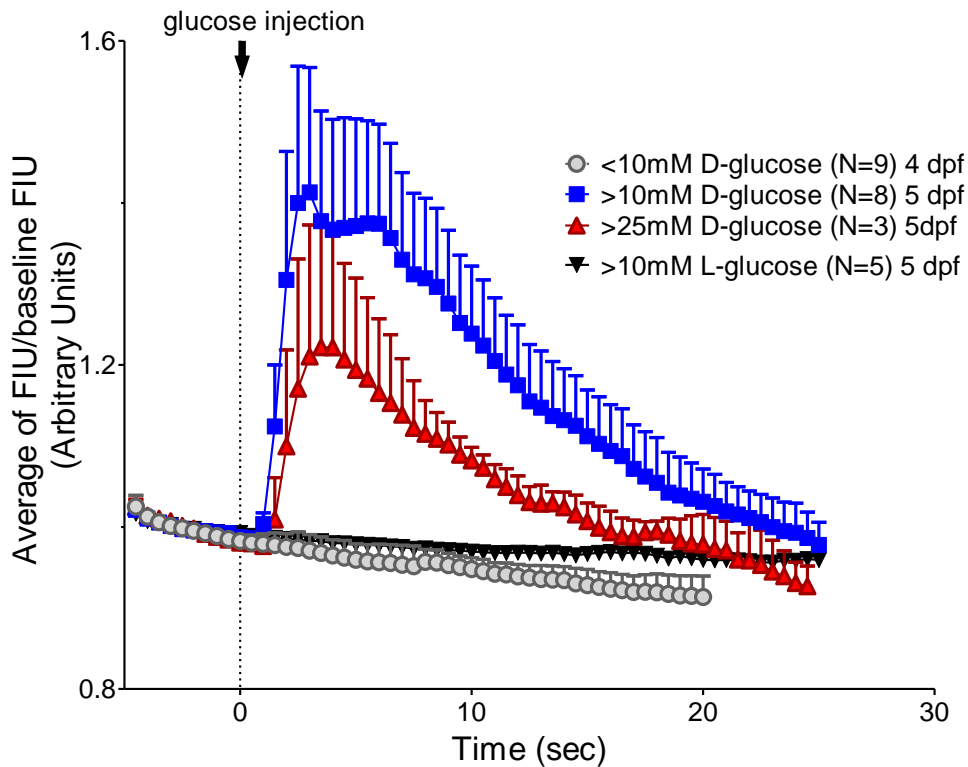
Figure 2: *Zebrafish β-cells show glucose dependent Ca²⁺ dynamics in vivo. Experiments in larvae revealed rapid fluorescence signal alteration (peak within 10 sec) in response to intravenous injection of higher concentrations of D-glucose (>10 mM (blue line) and >25 mM (red line)) (5 dpf). There was no response at 4 dpf, although only a lower concentration of glucose was injected (<10 mM) (gray line) (4 dpf). Again, to confirm that the response is due to D-glucose metabolism, L-glucose (>10 mM) (black line) using same concentration was also injected and showed no response.*

In order to confirm the sensitivity and specificity of our generated Tg(ins:GCaMP6s) imaging line in disease phenotypes and also to further investigate the role of the L-type Ca²⁺ channels in the islet function during embryonic pancreas development, island beat/Ca$_V$1.2 mutants crossed with Tg(ins:GCaMP6s) imaging line fish were also studied. Island beat (isl) is an ethylnitrosourea (ENU) induced, embryonic lethal recessive mutation in the L-type calcium channel alpha1 subunit (Ca$_V$1.2), which was isolated in a large scale genetic screen. The island beat mutant has a change from glutamine to a stop codon at the isl m458 allele that abolishes L-type calcium currents in isl cardiomyocytes and leads to severe cardiac edema (*Figure 3.*). Consequently, cardiomyocytes fail to contract periodically and thus never generate a functional heartbeat. At day two of development ventricular myocytes stay silent, although atrial myocytes still contract sporadically. The absent blood flow does not affect the mutant embryos until 4 dpf, the period when they begin to die [41,42]. Based on our measurements the island beat mutants show

higher glucose levels at 2, 3 and even at 4 dpf (*Figure 3/B.*). The hyperglycemic phenotype makes these mutants - in combination with Tg(ins:GCaMP6s) imaging line - excellent tools to demonstrate that the GCaMP6s imaging line is applicable in studying glucose-induced Ca^{2+} dynamics during early pancreatic islet development and the potential for iuts use in diabetes research. However, the fact that these mutants lack blood circulation necessitates *ex vivo* experiments on isolated larvae islets. This I aimed to carry out in the host laboratory during my 3 months stay.
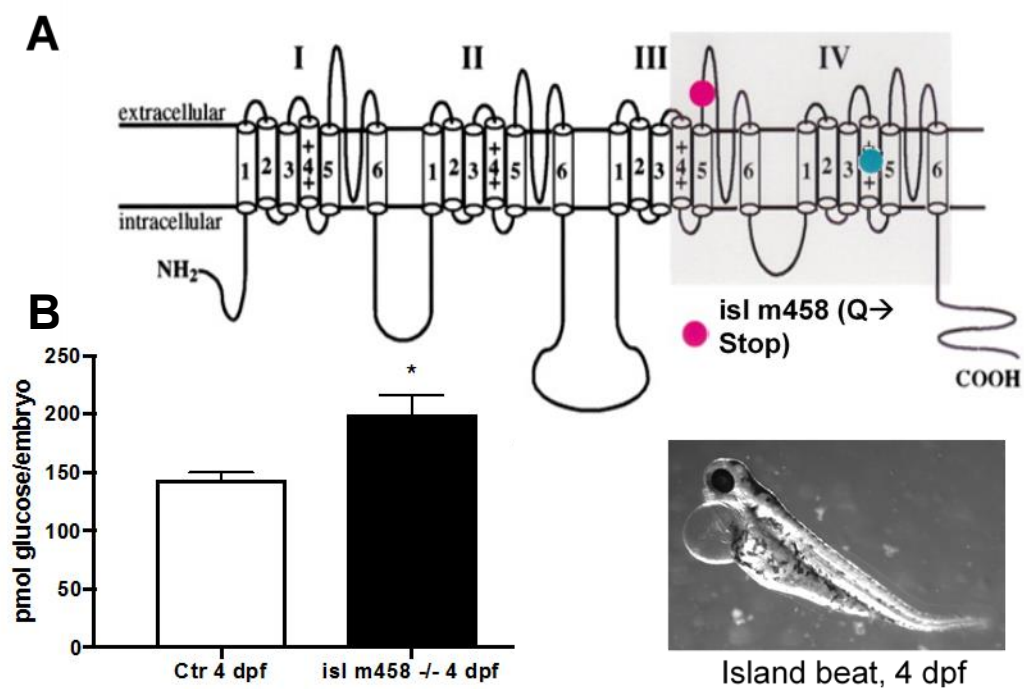

Figure 3: (A) Structural model of L-type voltage gated calcium channels showing the $Ca_V\alpha1.2$ subunit. The island beat mutants (isl m458-/-) have a Glutamine (Q) to Stop codon exchange at the position indicated by red dot. (B) isl m458-/- shows elevated whole larval glucose levels and develops severe heart edemas, dying around 5-6 dpf.

An ultimate goal is to enhance our understanding of β-cell function in health and disease during embryonic pancreas development in model zebrafish. This Research Project along with my ongoing PhD study may open new perspectives in pancreatic endocrine cell physiology that are necessary for understanding an integral comprehension of the regulation of glucose homeostasis and the development of diabetes.

2. Material and Methods

2.1   Zebrafish maintenance and fish lines

Zebrafish (*Danio rerio*) were maintained according to standard protocols. Developing embryos were staged by hours post fertilization (hpf) when incubated at 28 °C [43]. Zebrafish expressing either membrane or cytosolic localized, beta-cell specific GCaMP6s Ca^{2+} sensor (Tg(ins:GCaMP6s ins:H2B:RFP) and Tg(ins:cytoGCaMP6s cmlc2:eGFP), respectively) were used for islet isolation studies. The membrane localized GCaMP6s imaging line contains (generated by Institute of Molecular Biology, University of Innsbruck) an ins:H2B:RFP sequence resulting in red fluorescent beta-cell nuclei in order to ease screening and isolation of the islets. The cytosolic localized GCaMP6s imaging line (generated by the Department of Medicine and Cell Biology & Physiology, Washington University) contains cmlc2:eGFP sequence to induce eGFP expression in the heart and facilitating the screening. In both cases, the highly Ca^{2+} sensitive GCaMP6s was used to create a new beta-cell specific GECI (genetically encoded Ca^{2+} indicators). These fish were maintained in the Washington University zebrafish facility. Details of standard operating procedures for the facility can be found at http://zebrafishfacility.wustl.edu/documents.html. All procedures were approved by the Washington University in St Louis IACUC.

2.2   Adult islet isolation

Adult zebrafish islets were isolated as described previously [32,44], with minor modifications. In short, fish were first euthanized using cold-shock (8°C water immersion) followed by decapitation. A dissection microscope equipped with fluorescence (Leica) was used to perform the surgeries. Fish were rolled onto their right sides, skin was removed using surgical forceps to penetrate the body and to remove all visceral organs. The islets were identified at the intersection of hepatic and bile ducts with the intestine and confirmed by either GCaMP6s green fluorescence or RFP red fluorescence. Islets were removed by gently pinching ducts with forceps and separating the islets from the surrounding tissues.

Exocrine tissues surrounding islets were digested with collagenase (Sigma C9263, 0.4mgml−1 in Hank's buffered salt solution, 0.5 ml/5–10 islets), during incubation at 29°C for 20 min, shaking gently every 5 min. Islets were then placed in RPMI (ThermoFisher 11875-093) supplemented with 1mM HEPES, antibiotic solution (Sigma A5955, 10mll−1 solution), 10%

fetal bovine serum and diluted with glucose-free RPMI to final glucose concentration of 6.67mM (zebrafish islet media).

2.3   Larvae islet isolation

Zebrafish embryos at 2, 3, 4 and 5 dpf were euthanized using cold-shock (embryos in egg water-containing dishes are set directly on ice). Fish were rolled onto their left sides and using 26 G sterile hypodermic-needles, yolk and visceral organs were removed by gently applying pressure using needles until fully separated. The islets were identified and confirmed by either GCaMP6s green fluorescence or RFP red fluorescence. Islets were removed by separating the islets from the surrounding tissues, leaving some surrounding tissue around the islet in order to help to avoid the damage of the islet. Islets were transferred by glass pipette to the zebrafish islet media containing 12-well culture plate. The islets were incubated at 29°C until imaging.

2.4   Chemicals

Salts and glucose were purchased from Sigma Aldrich.

2.5   Calcium imaging and statistical analysis

Islets from either Tg(ins:GCaMP6s ins:H2B:RFP) or Tg(ins:cytoGCaMP6s cmlc2:eGFP) were placed into glass-bottomed culture plates containing 2 mM glucose solution (2 mM glucose in KRBH salt solution: 114 mM NaCl, 4.7 mM KCl, 1.16 mM $MgSO_4$ heptahydrate, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$ dihydrate, 5 mM $NaHCO_3$, 20 mM HEPES and 0.1 g/100ml BSA) with agarose gel wells. Till Photonics and Zeiss Axiovert 200 M microscopes with air lens (10x or 20x) were used for calcium imaging (time lapse with 500ms-1s time interval, 100 ms exposure for GCaMP6s, 2x2 binning), while solutions (glucose to glucose and glucose to KCl) were changed using handmade perfusion system (5 ml syringes connected to plastic tubes through 26 G needles). Images were collected using Slidebook 5.00 software and analyzed with ImageJ (Fiji) ROI Manager. Statistical comparisons between the various datasets were performed in GraphPad PRISM. Datasets were tested for normality (Shapiro– Wilk) and whether variances were statistically different between groups (Bartlett's test) and afterwards, unpaired t-test (Student's t-test) was used.

3. Results

3.1   Zebrafish pancreatic β-cells possess glucose-responsive calcium currents

To test our hypothesis that zebrafish pancreatic β-cells possess glucose-responsive calcium current, we first isolated pancreatic islets from adult (4-6 months old) zebrafish using Tg(ins:lynGCaMP6s ins:H2B:RFP) and Tg(ins:cytoGCaMP6s cmlc2:eGFP) lines. The genetically encoded Ca^{2+} sensor under the promoter of insulin enables examination of the calcium levels specifically in the pancreatic β-cells. GCaMP6s fluorescence increases as the [Ca^{2+}] also increases in the cells.
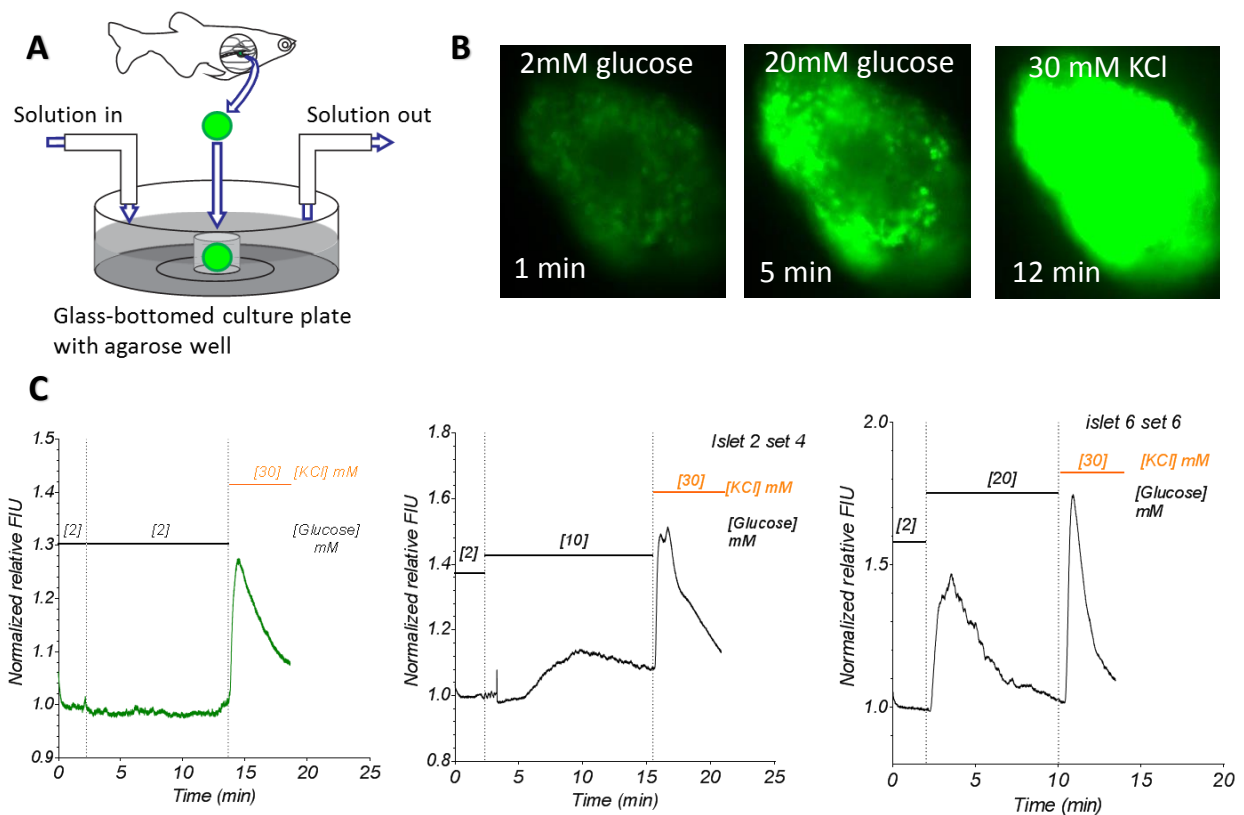

Figure 4: Zebrafish pancreatic β-cells possess glucose-responsive calcium currents (A) *Islets from Tg(ins:lynGCaMP6s ins:H2B:RFP) and Tg(ins:cytoGCaMP6s cmlc2:eGFP) fish are isolated and placed into glass-bottomed culture plates with agarose gel wells for calcium imaging in islets as solutions are changed. **(B)** Fluorescence image series of an isolated adult zebrafish islet showing beta cell-specific GCaMP6 signal at the indicated time-points in 2 mM glucose solution, in 20 mM glucose solution (as glucose concentration was increased), and in 30 mM KCl (potassium chloride) solution. KCl is used to assess maximum excitability-dependent Ca elevation of the whole islet, facilitating the quantification of the response to glucose in comparison to KCl. **(C)** Representative time-courses of relative FIU (Fluorescence Intensity Unit), normalized to the baseline when 2 mM glucose solution is applied, within 3 individual isolated adult zebrafish islets when 2 mM, 10 mM and 20 mM glucose solution are applied followed by KCl. Fluorescence intensity is measured in the whole islet using ROI Manager of ImageJ. FIU= Fluorescence Intensity Units (Arbitrary Units).*

Individual zebrafish pancreatic islets were placed into glass-bottomed culture plates with agarose gel wells containing 2 mM glucose in KRBH solution, the same concentration that we also used at the beginning of the recordings. The islets were then stimulated for 10–30 minutes with different glucose concentration (2, 6, 8, 10, 12, 16 and 20 mM) and at the end of the recording, 30 mM KCl (potassium chloride) was added in order to assess the maximum excitability-driven Ca elevation of the whole islet and to facilitate the quantification of the respond to glucose in comparison to KCl. KCl causes membrane depolarization and chronic activation of Ca currents in the β-cells. Figure 4 Illustrates recordings from representative islets, encompassing types of responses at different glucose concentration within a zebrafish pancreatic islet. There was no significant change in the normalized relative FIU (Fluorescence Intensity Unit) of the whole islets when 2 mM glucose was applied, however, responses of the β-cells could be observed to stimulation starting with 10 mM glucose (*Figure 5.*) and increased as we elevated the glucose concentration. The drop in the normalized relative fluorescence intensity at 16mM may be explained by the variability in response, a lower number of samples that we analyzed in this case (4 islets, whereas at 12 and 20 mM glucose concentration at least 8-11 islets were measured).

Taken together, we showed that zebrafish pancreatic β-cells possess glucose-dependent calcium oscillations starting around 10 mM glucose concentration. In mammals, stimulatory glucose concentrations where the calcium signal is oscillatory is above ~7 mM [45]. In zebrafish isolated islets Ca^{2+} oscillations are proportional to the glucose concentration, as glucose concentration was increased, the normalized relative FIU was also elevated (*Figure 5.*). These results are in line with results found in mouse islets of Langerhans *in vivo*, where duration of the intracellular Ca^{2+} oscillations are directly proportional to the blood glucose concentration. One of the most interesting properties of these oscillations is their synchronicity throughout the mouse islet. Such synchronicity is preserved when the islets are challenged with sudden increases in blood glucose [20] and maintained by gap junction (Connexin 36) mediated electrical communication [46]. In Cx36−/− (Connexin 36) mouse islets, after reduction in electrical coupling due lack of Cx36, calcium waves are slowed as well as disrupted, and there is reduced number of β-cells that show synchronous calcium oscillations. The gap junction coupling coordinates electrical oscillations that results in more effective pulsatile insulin release (instead of continuous level of insulin) [46]. We did not observe uniform bursting behavior and synchronicity in zebrafish islets, which leads us to the hypothesis that zebrafish islets are

electrically uncoupled, perhaps due to lack of connexin-mediated gap junctions within the islet. We further hypothesize that, in zebrafish islets, the lack of coupling may result in decreased or no insulin pulsatility which is consistent with the finding that fish species including zebrafish have a lower glucose tolerance than mammals: intravenous injection of glucose in zebrafish produces peak blood glucose levels within 30 min following injection and blood glucose levels returns to the control level within 6 h, which is much longer than in mammals (30-40 min) [4,47].
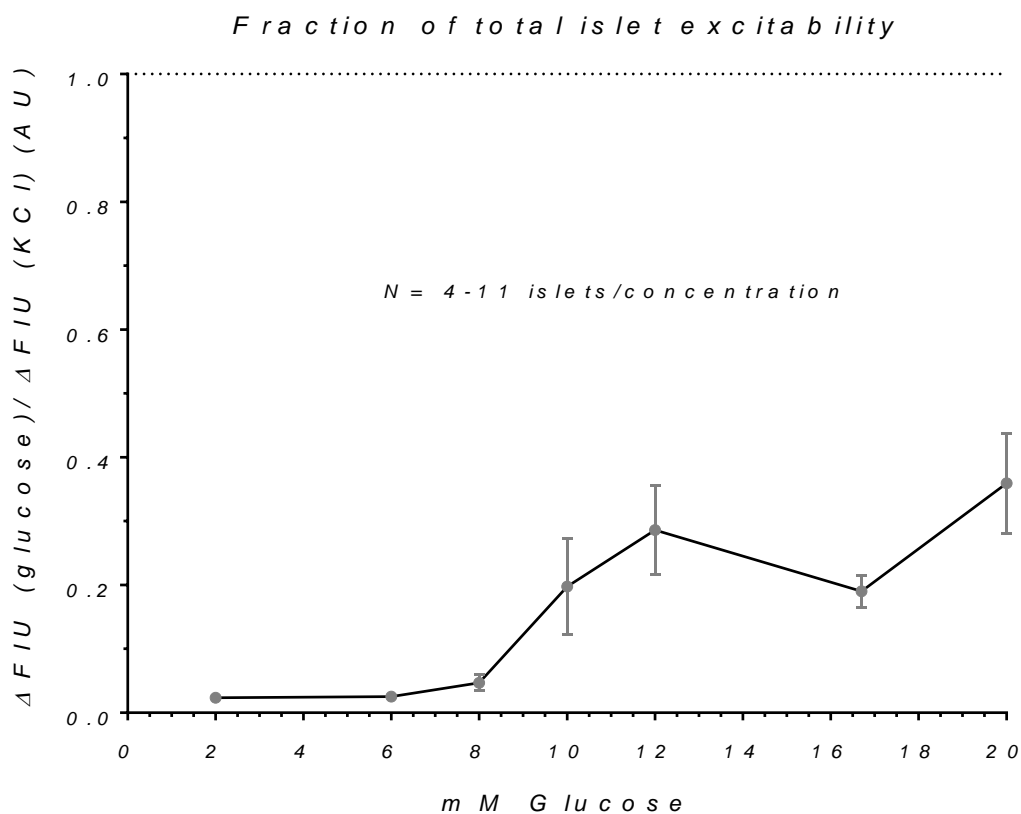

Figure 5: Zebrafish pancreatic β-cells possess glucose-dependent calcium oscillations. *Normalized relative FIU (Fluorescence Intensity Unit) (normalized to baseline (response to 2 mM glucose) and relative to response to KCl) within each islets are showed as a fraction of total islet excitability at each glucose concentration (2, 6, 8, 10, 12, 16 and 20 mM). At each glucose concentration, 4-11 islets were measured.*

In the future, closer examination of gap junction coupling in the zebrafish pancreatic islet is needed to help to determine why the zebrafish islet is electrically uncoupled and to understand how the unsynchronized behavior characterized by oscillatory $[Ca^{2+}]_i$ relates to the degree of gap junction coupling. Gap junction conductance is predicted to affect calcium wave propagation. Although gap junctions are not required for insulin secretion in response

to glucose, but modified electrical coupling could result in modified pattern of insulin release [46,48,49], adding relevance to further study of whether zebrafish islets are electrically uncoupled.

3.2 β-cells differ in their glucose responsiveness during embryonic development

Based on our preliminary *in vivo* results - zebrafish larvae at 4 dpf do not respond to intravenous injection of D-glucose but shows increased FIU/FIU baseline of GCaMP6s signal within 10 seconds at 5 dpf *(Figure 2.)* - we also predicted that zebrafish pancreatic β-cells differ in their glucose responsiveness during embryonic development. In order to confirm this hypothesis, we isolated pancreatic islets from zebrafish larvae at different stages (2 dpf, 3 dpf, 4 dpf and 5 dpf). In all cases, we performed Ca^{2+} imaging on the isolated larval islets (*ex vivo*) stimulating them with 20 mM glucose solution and at the end of the recording 30 mM KCl solution was applied to confirm that the islet was excitable and to calculate the total islet exci-
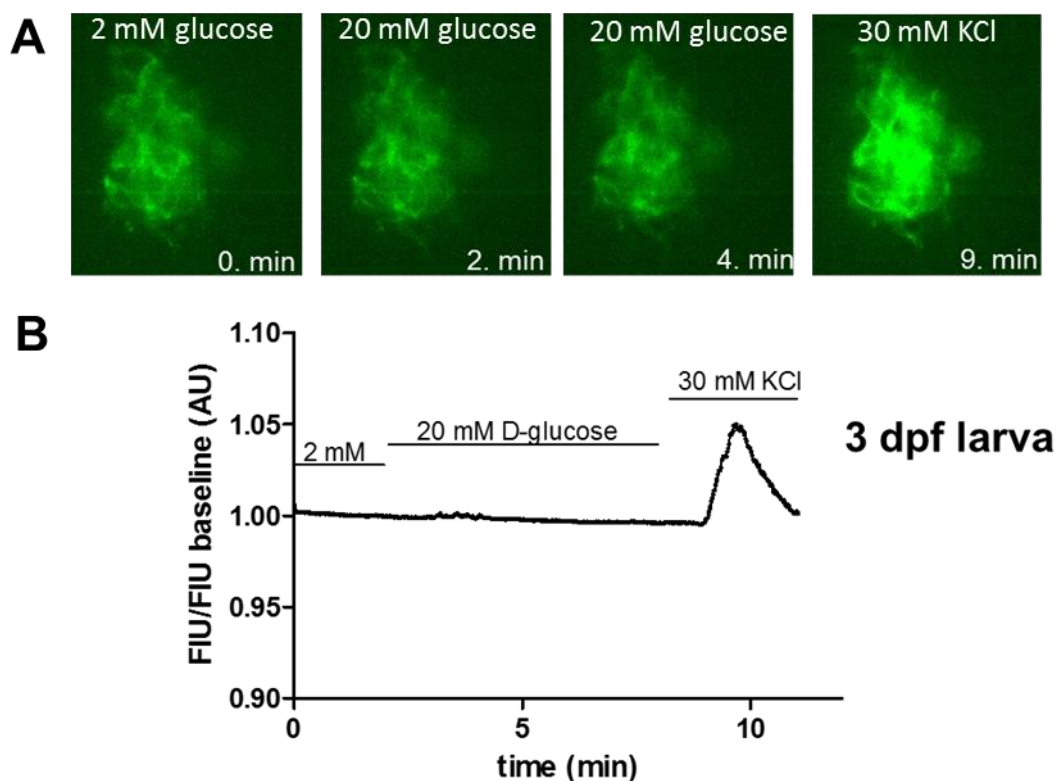

Figure 6: (A) *Fluorescence image series of an isolated islet from 3 dpf old Tg(ins:GCaMP6s ins:H2B:RFP) larva. There is no calcium influx after either 2 mM or 20 mM glucose stimulation but there is calcium influx after 30 mM KCl. This images series were taken by Zeiss spinning disk microscope. (B) Quantitation of GCaMP signal intensity in the time series shown in (A). After stimulation with 20 mM glucose solution, there is no FIU/FIU baseline changes, however, the islet was excitable (beta cell-specific GCaMP6s FIU/FIU baseline intensity showed increased level) by membrane depolarization by 30 mM KCl. FIU intensities for the whole islet were determined using Image-J and normalized to the FIU baseline (intensities measured in the first 2 min when 2 mM glucose was applied). FIU= Fluorescence Intensity Units (Arbitrary Units).*

Figure 7: (A) *Fluorescence image series of an isolated islet from 4.5 dpf old Tg(ins:GCaMP6s ins:H2B:RFP) larva. After stimulation with 20 mM glucose solution there is calcium influx directly after applying of high glucose solution. Membrane depolarization by KCl also showed calcium influx as expected. This images series were taken by Zeiss spinning disk microscope.* ***(B)*** *Quantitation of GCaMP6 signal intensity in the time series shown in (A), showing fast FIU/FIU baseline change after 20 mM glucose as well as after 30 mM KCl stimulation but not after 2 mM glucose. The total islet excitability was examined by membrane depolarization by 30 mM KCl that showed higher GCaMP6s signal intensity change (FIU/FIU baseline) in comparison to 20 mM glucose. FIU intensities for the whole islet were determined using Image-J and normalized to the FIU baseline (intensities measured in the first 2 min when 2 mM glucose was applied). FIU= Fluorescence Intensity Units (Arbitrary Units).*
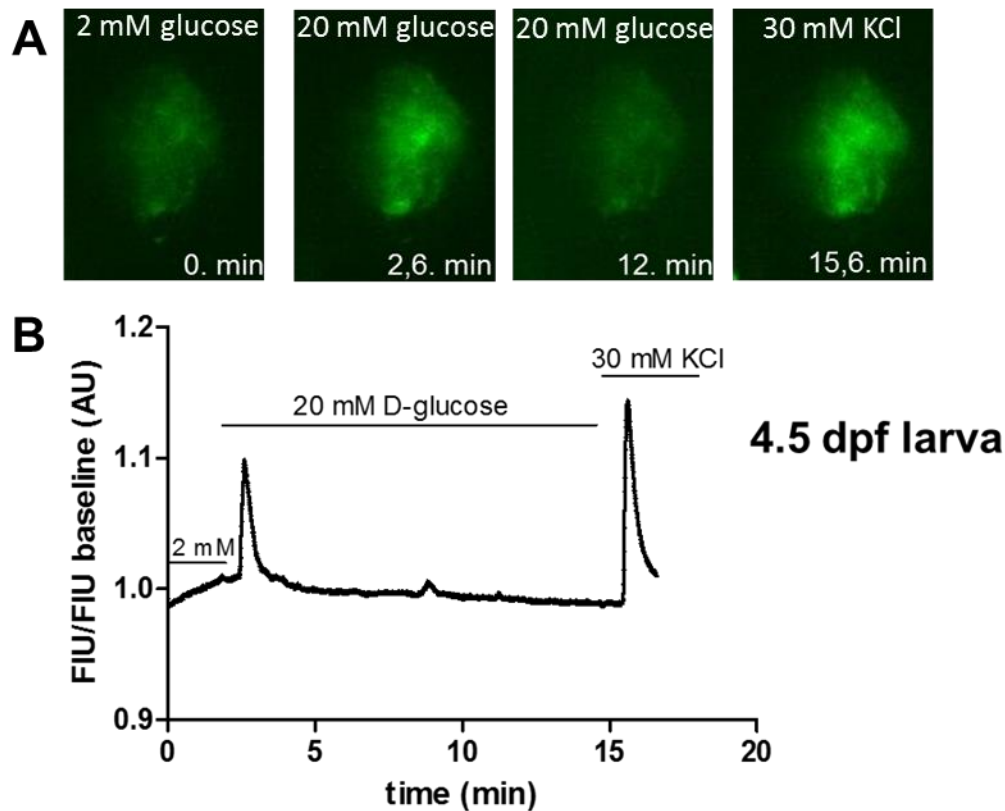
tability. We did not observe glucose responsiveness of isolated islets from 3 dpf larva but islets isolated from 4.5 dpf larva showed response to glucose (*Figure 6. and 7.*). Next, we also carried out *ex vivo* Ca^{2+} imaging on islets isolated from 4 and 5 dpf larvae. At 4 dpf we did not detect any significant changes in the GCaMP6s fluorescence intensity measured in the whole islet (*Figure 8/A.*). However, whole islets isolated from 5 dpf larvae showed rapid increase in the GCaMP6s FIU/FIU baseline intensity (*Figure 8./B*), similar to the glucose-response that we observed *in vivo* after injection of D-glucose into the circulation of the same age larvae (*Figure 1. and 2.*). These results confirm our hypothesis that β-cells indeed differ in their glucose responsiveness during embryonic development. In addition, the quantitative results (showing as the fraction of the total islet excitability) (*Figure 8/C.*) also show significant differences in

glucose responsiveness between 5 dpf (as well as 4.5 dpf) and younger embryos (4, 3 and 2 dpf). All the fluorescence intensities were measured within the whole islet. Therefore, the possibility of glucose-responses in single β-cells, when only a few cells are firing, cannot be excluded. However, analysis of individual β-cell responses requires higher-resolution images, which may be a future focus of this project. Moreover, we found the same glucose-stimulated Ca^{2+} dynamics in the pancreatic β-cells *ex vivo* that we previously observed in living animals *in vivo*. However, it is important to mention that the time scale of this Ca^{2+} dynamics is different *in vivo* in living animal than *ex vivo* in extracted islet. *In vivo*, we could see the peak of the GCaMP6s fluorescence intensity within the first 7 sec after injection of D-glucose, while *ex vivo* the time to peak is in average 70 sec (*Figure 2.* and *Figure 8.*). This time delay in the isolated islet could be explained by the used perfusion system. We put the islet samples into a glass-bottom culture plates that contain 1% low melt agarose gel almost in the entire plate except a hole where the islet is located during the imaging in order to avoid movements that would be caused by changing of the different solutions. However, glucose diffuse freely within agarose gel matrix, it does it with high diffusion coefficient meaning far slower than for example in water (and in blood) [50]. Injection of a component directly to the circulation of a living animal has obviously faster effect in comparison to a perfusion system where solution needs to reach the cells in the extracted islet that misses *in vivo* environment (circulation). At the same time, based on our results it seems that *ex vivo* circumstances with all its advantages (manipulation of the conditions) are suitable for studying glucose-responsiveness of the pancreatic β-cells without having the problems that most pancreatic beta cell lines have including non-physiological glucose-responsiveness and defective insulin secretory characteristics [51]. However, based on the experiments that we conducted on extracted islets (from 4 dpf as well as 3 dpf larvae) that were incubated at 29°C overnight and imaged on the next day after isolation (*Figure 9.*), we could conclude that overnight cultures of the pancreatic islets show different glucose responses in comparison to the corresponding controls. Figure 9. show that we did not observe significant glucose response on extracted islets isolated from 3 dpf embryos, however, islets that were isolated at 3 dpf and incubated at 29°C overnight showed significant higher glucose response. This was the case on islets that were isolated from 4 dpf old larvae (*Figure 9.*). These data are also in line with those results that were revealed from freshly isolated *Sur1 knockout* (lacking the SUR1 subunit of the K_{ATP}-channels) mouse islets that showed no elevated $[Ca^{2+}]_i$ levels and only poorly secreted insulin, however,

overnight culture of Sur1KO islets in 10 mM glucose resulted in 7-8 fold higher stimulated secretory rate than in fresh islets [52]. They also conclude that this refractoriness is not because of defect of β-cells lacking K_{ATP} channels. They explain it that the Ca^{2+} signal is insufficiently triggered after shorter incubation time and this cannot be compensated but can be improved by overnight culture of the islets. The underlying mechanism is still unclear, but they suggest that the reason is a K_{ATP}-independent pathway meaning that biophysical sensor other than K_{ATP} channels can mediate the effects of glucose on the production of the triggering Ca^{2+} signal. However, they studied this novel mechanism only in islets that lack of K_{ATP} channels and not in normal β-cells [52].
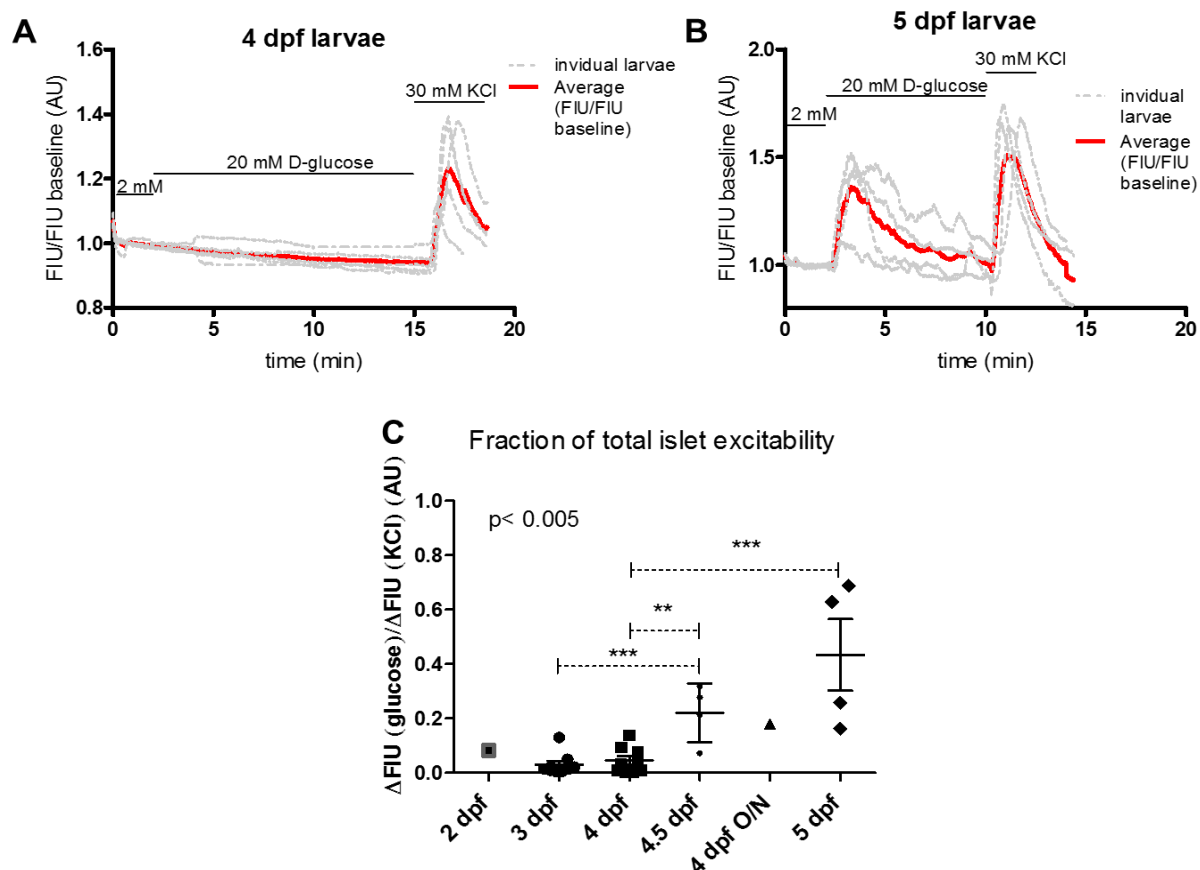
Figure 8: *Quantitation of GCaMP signal intensity in islets isolated from 4 dpf larvae **(A)**, and from 5 dpf larvae **(B)** showing no FIU/FIU baseline change at 4 dpf **(A)** but rapid change at 5 dpf **(B)** after 20 mM glucose. The total islet excitability was examined by membrane depolarization by 30 mM KCl that showed higher GCaMP6s signal intensity change (FIU/FIU baseline) in both cases. FIU intensities for the whole islet were determined using Image-J and normalized to the FIU baseline (intensities measured in the first 2 min when 2 mM glucose was applied). **(C)** Normalized relative FIU (normalized to baseline (response to 2 mM glucose) and relative to response to KCl) within each islets are showed as a fraction of total islet excitability at different developmental stages (2, 3, 4, 4.5, 4 O/N and 5 dpf). FIU= Fluorescence Intensity Units (Arbitrary Units)*

In conclusion, our *in vivo* and *ex vivo* experiments showed that the glucose-responsiveness within the zebrafish pancreatic islet changes during embryonic development. The biggest change occurs between 4 and 5 dpf, probably around 4.5 dpf (111 hpf), as we saw significant increase in FIU/FIU baseline intensities at this stage in comparison to 4 dpf (96 hpf) (*Figure 8./C*). It has been shown that expression of metabolic genes related to glucose metabolism (glucokinase, hexokinase, pyruvate kinase, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase), transport (sodium-dependent glucose cotransporter 1) and carbohydrate digestion (amylase) are lower at 0.2 dpf in comparison to later stages of zebrafish development (4, 6 and 10 dpf) [53]. However, these genes were analysed in total RNA extracted from whole larvae and not specifically in the pancreatic β-cells. Recently, transciptome

analysis of pancreatic cells across distant species, including zebrafish, highlights not just novel important regulator genes but also shows striking differences between the transcriptomes of fetal and adult β-cells. Among the 1853 genes enriched in adult endocrine cells, there are genes associated with glucose metabolism and transport (pyruvate kinase, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, glucose transporter type 2) that are not detected in embryonic cells at 27 hpf [54]. Interestingly, most of these genes show significant expression level changes during early embryonic development within whole zebrafish larvae (Rocha et al). Specifically, PK-L (pyruvate kinase, liver), PEPCKc (phosphoenolpyruvate carboxykinase) and AMY (amylase) genes show a higher induction of expression at 6 dpf compared with 4 dpf, potentially because, during this period, the formation and differentiation of two main organs involved in glucose homeostasis (liver and pancreas) is still ongoing [53]. It should also be noted that at 4 dpf yolk is diminishing which stimulates a starved state and at 5 dpf a transitional period from endogenous to exogenous feeding starts when larvae undergo a remarkable metamorphosis in preparation for ingesting food [55]. Recently, in rat β-cells, it has been shown that a drastic nutritional shift (fat-enriched maternal milk is replaced by a carbohydrate-rich diet) that occurs at weaning (20-day-old) results in a switch in the expression of metabolic enzymes and the acquisition of glucose-induced insulin release. Newborn rat β-cells lack glucose induced insulin secretion and show very low expression of genes involved in glucose metabolism [26]. We also showed that overnight culture of the zebrafish pancreatic islets increase the glucose responsiveness of the β-cells that can be due to K_{ATP}-independent pathways. However, the underlying mechanism should be further investigated. For this purpose, a mutant that lack K_{ATP} channel and crossed with the Tg (ins:GCaMP6s) imaging line could help to investigate whether the β-cells indeed show K_{ATP}-independent glucose responses already at early developmental stages (data not shown). A mutant that lack 187 bp (basepair) at the C-terminal of the Kir6.2 subunit of the K_{ATP} channel is already generated and crossed with the Tg(ins:GCaMP6s) imaging line, however, studies are needed to find out if these mutants show defect in the glucose responsiveness at different developmental stages.
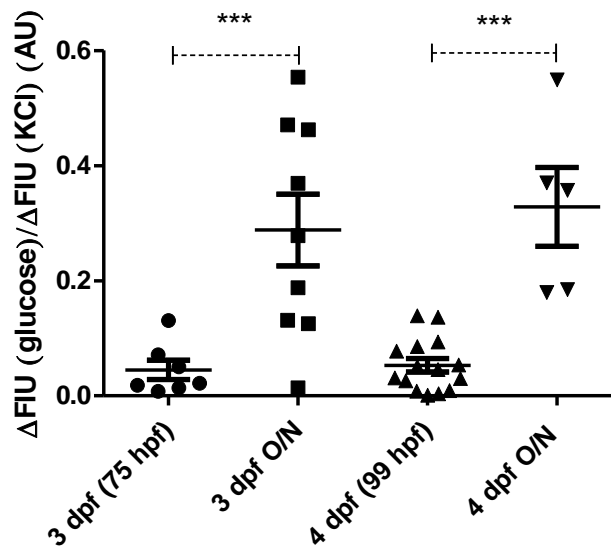
Figure 9: *Quantitation of GCaMP signal intensity in islets isolated from 3 dpf (75 hpf), 3 dpf O/N (overnight cultured), 4 dpf (99 hpf), and 4 dpf O/N (overnight cultured) larvae. Islets extracted from 3 dpf or 4 dpf old larvae and incubated at 29°C overnight show significant higher normalized relative FIU values ((ΔFIU (glucose)/ΔFIU (KCl)) in comparison to those islets that were isolated and imaged at 3 dpf (75 hpf) or at 4 dpf (99 hpf), respectively. FIU intensities for the whole islet were determined using Image-J and normalized to the FIU baseline (intensities measured in the first 2 min when 2 mM glucose was applied). Normalized relative FIU (normalized to baseline (response to 2 mM glucose) and relative to response to KCl) within each islets are showed as a fraction of total islet excitability at different developmental stages. FIU= Fluorescence Intensity Units (Arbitrary Units)*

3.3 Dysregulation of the L-type Ca_V1 channels impairs β-cell function in zebrafish

In order to confirm the sensitivity and specificity of our Tg(ins:GCaMP6s) imaging line in disease phenotypes and also to further investigate the role of the L-type Ca^{2+} channels in the islet function during embryonic pancreas development, Ca^{2+} imaging of the island beat/$Ca_V1.2$ mutants crossed with Tg(ins:GCaMP6s) imaging line was carried out. Since these mutants lack blood circulation, isolation of embryonic pancreatic islets is necessary to study potential changes in Ca dynamics as a consequence of the glutamine to stop codon alteration (isl m458) in the L-type calcium channel alpha1 subunit ($Ca_V1.2$). Based on our observation that zebrafish pancreatic β-cells do not show glucose-responsiveness until 4.5 dpf (*Figure 8.*), we decided to study the $Ca_V1.2$ mutant at 5 dpf. At this stage these mutants begin to die, but there are still
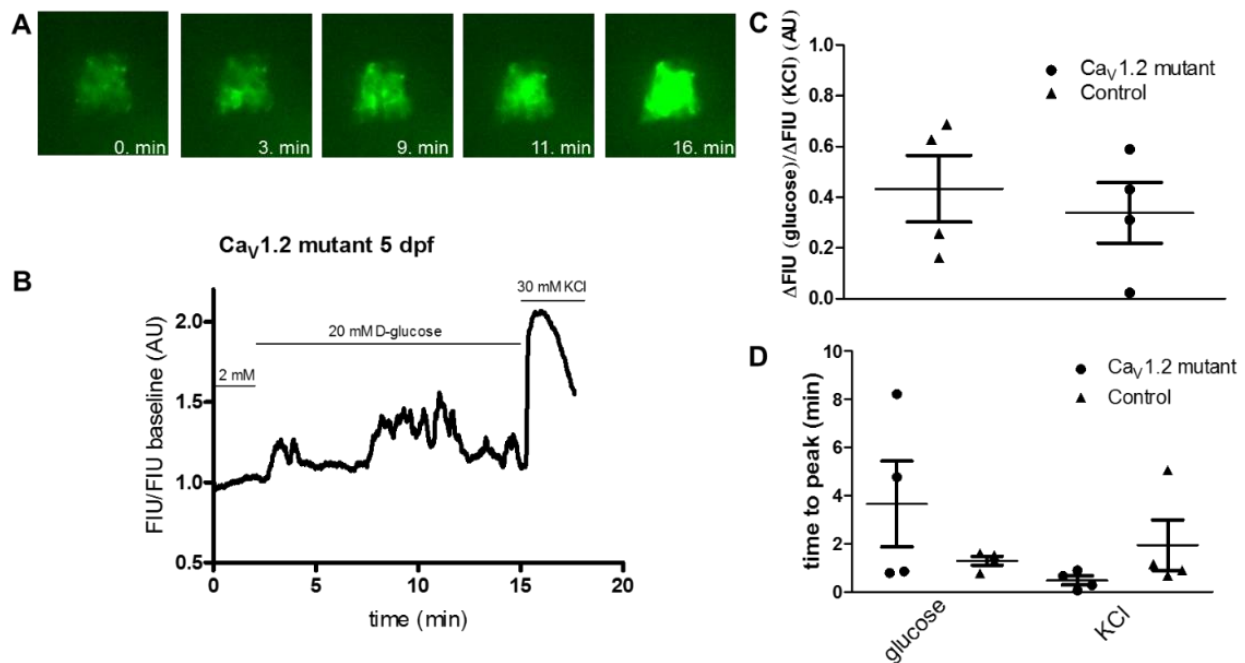
Figure 90: (A) *Fluorescence image series of an isolated islet from 5 dpf old CaV1.2 mutant crossed with Tg(ins:GCaMP6s ins:H2B:RFP) larva. After stimulation with 20 mM glucose solution there is calcium influx but not similar to the Wild type case. Membrane depolarization by KCl also showed calcium influx as expected.* **(B)** *Quantitation of GCaMP signal intensity in the time series shown in (A), showing FIU/FIU baseline changes after 20 mM glucose as well as after 30 mM KCl stimulation but not after 2 mM glucose. The total islet excitability was examined by membrane depolarization by 30 mM KCl that showed higher GCaMP6s signal intensity change (FIU/FIU baseline) in comparison to 20 mM glucose. FIU intensities for the whole islet were determined using Image-J and normalized to the FIU baseline (intensities measured in the first 2 min when 2 mM glucose was applied). FIU= Fluorescence Intensity Units (Arbitrary Units).* **(C)** *Normalized relative FIU (normalized to baseline (response to 2 mM glucose) and relative to response to KCl) within each islets are showed as a fraction of total islet excitability in Ca$_V$1.2 mutant and in Control larvae.* **(D)** *Time to peak values (minutes) in Ca$_V$1.2 mutant and in Control larvae calculated from the time point when higher glucose or KCl, respectively was changed on the isolated islets.*

living embryos that can be analyzed. The quantification of our dataset as the fraction of the total islet excitability does not show significance difference between Ca$_V$1.2 mutant and control larvae (*Figure 10./C*), however, the Ca^{2+} dynamics are distinctly different from those in control larvae at 5 dpf (*Figure 10./B* and *Figure 8./B*, respectively). It appears that in the Ca$_V$1.2 mutants the Ca^{2+} influx occurs more frequently (hyperactivation) after high glucose stimulation and the GCaMP6s signal does not return to the baseline after 1-2 minutes as in controls. The time to peak value after glucose stimulation also shows greater variability than in controls, although the difference is not significant (p=0,23). These observations are consistent with the findings that ablation of Ca$_V$1.2 in mice had no detectable effects on intracellular Ca^{2+} signaling except that the glucose-induced increase in cytoplasmic Ca^{2+} was delayed by ~1 min relative to that observed in control β-cells [35]. However, the mouse studies did not reveal modified Ca^{2+} influx patterns as we did. Based on our findings, disruption in the

Ca$_V$1.2 gene perturbs the intracellular Ca^{2+} dynamics in zebrafish pancreatic islet. The reason for the observed ''hyperactivity'' of the calcium channel-mediated Ca^{2+} influx in the zebrafish mutants remains unclear but we hypothesize that the long lasting hyperglycemia may hyperactivate the other Ca$_V$1 channels, as it does it in streptozocin-induced diabetic rats despite the blunted Ca^{2+} elevation [33]. We cannot exclude the possibility that there is a compensatory overexpression of other Ca$_V$ channels that show increased activity when Ca$_V$1.2 is ablated, as overexpression of Ca$_V$1.2 subunit proteins were observed in Ca$_V$1.3 subunit knockout mice [34]. Importantly, the obvious difference in the pattern of the voltage gated calcium channel-mediated Ca^{2+} influx in the Ca$_V$1.2 mutants in comparison to the control suggest that these mutants has an impaired β-cell function. Based on our measurements the Ca$_V$1.2 mutants show higher glucose level at 2,3 and even at 4 dpf *(Figure 3.)* and decreased insulin mRNA expression at 4 and 5 dpf (data are not shown) which also assume decreased insulin secretion. Further analyses are needed to investigate whether, similarly to mice [35], deletion in the Ca$_V$1.2 subunit also causes reduction in first phase insulin secretion and results in glucose intolerance in zebrafish larvae. However, our study makes clear that, as in mammals, dysregulation of L-type Ca$_V$1 channels also impairs β-cell function in zebrafish. Our findings show not only that the Tg(ins:GCaMP6s ins:H2B-RFP) imaging line is an excellent tool to study glucose-induced Ca^{2+} dynamics in zebrafish but also that zebrafish may be a convenient small animal model for further investigations of physiology and pathophysiology of β cell Ca$_V$1 channels.

4. Conclusion

Studying the response of pancreatic islet cells to glucose stimulation is important for understanding β-cell function in healthy and diseased states. However, imaging of insulin secretion, as well as Ca^{2+} dynamics in pancreatic β-cells has thus far been restricted to cell culture systems and extracted islets, preparations that might show different responses when compared to cells in an *in vivo* environment. Here we establish a transgenic zebrafish line encoding a membrane-localized beta-cell specific GCaMP6s Ca^{2+} sensor that allows us to monitor the intracellular Ca^{2+} dynamics not just in isolated pancreatic islets but also for the first time *in vivo*. We demonstrate that glucose-stimulated Ca^{2+} dynamics in pancreatic β-cells *ex vivo* is similar but not identical (different time-scale) to what we observe in living animals *in vivo*. We also show that adult zebrafish pancreatic β-cells possess glucose-dependent calcium oscillations starting above 10 mM glucose concentration. Furthermore, wildtype zebrafish pancreatic β-cells show different glucose-responsiveness during embryonic development, starting to response to D-glucose after 4.5 dpf, and we predict that metabolic pathways associated with glucose homeostasis start to be fully functional at 5 dpf, at onset of exogenous feeding. Furthermore, *ex vivo* physiological studies performed on islets from Cav1.2 mutants reveal that dysregulation of the L-type Ca_V1 channels impairs β-cell function in zebrafish, similar to mammals, and make zebrafish larvae a convenient small animal model for further investigations of physiology and pathophysiology of β-cell Ca_V1 channels. This study to monitor the intracellular Ca^{2+} dynamics in pancreatic β-cells *ex vivo* and for the first time *in vivo* expands the understanding of β-cell physiological function in healthy and diseased states of zebrafish. In addition, it may open new perspectives in pancreatic β-cell physiology during embryonic development that are necessary for a more complete understanding of the regulation of glucose homeostasis and the development of diabetes.

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