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The effect of amylin on NeuroD1 expression: from pancreas to the brain

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ABBREVIATIONS

AMY	amylin receptor
AP	area postrema
BBB	brain blood barriers
bHLH	beta helix-loop-helix
CNS	central nervous system
CTR	the calcitonin-receptor
DBD	DNA-binding domain
ECM	extracellular matrix
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSIS	glucose-stimulated insulin secretion assay
IAPP	islet amyloid polypeptide
IGT	impaired glucose tolerance
INS	insulin gene
INS-R	insulin receptor
KRBB	Krebs-Ringer bicarbonate buffer
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homologue A
MAPK-ERK	mitogen-activated protein kinase
NeuroD1	neurogenic differentiation-1
pro-IAPP	pro-islet amyloid polypeptide
PDX-1	pancreatic and duodenal homeobox-1
RAMPs	receptor activity modifying proteins
S18	ribosomal protein S18
TAD	trans-activating domain
T2D	type 2 diabetes

ABSTRACT

Transcription factors are involved in several processes such as the cell survival and differentiation, the embryonic development, neurogenesis and also in the regulation of the transcription of important peptides, such as insulin and amylin. Insulin gene transcription is mostly regulated by three β-cell-specific transcriptional regulators NeuroD1 (neurogenic differentiation-1), PDX-1 (pancreatic and duodenal homeobox-1) and MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homologue A), that have been demonstrated to play a crucial role in glucose induction of insulin gene transcription and pancreatic β-cell function.

The beta-helix-loop-helix transcriptional factor NeuroD1 controls insulin synthesis in pancreatic β-cells in combination with PDX-1 and MafA. Furthermore, NeuroD1 is known for its role in neuronal differentiation.

The satiating hormone amylin is co-secreted with insulin by pancreatic β-cells and it exerts its actions mainly in the *area postrema* (AP). Recent studies in our laboratory demonstrate that amylin upregulated NeuroD1 expression in the rat AP and that this response was blocked by the administration of amylin and the amylin receptor antagonist AC187. Thus, suggesting a potential role of amylin in the transcriptional regulation of NeuoD1 in the brain. Whether amylin has the potential to affect insulin transcription *via* NeuroD1 in the pancreas require further investigation.

Therefore, by using qPCR we investigated the transcriptional expression of NeuroD1, MafA, PDX-1 and insulin receptor (INS-R) in the AP and in the pancreas of mice. First, we confirmed the presence of NeuroD1, PDX-1 and MafA transcripts in the pancreas and in the AP of mice. Further, our results demonstrate that NeuroD1 mRNA expression increased after acute amylin treatment in the pancreas and that NeuroD1 transcript levels are also elevated in the mouse AP.

Since NeuroD1 acts in a co-ordinated and synergistic manner with PDX-1 and MafA to regulate insulin expression in β-cells, we then investigated the presence of PDX-1 and INS-R mRNAs in the pancreatic islets. Our results demonstrate that when glucose was administered at a high levels, PDX-1 and INSR transcripts were downregulated.

Thus, we reported that, in addition to its role in the regulation of insulin synthesis, NeuroD1 is a potential key mediator of the amylin signalling in the mouse AP.

RIASSUNTO

I fattori di trascrizione sono coinvolti in diversi processi come ad esempio la sopravvivenza e la differenziazione cellulare, lo sviluppo embrionale, la neurogenesi e anche nella regolazione della trascrizione di importanti peptidi, come l'insulina e l'amilina.

La trascrizione del gene dell' insulina è in gran parte regolata da tre specifici fattori di trascrizione beta cellulari quali NeuroD1 (neurogenic differentiation-1), PDX-1 (pancreatic and duodenal homeobox-1) e MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homologue A) che svolgono un ruolo fondamentale nell'induzione da parte del glucosio della trascrizione genica dell'insulina e della funzionalità delle cellule β del pancreas.

Il fattore di trascrizione NeuroD1, con struttura beta-elica-ansa-elica, controlla la sintesi di insulina nelle cellule β pancreatiche in combinazione con PDX-1 and MafA. Inoltre, NeuroD1 è noto per il suo ruolo nella differenziazione neuronale.

L'amilina, ormone che induce il senso di sazietà, è co-secreta con l'insulina dalle cellule β pancreatiche ed esercita la sua azione principalmente nell'*area postrema* (AP). Recenti studi condotti nel nostro laboratorio hanno dimostrato che l'amilina aumenta l'espressione di NeuroD1 nell'AP del ratto e che questa risposta viene bloccata se la somministrazione di amilina è accompagnata da quella con l'AC187, antagonista del recettore per l'amilina.

Pertanto, questi risultati suggeriscono un ruolo potenziale dell'amilina nella regolazione trascrizionale di NeuoD1 nel cervello. Laddove l'amilina possa potenzialmente influenzare la trascrizione di insulina nel pancreas attraverso la *via* di NeuroD1 richiede ulteriori indagini.

Pertanto, utilizzando la tecnica di qPCR abbiamo studiato l'espressione trascrizionale di NeuroD1, MAFA, PDX-1 e del recettore dell'insulina (INS-R) nell'AP e nel pancreas in modelli murini. In primo luogo, abbiamo confermato la presenza dei trascritti di NeuroD1, PDX-1 e MafA sia nel pancreas e nell'AP di topi. Inoltre, i nostri risultati dimostrano che l'espressione dell'mRNA di NeuroD1 aumenta dopo il trattamento intensivo con amilina nel pancreas e che i livelli di trascrizione di NeuroD1 sono elevati nell'AP di topo.

Siccome NeuroD1 agisce in maniera coordinata e sinergica con PDX-1 e MafA per regolare l'espressione dell'insulina nelle cellule β pancreatiche, abbiamo voluto studiare anche la presenza dei trascritti di mRNA di PDX-1 e INS-R nelle isolette pancreatiche. I nostri risultati dimostrano che quando il glucosio viene somministrato in elevate concentrazioni, la trascrizione di PDX-1 e INS-R viene inibita.
In conclusione, abbiamo riportato che, oltre al suo ruolo nella regolazione della sintesi di insulina, NeuroD1 è potenzialmente un mediatore che esercita un ruolo fondamentale nella via di signalling dell'amilina nell'AP di topo.

The research activity was performed in the Professor Thomas Alexander Lutz Laboratory at the Institute of Veterinary Physiology, Zurich, Switzerland (Sector: Zurich Center for Integrative Human Physiology, ZIHP).

L'attività di ricerca è stata svolta presso il laboratorio del Professore Thomas Alexander Lutz nell' Istituto di fisiologia veterinaria, Zurigo, Svizzera (Settore: Fisiologia Integrativa Umana, ZIHP).

1. INTRODUCTION

1.2 The pancreas

The pancreas is an endocrine and exocrine organ located across the back of the abdomen, behind the stomach. Anatomically, it is divided in two parts called the head and the body, respectively. The head is the large part of the organ and lies in the curve of the duodenum, which is the first section of the small intestine, while the body of the pancreas ends near the spleen. The pancreas is a dual-function gland, with both endocrine and exocrine functions. The exocrine part of the pancreas secretes digestive enzymes such as trypsin, lipase, amylase and many others. Those enzymes are secreted into a network of ducts that join the main pancreatic duct. The exocrine gland it is constituted by acini, which are formed of zymogenic cells around a central lumen and are arranged in lobules (Edlund H., 2001; Sander M. and German M.S., 1997; Slack J.M., 1995).

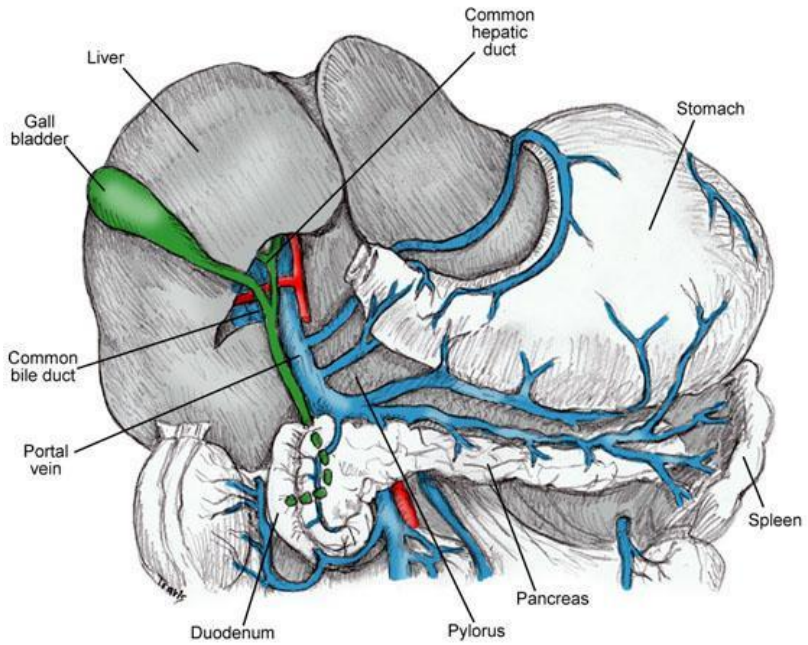


Figure 1. *Anatomy of the pancreas*. The pancreas is located behind the stomach with its head surrounded by the duodenum. The main pancreatic ducts, such as the common hepatic duct and the common bile duct are visible. (http://emedicine.medscape.com)

The endocrine pancreas relies on clusters of cells called islets of Langerhans (Edlund H., 2001; Wilson M.E. et al., 2003). One-two % of the total mass of the adult pancreas is constituted by mature pancreatic islets (Naya F. et al.; 1997).

Four principal endocrine cell types exist in the islets and their secretion can be used to classify them: the α-cells secrete the peptide hormone glucagon, the β-cells store and release insulin and amylin, the δ-cells are somatostatin-producing cells and the PP cells produce the pancreatic polypeptide (Naya F.et al., 1997; Slack J.M. 1995; Wilson M.E. et al., 2003).Therefore, in this micro-organ, this cluster of cells works in a co-operative manner to maintain euglycemia. The ~65–80% of the total number of endocrine cells is composed by the β-cells, since those are the most abundant, while the remaining endocrine cell types are more distributed at the periphery of the islets (Naya F. et al.; 1997). In rodents, it has been reported that the islet architecture is organised when the β-cells are located predominately in the central core with α-cells and δ-cells localized in the periphery forming a mantle (Kim A. et al., 2009; Striegel D.A. et al., 2015).

Several studies suggested that the importance of the structure of the islets must be intact, to ensure a proper functionality of the normal islets, which is regulating glucose homeostasis. An impairment of pancreatic β-cell function readily causes disorders of glucose homeostasis such as diabetes (Halban P.A. et al., 1982; Lucas-Clerc C. et al., 1993; Orci L., 1982; Pipeleers P. et al., 1982).

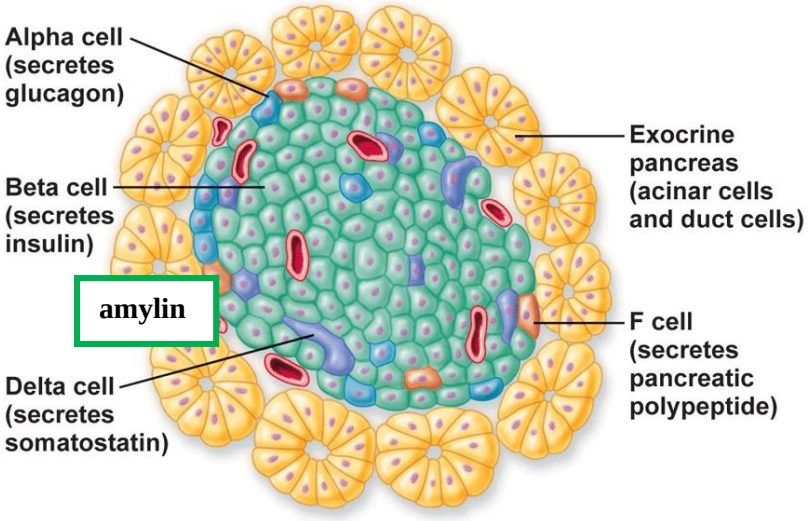


Figure 2. *Anatomy of the islet of Langerhans*. Picture showing the disposition of the four different cells types and their secretions. (http://quasargroupconsulting.com**)**

1.3 Amylin

Amylin (also known as "islet amyloid polypeptide", IAPP) is co-secreted with insulin by pancreatic β-cells (Lutz T.A., 2013; Woods S.C. et al., 2006). Amylin acts as a satiation signal in the central nervous system (CNS) to reduce food intake by activating specific amylin-sensitive neurons in the *area postrema* (AP) (Lutz T.A.; 2010). The AP belongs to the family of sensory circumventricular organs, which lack a brain blood barrier (BBB) and thus is in direct contact with toxins and potential harming substances transported in the blood. The AP is a major brain area that mediates the amylin effects on eating, gastric emptying, glucagon secretion and potentially on energy expenditure (Potes C.S. and Lutz T.A., 2010; Riediger T. et al., 2001). Following a substantial meal, amylin slows gastric emptying and inhibits glucagon secretion. Glucagon is the peptide hormone produced by alpha cells of the pancreas, which is known to exert the opposite effect of insulin (Young A.; 2005).

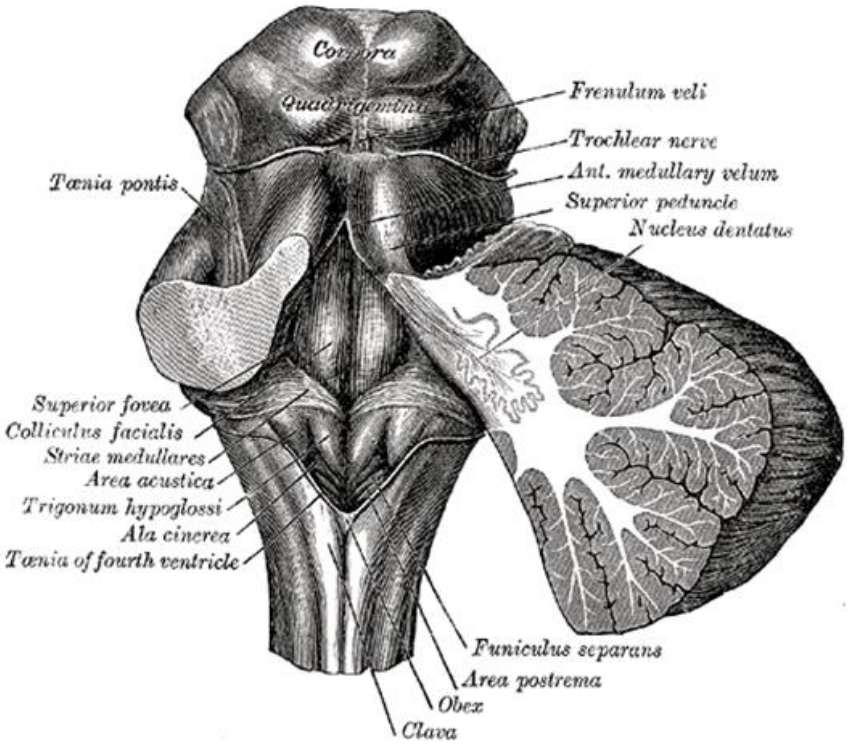


Figure 3. *Rhomboid fossa. (Area postrema labeled at bottom center).* The privileged location of the *area postrema* in the brain also allows it to play a vital role in the control of autonomic functions by the central nervous system. (Henry Gray; 1918: Anatomy of the Human Body)

Amylin is a 37 amino acid peptide and it is amidated on its carboxyl terminus and has a disulphide bond between cysteine residues at positions 2 and 7 (Betsholz C. et al., 1989; Roberts A.N. et al.,1989; Sanke T. et al.; 1988). Amylin is processed from the precursor pro-islet amyloid polypeptide (pro-IAPP, pro-amylin, pro-islet protein) that consists of 67 amino acids (Suva M. et al.; 2015). In the β-cells, amylin undergoes a series of post-translational modification, including cleavage that lead the peptide to its biologically active form IAPP (Higham C.E. et al., 2000; Sanke T. et al., 1988). Amylin is the principal constituent of the amyloid deposits that form in the islets of Langerhans in patients with type-2 diabetes mellitus (Cooper G.J. et al., 1987; Lorenzo A. et al., 1994; Westermark P. et al., 1987; Westermark P., 1986), but its role in the pathogenesis of this disease is unresolved (Leighton B. et al., 1988; O'Brien T.D. et al., 1993; Johnson K.H. et al.; 1989). Amylin and insulin are co-secreted from pancreatic β-cells in response to meals in a molar ratio of approximately 1:100 (amylin: insulin).

Once secreted by pancreatic islets, amylin goes into the bloodstream and it is finally eliminated in the kidney by the proteolytic activity of IAPP degrading enzymes (Bellia F., Grasso G.; 2014). The half-life of the secreted amylin is therefore 15 minutes (Young A.A. et al.; 1993). Although little is known about amylin regulation, its connection to insulin indicates that regulatory mechanisms that affect insulin secretion also affect amylin secretion.

Amylin binds to the amylin receptors (AMY) which consist of a heterodimer of the calcitonin-receptor (CTR) and one or several member of the receptor activity modifying proteins (RAMPs) (Hay D.L and Christopoulos G. et al., 2004; Liberini C.G. et al., 2016). It has been demonstrated that amylin is involved in the eating-inhibitory effects by activating the ERK signalling pathway in AP- neurons expressing AMY (Potes C.S. et al.; 2012). The MAPK-ERK (mitogen-activated protein kinase) family includes ERK1/2 cascade member which is involved in various cellular processes (Chang L. and Karin M., 2001; Torii S. et al., 2004). The ERK phosphorylation is important for the activation of gene transcription and also leads to acute neuronal responses, such as activation or inhibition of ion channels that directly and quickly affect neuronal excitability (Nishimoto S. and Nishida E., 2006; Yuan L.L. et al., 2002). Furthermore, previous studied reported that amylin time and dose-dependently activates the extracellular signal-regulated kinase 1 and 2 (ERK 1/2) cascade by inducing ERK

phosphorylation (Potes C.S. et al.; 2012). In addition, after 10-15 minutes of acute amylin administration, the number of pERK-positive AP-neurons is highest which also matches the satiation effect of amylin because the latter also has its maximum at this time window (Lutz T.A. et al., 1995; Potes C.S. et al., 2012).
All these findings support the hypothesis that ERK signalling cascade is employed by amylin to induce meal termination in the AP.

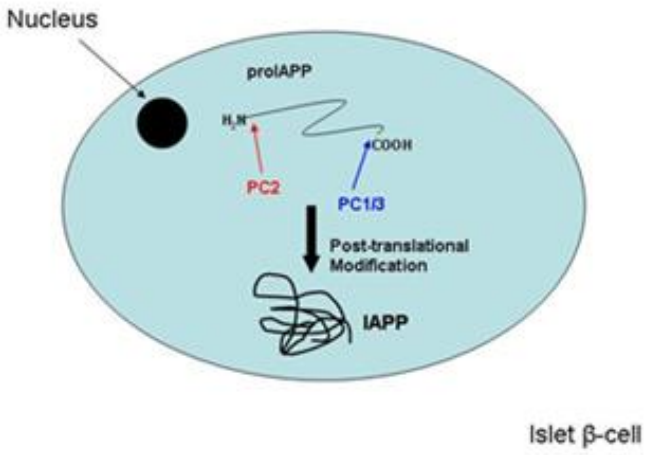


Figure 4. *Post-translational modification of pro-IAPP to form IAPP*. The pro-IAPP, after released from the signal peptide, undergoes additional proteolysis and post-translational to form active IAPP. (https://www.researchgate.net)

Amylin and insulin share a common regulatory promoter motif and consequently, both are regulated by similar factors (Butler P.C. et al., 1990; Cooper G.J., 1994; Höppener J.W. et al., 2000). These hormones enforce the nutritional flow and seem to have an important role in the physiological process of the organism (Lutz T.A.; 2013).

1.4 Insulin

Insulin is a peptide hormone produced by β-cells in the pancreas which plays a major role in the regulation of glucose and thus in the maintenance of energy homeostasis. It regulates the metabolism of carbohydrates and fats by promoting the absorption of glucose from the blood to skeletal muscles and fat tissue (Sonksen P. and Sonksen J., 2000; Zhuo F. et al., 2013).

In the case of the Types 2 diabetes (T2D), there is an insulin resistance: the body does not properly regulate insulin production and this causes an increase of this hormone into the bloodstream (Guanyu W.; 2014). The hyperglycaemia, that characterizes chronic diabetes, is combined with an insufficient secretion of insulin from pancreatic β-cells (Poitout V. et al.; 2006). The β-cells are unable to secret adequate amounts of insulin to compensate the decrease of insulin sensitivity, and this involves in a significant loss of functional β-cells and into an insulin secretory dysfunction (Butler A.E. et al., 2003; Cozar-Castellano I. et al., 2006; Kahn S.E. et al., 2006). The progression of the diabetes disease leads in an increase of β-cells apoptosis.

Diabetes and the insulin secretory dysfunction is also associated to other pathological cases including impaired glucose tolerance (IGT) and obesity (Boden G., 1997; Nolan C.J, 2006; Pietropaolo M. and Le Roith D., 2001; Polonsky K.S., 2000; Prentki M. and Rhodes C.J., 2005).

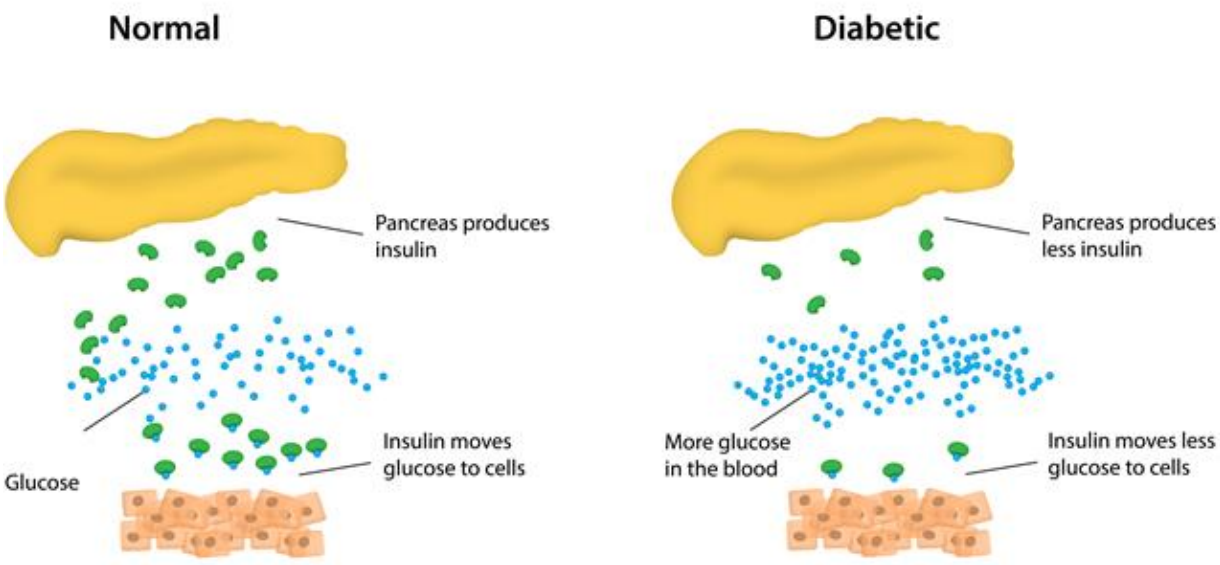


Figure 5. *Type 2 diabetes condition*. Insulin resistance is the inability of the body to properly use insulin. During normal condition, insulin is produced and moves efficiency glucose to cells, while in the diabetic condition the insulin release is strictly compromised. This involves an overtime blood glucose that will build up causing loss of body energy and tissue damage. (http://diabetesnewsjournal.com)

Insulin is synthesized in the pancreas within the β-cells of the islets of Langerhans. It is however first synthesized as a single polypeptide called pre-pro-insulin and processed to pro-insulin: the signal peptide is cleaved as the polypeptide is translocated into lumen of the endoplasmic reticulum, forming proinsulin (Ronald Kahn C. et al.; 2005).

Proinsulin is then converted to insulin and C-peptide and stored in secretary granules awaiting release on demand (Zhuo F. et al.; 2013).

Glucose metabolism plays an important role in stimulating the production of insulin and is the major physiologic regulator of the insulin gene transcription and mRNA translation (Poitout V. et al.; 2006). Moreover, the release of the insulin hormone is characterized by a specific binding with its receptor, the insulin receptor (INS-R).

The INS-R is a tyrosine kinase receptor that plays a key role in the regulation of glucose homeostasis (Chiu S.L. et al., 2008; Ward C.W. and Lawrence M.C., 2009).

Furthermore, previous studied demonstrated that in normal β-cells the mRNA for the insulin receptor was expressed (Harbeck M.C. et al.; 1996).

In addition, considering the molecular level, several processes altered the insulin action cascade and this implied a downregulation of the receptor and a decrease in the receptor kinase activity (Kulkarni R.N. et al.; 1999). Moreover, it has been reported that transcription factors such as PDX-1, MafA and NeuroD1 regulate insulin production based on glucose concentration (Andrali et al., 2008; Hay C.W. and Docherty K., 2006; Zhuo F. et al., 2013).

Finally, in the brain, it has been reported that insulin receptor signalling is involved in neuronal survival, synaptic plasticity, learning and memory (Dou J.T. et al., 2005; Grillo C.A. et al., 2015; Valenciano A.I. et al., 2006; Zhao W. et al., 1999).

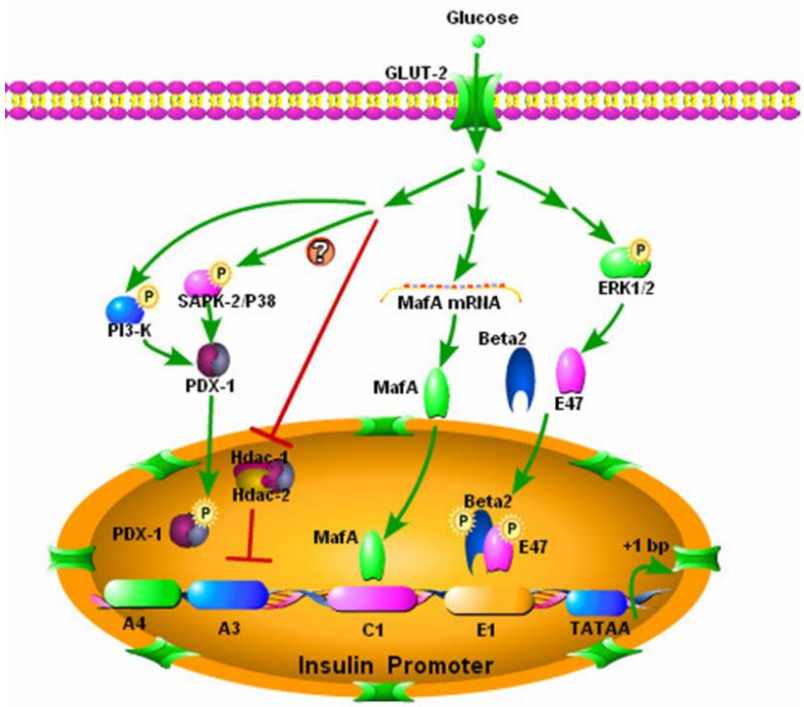


Figure 6. *The main transcriptional factors involved in insulin synthesis.* In response to glucose concentration insulin activity is regulated by three main transcriptional factors NeuroD1 (BETA2/Neurogenic differentiation 1), PDX-1 (pancreatic and duodenal homeobox-1, and MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homologue A). Glucose metabolism causes a shift of transcription factors NeuroD1 and PDX1 from the cytoplasm to the nucleus inducing post translation modifications. Glucose affects MafA at the mRNA level. Stimulatory glucose levels increase MafA transcription and result in increased MafA protein. (Ren J. et al.; 2007)

1.5 The transcription factors

Transcription factors are proteins that bind to specific DNA sequences, thereby controlling the rate of transcription of genetic information from DNA to messenger RNA (Latchman D.S.; 1997).

The main role of these proteins is to promote the activity of the RNA polymerase to specific genes. Transcription factors can act alone or in combination with other factors and they can act as activators or repressors by promoting or blocking the induction of the RNA polymerase, respectively (Lee T.I. and Young R.A., 2000; Roeder R.G., 1996).

The mechanism of action is controlled by their DNA-binding domains, that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. The transcription of the adjacent gene is either up- or down-regulated by the activity of the transcription factor (Latchman D.S.; 1997).

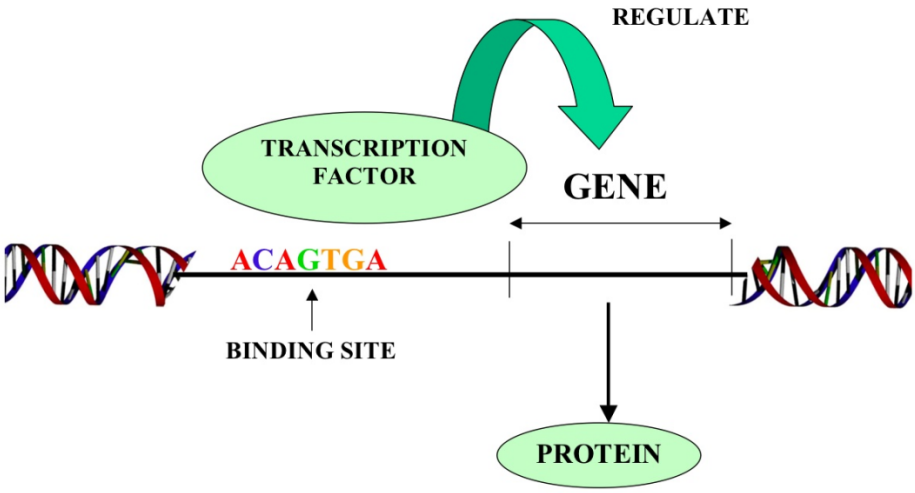


Figure 7. *The activity of the transcription factors.* Picture showing a transcription factor molecule that binds to the DNA at the binding site, and thereby regulates the production of a gene. (http://www.bio.miami.edu)

Transcription factors are modular in structure and contain two main domains: the DNA-binding domain (DBD) and the trans-activating domain (TAD). The DBD attaches to specific DNA-sequences: in general transcription factors bind to core promoter regions through recognition of common elements such as TATA boxes and initiators (Farnham P.J.; 2009). The trans-activating domain contains binding sites for other proteins such as transcription co-regulators (Wärnmark A. et al.; 2003).

These additional proteins interact with transcription factors to either activate or repress the transcription of specific genes. Although they play crucial roles in gene regulation, they are not classified as transcription factors because they lack DNA binding domains (Wärnmark A. et al.; 2003).

Transcription factors could exert their function alone or in a co-ordinated and synergistic manner with others.

In the pancreas, NeuroD1, PDX1 and MafA cooperate together to stimulate INS expression in response to increased glucose levels (Matsuoka T.A. et al., 2007; Ohneda K. et al., 2000; Song Y.D. et al., 2007).

Moreover, transcription factors control biological processes such as differentiation, proliferation, and apoptosis (Guo Q.S. et al.; 2012). Their actions allow for unique expression of each gene in different cell types, during development and adult life (Guo Q.S. et al.; 2012).

Consequently, this essential function for the regulation of gene expression is found in all living organisms. Therefore, it is not surprising that failure of transcription factor function can result in several diseases, most of which are characterized by malformation syndromes. (Latchman D.S., 1997; Villard J., 2004).

1.5.1 NeuroD1

1.5.1.1 NeuroD1 in pancreas development

NeuroD1 is a member of the NeuroD family of (bHLH) transcription factors (Naya F. et al; 1997). The protein forms heterodimers with other bHLH proteins and activates the transcription of genes that contain a specific DNA sequence known as E-box (Chae J.H.; 2004). bHLH mainly control determination, differentiation and cell fate in various tissues types during embryonic development (Naya F. et al.; 1995). Specifically, NeuroD1 is involved in β-cells survival during development and directly binds to the insulin gene (INS) promoter to regulate INS mRNA transcription in the pancreas, in adults (Chae J.H., 2004; Guo Q.S. et al; 2012). It has been shown that NeuroD-null mice exhibited severe glucose intolerance when the expression of NeuroD1 is lacking in insulin-expressing cells (Gu C. et al.; 2010). Moreover, after birth, these mice die immediately due to severe diabetes. In addition, both the β-cells architecture and their majority are poorly differentiated or lost (Naya F. et al.; 1997).

NeuroD1 is also a very important regulator for the proper development, differentiation and survival of pancreatic endocrine cells, especially of insulin and glucagon gene transcription (Andrali S.S. et al., 2008; Naya F. et al., 1997).

Furthermore, it has been shown that glucose regulates the nuclear localization as well as the transactivation capacity of NeuroD1 via post-transcriptional modification (Andrali S.S. et al.; 2008).

To define how NeuroD1 activity is controlled in glucose-stimulated β-cells, previous studies in MIN6 β-cells suggested that NeuroD1 is in the nucleus under stimulating glucose conditions (20mM) and predominantly cytoplasmic at low, non-stimulating glucose concentration (3mM) (Petersen H.V. et al.; 2002). Moreover, NeuroD1 is modified by phosphorylation through MAPKs-ERK1/2 signalling (Arnette D. et al., 2003; Khoo S. et al., 2003; Lawrence M. et al., 2008). In the presence of high glucose, specifically ERK2 enhanced the transactivation capacity of NeuroD1 by acting at multiple sites (Khoo S. et al.; 2003).

Although NeuroD1, PDX-1 and MafA have been shown to be crucial for glucose regulation of INS transcription, the exact mechanisms by which glucose modulates the function of these transcription factors still remains to be elucidated.

Figure 8. *Co-ordinated and synergistic activation of insulin gene expression by Pdx-1, NeuroD1 and MafA.*

Picture shows the three transcription factors with their DNA-binding domains, that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. In this picture, Pdx-1 binds to the A-boxes (A3), NeuroD1 to the E-boxes (E1) and MafA to the C1 element within the 400 bp region of the insulin promoter, and activate insulin gene expression depending on different concentration in blood glucose levels. (Andrali S.S. et al.; 2008)

1.5.1.2 NeuroD1 in neuronal development

NeuroD1 is also involved in neurogenesis (Boutin C. et al., 2010; Moore K.B. et al., 2002). Several studies demonstrated that NeuroD1 promotes premature cell cycle exit and differentiation of neural precursor cells into neurons, both during embryonic life and adulthood (Lee J.E. et al., 1995; Miyata T. et al., 1999; Schwab M. H. et al., 2000). Thus, NeuroD1 acts as differentiation factor (Lee J.E. et al., 1995; Miyata T. et al., 1999; Schwab M. H. et al., 2000). NeuroD1 is expressed widely throughout the developing CNS, the auditory and the vestibular systems (Kim W. I. et al.; 2001).
Recently, preliminary work in our laboratories demonstrated that, in rats fasted for 12 hours and treated with either amylin, amylin plus the amylin receptor antagonist AC187 or vehicle, NeuroD1 mRNA was strongly upregulated (15 fold change) in the AP of adults rats after amylin treatment. Moreover, this response was successfully blocked by the administration of AC187. Further, the up-regulation of NeuroD1 mRNA was positively correlated with an increase (2 fold change) in the transcriptional expression of insulin receptors (INS-R) in the AP.

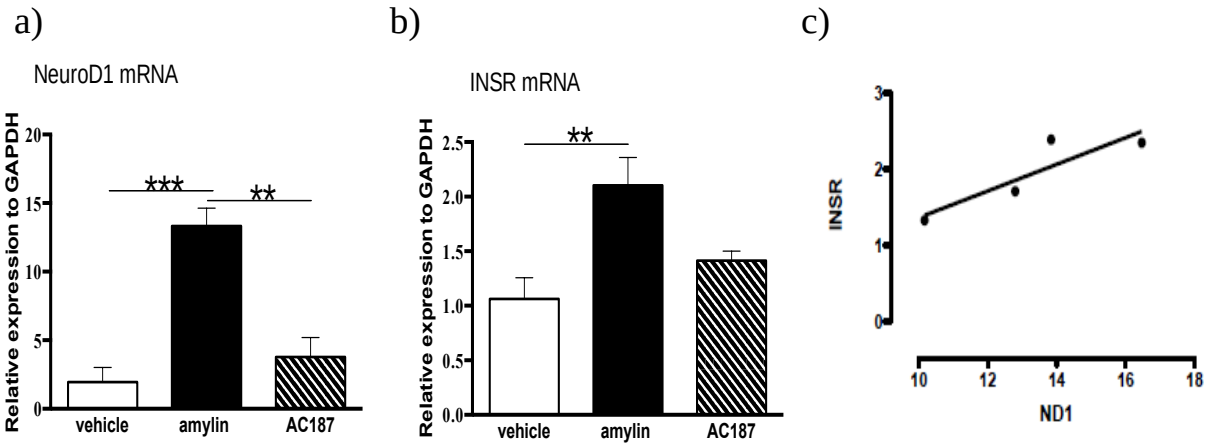


Figure 9. In rats (n= 8-10/treatment group) fasted for 12 hours, (a) amylin-treatment increased ND1 mRNA expression (13-fold change) in the AP, and this effect was reversed when rats were treated with amylin and AC187 (one-way-ANOVA; p value = 0.0002.) (b) Amylin-treatment also affected the expression of INS-R (2-fold change) in the AP (one-way-ANOVA; p value = 0.0102). (c) Increasing ND1 mRNA expression is moderately correlated with an increase in the expression of the insulin receptor (linear regression, p value = 0.1143) (Liberini CG. et al; 2016).

1.5.2 PDX-1

PDX-1 is a transcription factor that belongs to the homeodomain family of proteins and it is important for the embryonic development of the pancreas, including β-cell maturation and duodenal differentiation (Hui H. and Perfetti R., 2002; Jonsson J. et al., 1994; McKinnon C. M. and Docherty K., 2001; Mosley A.L. et al., 2004; Offield M.F. et al., 1996). In mice embryonic development, PDX-1 is first expressed after the endoderm begins to form a primitive gut tube around the embryonic day 8.5 (Jonsson J. et al.; 1994).

It has been reported that homozygous PDX-1 knockout mice fail to develop a pancreas and die immediately, because of a lack of insulin. Moreover, heterozygous PDX-1 mice are hyperglycaemic although they developed a normal pancreas, and this is caused by a decrease of insulin production (Ahlgren U. et al, 1996; Andrali S.S. et al., 2008; Jonsson J. et al., 1994). Thus, these findings indicate that PDX-1 is essential for the early pancreatic development and it plays a fundamental role in β-cell maturation and function.

Once the full development of the pancreas is completed, PDX-1 expression is mainly restricted to the insulin-producing β-cells and somatostatin producing δ-cells within the pancreatic islets (Andrali S.S. et al.; 2008).

Moreover, PDX-1 has been shown to be fundamental for the transactivation of several pancreatic genes including insulin, somatostatin, islet amyloid polypeptide (IAPP), glucose transporter GLUT-2 and MafA, as well as auto-regulating its own expression (Miller C. P. et al. , 1994; Raum J. C. et al., 2006; Serup P. et al., 1996; Waeber G. et al., 1996; Watada H. et al., 1996).

1.5.3 MafA

MafA is a basic- leucine- zipper (bLZ) transcription factor, belonging to the large Maf transcription family. It controls β-cell-specific expression of the insulin gene through a cis-regulatory element called RIPE3b1 and functions as an important transactivator for the insulin gene (Kataoka K. et al., 2002; Matsuoka T. A. et al., 2003; Olbrot M. et al., 2002). Previous studies reported that MafA deficient-mice developed diabetes as the result of reduced insulin expression.

Further, MafA deficient-mice showed deficits in insulin secretion and deterioration of islets architecture (Kaneto H. et al., 2008; Zhang C. et al., 2005).

During murine pancreas development, MafA begins to be expressed in the principal phase of insulin-producing cells production and becomes detectable at embryonic day 13.5, suggesting a role for MafA in β-cells maintenance (Matsuoka T. A. et al., 2004; Nishimura W. et al., 2006). Moreover, MafA is expressed later in development than NeuroD1 and PDX-1 and, as a peculiarity, it is only expressed in β-cells. Therefore, MafA is another key activator of the insulin gene transcription and a transcription factor that plays a crucial role for islet β-cell formation and function.

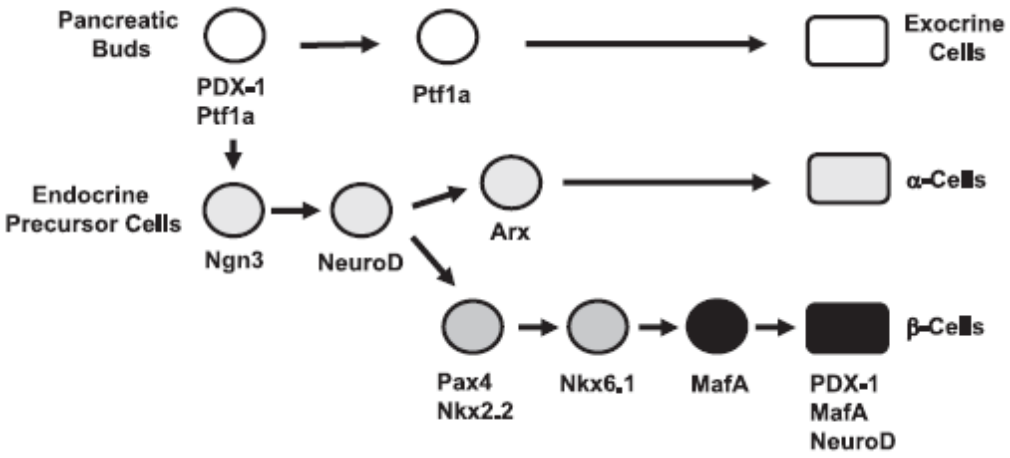


Figure 10. *A simplified model of pancreatic transcription factor hierarchy during pancreas development.* The development of the mature pancreas involved several transcription factor and many interaction among them. Picture shows that PDX-1 and NeuroD1 are expressed from the early stage of the pancreas, instead MaFa expression is induced at the final stage of β-cells differentiation. (Kaneto H. et al.; 2008)

1.6 Clinical relevance: the Type-2 diabetes (T2D)

T2D is the most common form of diabetes in humans, accounting for about 90% of all cases. The disease primarily relates to obesity and affects more than 200 million people worldwide resulting in more than a million deaths from diabetes annually (Whiting D.R. et al., 2011; Zimmet P. et al., 2001). T2D is mainly due to a combination of two physiological defects: impaired insulin secretion and reduced peripheral and hepatic

insulin sensitivity (Bajaj M. and Defronzo R.A., 2003; Beck-Nielsen H. and Groop L.C., 1994). Moreover, those defects are followed by a compensatory increase in β-cell mass and insulin and amylin secretion to maintain normal blood glucose levels.

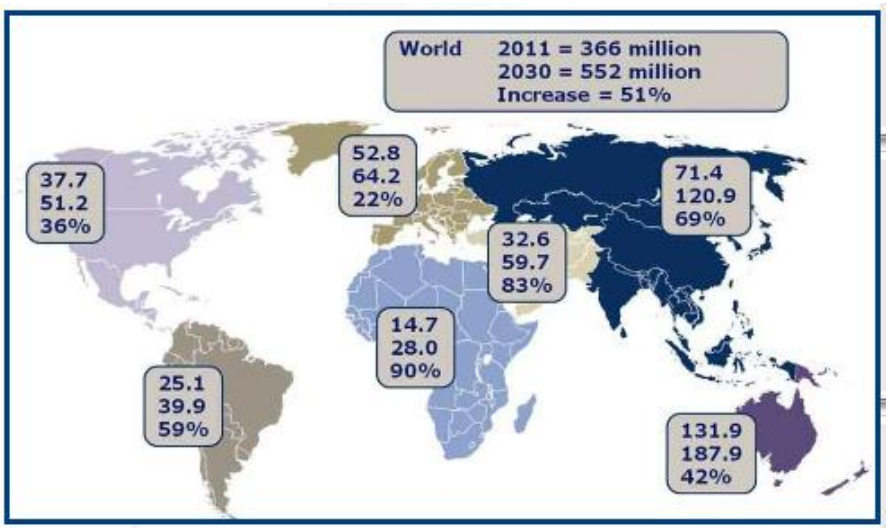


Figure 11. *Global projection for diabetes epidemic: 2011-2030.* (Whiting D.R. et al.; 2011).

In patients with TD2, amylin is the principal constituent of the amyloid deposits that form in the islets of Langerhans: high concentration of amylin favour the formation of toxic amylin oligomers and deposition of amylin fibrils (Zhang X.; 2016). In addition, the deposits of islet amyloid present in T2D diabetic pancreas may lower the functional β-cell mass (Kahn S.E. et al. 1999; Marzban L. et al., 2003). Importantly, only amylin in primates and cats has the propensity to form these deposits, and this role of amylin in the pathogenesis of TD2 has to be distinguished from its role as circulating hormone that has been described before. It is therefore not a contradiction that the non-amyloidogenic amylin analogue pramlintide is currently used in the treatment of both T2D and obesity because the pramlintide exerts its effect by inducing weight loss and by decreasing eating (Chapman I. et al.; 2005). To better characterize the mechanism of amylin action in the brain and in the pancreas will be clinically relevant for the treatment of obesity and possibly of TD2.

1.7 Aims of the project

1.7.1 Confirm the presence of NeuroD1, PDX-1, MafA and INS-R transcripts in the pancreas and in the AP of mice

Several studies reported that gene expression programs for developing neurons and pancreatic β-cells are remarkably similar and, as a consequence, the transcription factors that regulate the endocrine development are analogue to those that regulate brain development (Edlund H., 2002; Habener J.F. et al., 2005; Wilson M.E. et al.; 2003). One of the most important transcription factors is NeuroD1. Since NeuroD1 activates the transcription of the insulin promoter in pancreatic β-cells and because insulin and amylin are co-secreted by the pancreas, we investigated whether amylin could affect insulin transcription in the pancreas. Moreover, as a major brain area where amylin exerts its action is the AP, we also evaluate the potential role of amylin in the regulation of NeuroD1, PDX1 and MAFA mRNAs in the AP of mice. Finally, as the INS-R important for its specific bound with insulin hormone, we also investigate its mRNA expression after amylin treatment.

1.7.2 Investigate the presence of PDX-1 and INS-R mRNAs in pancreatic islets

Mature and functional islets of Langerhans regulate glucose homeostasis and are structured in four type of different cells that release specific hormones. We focused on β-cells that co-secrete amylin and insulin. Moreover, as the synthesis of insulin is activated by the action of several transcription factors, we investigate the presence of PDX-1 and INS-R mRNA to confirm the functionality of the islets upon glucose stimulation assay.

2. MATERIALS AND METHODS

2.1 Animals and tissue collection

Male C57/BL6 mice (Janvier, Le Genest Saint Isle, France; study 1; *n* = 4; study 2; *n* = 20; study 3; *n* = 8; all 20-25g) were group-housed in a temperature controlled environment (21±1°C) on an artificial 12h/12h light/dark cycle. Mice had *ad libitum* access to water and standard chow, except during fasting periods as described below. All procedures involving animals and their care were approved by the Veterinary Office of the Canton Zurich, Switzerland (license number 121/2012), and in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2 Experimental design

2.2.1 Study 1: Selection of the most appropriate housekeeping gene

The mRNA expression of three housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein S18 (RPS18) and Beta-actin, commonly used as internal controls in expression studies in mice, was measured in the AP, pancreas and liver of mice. Briefly, mice were euthanized by CO_2 inhalation and tissue samples were collected and snap-frozen in single-step RNA isolation reagent (the entire pancreas and liver in 800 µl; the AP in 100 µl of TRI Reagent®, Sigma-Aldrich, Switzerland) according to the manufacturer's instructions, and stored at -80°C until further investigation. Expression of candidate housekeeping genes in AP and pancreas were compared with liver samples used as internal control.

2.2.2 Study 2: Effects of amylin on NeuroD1, PDX-1, MAFA and INS-R expression levels in the pancreas and in the area postrema

Mice (n= 5 per group) were fed ad libitum or fasted for four hours. At the beginning of dark onset, mice were acutely treated with vehicle (0 .9% NaCl; 1ml/kg) or amylin (50µg/kg; i.p; Bachem AG, Bubendorf, Switzerland; catalogue number: H-9475.1000).

Ninety minutes after drug administration, mice were anesthetized with isoflurane and decapitated. Brains were promptly excised and the AP was surgically removed from the brainstem. At the same time, the pancreas and the liver were rapidly isolated from the body. All specimens were snap-frozen in single-step RNA isolation reagent TRI Reagent® (TRI Reagent®, Sigma-Aldrich, Switzerland) according to the manufacturer's instructions and stored at -80°C until further investigation.

2.2.3 Study 3: Pancreatic islet gene expression after culture at low-, and high-glucose concentrations

Islets were isolated from eight mice by collagenase digestion as described previously (Dong-Sheng Li et al.; 2014). Briefly, pancreata were perfused with a collagenase solution (Worthington, Lakewood, NJ) and digested in the same solution at 37°C, followed by filtration through 500- and 70-µm cell strainers, respectively. Islets were cultured in RPMI-1640 medium containing 11.1 mmol/L glucose, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamax, 50 µg/mL gentamicin, 1:1,000 Fungizone (Gibco, Thermo Fisher, Basel, Switzerland), and 10% FCS (Sauter et al.; 2015). Islets were allowed to adhere and spread on the extracellular matrix (ECM)-coated plates (at 12 islets/well) derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel) for 48 h before initiation of experiments (Sauter et al.; 2015). Islets were treated for 24 h with 2.8 or 16.7mmol/L glucose before RNA extraction or being used for glucose-stimulated insulin secretion experiments.

2.3 Reverse - transcription PCR

Total RNA was extracted from all tissues and pancreatic islets using the TRI Reagent® (300 µl per well/12islets) and then purified following the cleanup protocol of RNeasy® Mini kit (Quiagen, Germany), including the DNase step. The concentration and the integrity of the RNA were measured using a nanodrop system (NanoDrop 1000 Spectrophotometer, Thermo Scientific). cDNA was synthesized using random hexamer primers using the SensiFAST cDNA synthesis kit (Bioline, Switzerland).

The thermal cycler condition for the reverse transcription were the following: primers annealing step of 10 min at 25°C, followed by the reverse transcription step of 15 min at 42 °C and a final inactivation step for 5 min at 85 °C. RT-PCR amplification products were separated by 2% agarose gel electrophoresis.

2.4 Quantitative PCR (qPCR)

qPCR was performed using the 7500 Fast system (Applied Biosystem/Life Technologies) with QuantiTect® SYBR® green PCR kit (QUIAGEN).

To avoid amplification of contaminate DNA, intron-spanning primer pairs for mouse GAPDH, S18, Beta-actin, NeuroD1, PDX-1, MAFA and INS-R were designed with IDT (Integrated DNA Technologies). The primer sequences were the following; for mouse GAPDH, forward: 5'-AACAGCAACTCCCACTCTTC -3' and reverse: 5'-CCTGTTGCTGTAGCCGTATT'-3' (Accession: NM_008084.3), for mouse s18 forward 5'-GCGAGTACTCCACACCAACATC -3' and reverse 5'-CCTCAACACCACATGAGCATATC -3' (Accession: NM_011296.2), for mouse Beta-actin forward 5'- CCGTAAAGACCTCTATGCCAA -3' and reverse 5'-AGGAGCCAGAGCAGTAATCT -3' (Accession: NM_007393.3), for mouse PDX-1, forward: 5'- GATGTTGAACTTGACCGAGAGA -3' and reverse: 5'-TCTAAATTGGTCCCAGGAAAGAG -3' (Accession: NM_008814.3), for mouse MAFA, forward: 5'- TGGAGGATCTGTACTGGATGAG -3' and reverse: 5'-CCCGCCAACTTCTCGTATTT -3' (Accession: NM_194350.1) and for mouse INS-R, forward: 5'- GAAGTTGGGCAATGGGAATAAC -3' and reverse: 5'-ATAACCACCCAGGCACATAAA -3' (Accession: NM_010568.2).

100 ng of cDNA were subjected to an initial heat activation at 95°C for 2 minutes, followed by 40 cycles of denaturation 95°C for 5s then both the annealing and the final extension 60°C for 30 s.

In *study 2 and 3*, the relative transcriptional levels of NeuroD1, PDX-1, MAFA and INS-R mRNA were calculated using the comparative ΔΔCt method that generates relative NeuroD1, PDX-1, MAFA and INS-R mRNA levels adjusted for the GAPDH endogenous control mRNA. Each sample was run in duplicate.

2.5 Glucose-Stimulated Insulin Secretion (GSIS) Assay

To determine acute insulin release in response to glucose stimulation, islets were washed and preincubated (30 min) in modified Krebs-Ringer bicarbonate buffer (mKRBB; 115 mmol/L sodium chloride, 4.7 mmol/L potassium chloride, 2.6 mmol/L calcium chloride dihydrate, 1.2 mmol/L monopotassium sulfate, 1.2 mmol/L magnesium sulfate heptahydrate, 10 mmol/L HEPES, and 0.5% bovine serum albumin [pH 7.4]) containing 2.8 mmol/L glucose. The medium was then replaced with fresh mKRB containing 2.8 mmol/l glucose for 1 h for basal secretion, followed by an additional 1 h incubation in mKRB containing 16.7 mmol/l glucose (stimulated insulin release). Incubates were collected and frozen for insulin assays. Thereafter, islets were washed with PBS and extracted with 0.18N HCl in 70% ethanol for 24 h at 4°C to determine insulin content; the acid-ethanol extracts were collected and frozen for determination of insulin content. Insulin concentrations were determined using mouse insulin ultrasensitive ELISA (Mercodia, Uppsala, Sweden) (Sauter et al.; 2015).

2.6 Statistics

Data were analyzed with the GraphPad Prism program version 6.0 (San Diego, CA). Statistical significance was determined by one-way ANOVA with Bonferroni's or Dunnett's post hoc test for multiple-comparison analysis. Data is expressed as mean ± S.E.M. Significance was set at $P < 0.05$.

3. RESULTS

3. Selection of the most appropriate housekeeping gene

To select the most appropriate reference gene, the transcriptional expression of GAPDH, s18 and Beta-actin was measured in our target tissues AP and pancreas. The liver was used as internal positive control.

RT-PCR analysis revealed that GAPDH is the gene with the highest expression (e.g, lower CT values) compared to S18 and Beta-actin in the liver (Fig.1, One-way ANOVA; $P< 0.05$) and the AP (Fig.12, One-way ANOVA; $P< 0.05$). No significance difference between GAPDH, S18 and Beta-actin was found in the pancreas.

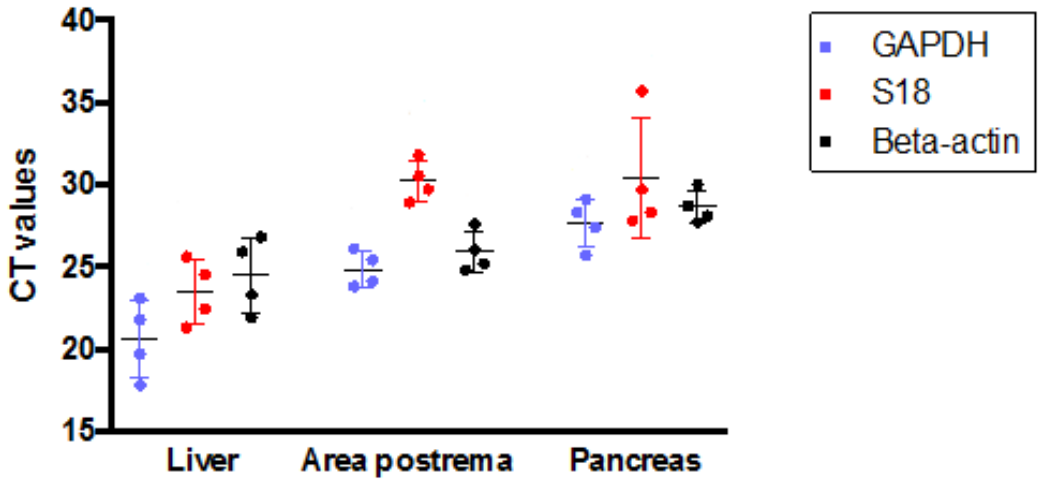
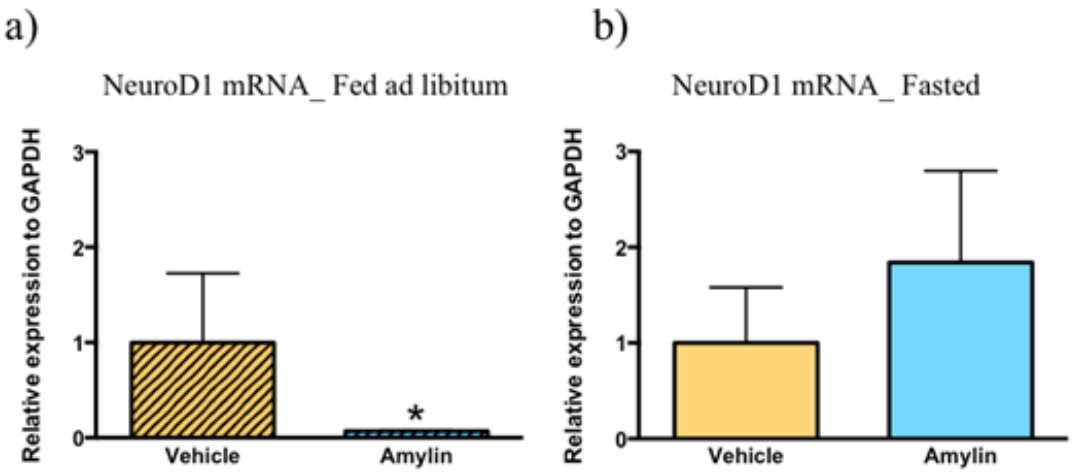


Figure 12. Differential expression of *GAPDH, s18 and Beta-actin in liver, AP and pancreas*. The mRNA level of target genes was measured in different tissues. Liver represents a reference-tissue positive control. In the liver GAPDH expression was significantly lowered compared to S18 and Beta-actin (One-way ANOVA; $P< 0.05$). In the AP GAPDH was significantly lowered compared to S18 and Beta-actin, respectively (One-way ANOVA; $P< 0.05$). Finally, no significance difference between GAPDH, S18 and Beta-actin was found in the pancreas.

3.2 Effects of acute amylin treatment on the transcriptional expression levels of NeuroD1, PDX-1, MAFA and INS-R in the AP and in the pancreas of mice fasted and fed ad libitum.

To investigate the effect of acute amylin and feeding *per se* on the mRNA levels of NeuroD1, PDX-1, MAFA and INS-R in the AP and in the pancreas of mice, we performed qPCR analysis. Mouse GAPDH was used as housekeeping gene and mRNA levels were expressed as fold changes.

In the AP of mice fed ad libitum, amylin significantly down-regulated the mRNA expression of NeuroD1 (Student's t-test; P=0.04, Fig. 13a) while no difference was found in the fasted status (Fig. 13b). Acute amylin administration significantly down-regulated the mRNA level of PDX-1 in animals that were fed ad libitum (Student's t-test; P=0.03, Fig. 13c) compared to controls. A potential trend in downregulation was observed for PDX-1 mRNA after fasting, although the difference did not reach the statistical significance (Fig. 13d). The mRNA expression of MafA in the AP was not statistically different after amylin in both fasted and fed status compared to the vehicle controls (Fig. 13e,f). Interestingly, INS-R mRNA expression was not measurable in any of the conditions tested in this study in the AP.



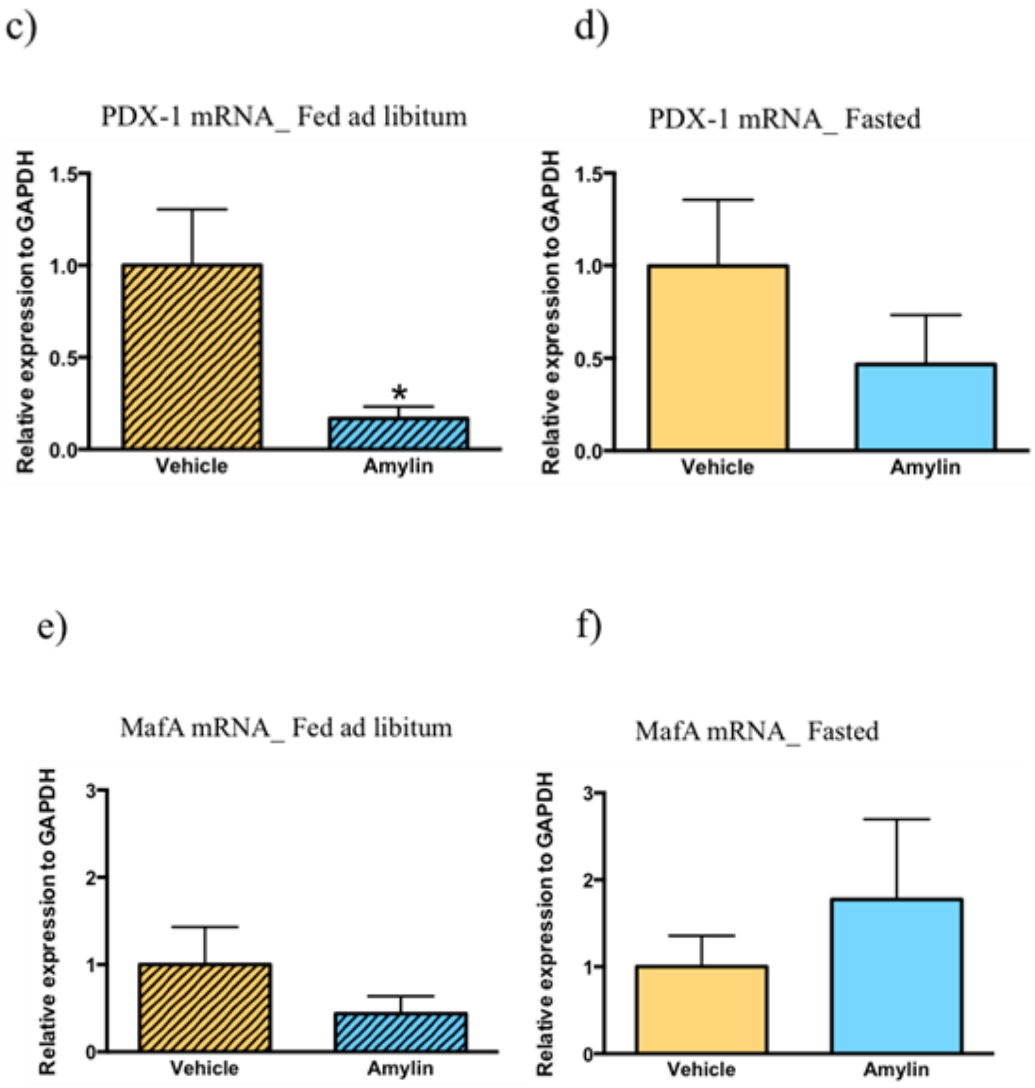
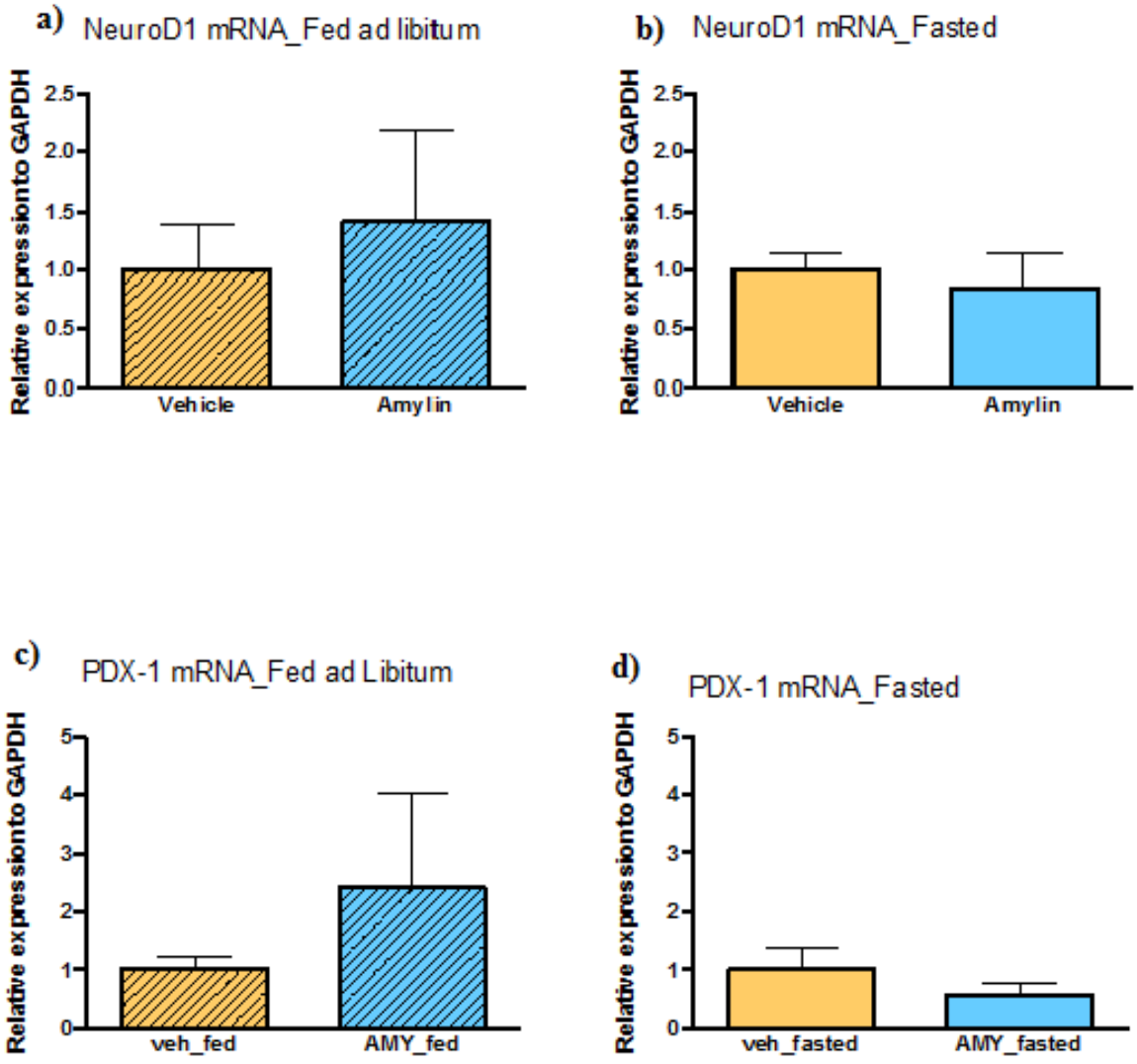


Figure 13. *Effects of amylin on NeuroD1, PDX-1 and MafA gene expression in the area postrema.*

Mice (n=5/group) were fed ad libitum or fasted for four hours and intraperiotoneally injected with either amylin (50 μg/kg) or vehicle (0.9% NaCl; 1ml/kg). mRNA expression level of NeuroD-1 at (a) fed or (b) fasted condition. mRNA expression levels of PDX-1 at (c) fed or (d) fasted condition. mRNA expression levels of MafA at (e) fed or (f) fasted condition Data is represented as mean ± SEM. Statistical significance was consider as $p < 0.05$.

In the pancreas of mice fed ad libitum or fasted, amylin did not exert any significant effect in the transcriptional regulation of NeuroD1 (Fig. 14a,b), PDX-1 (Fig. 14c,d) and MafA (Fig. 14e,f). However, acute amylin treatment significantly upregulated INS-R mRNA in the pancreas of mice fed ad libitum compared to control (Fig. 14g). No difference in INS-R transcriptional levels was observed in the fasted state (Fig. 14h).



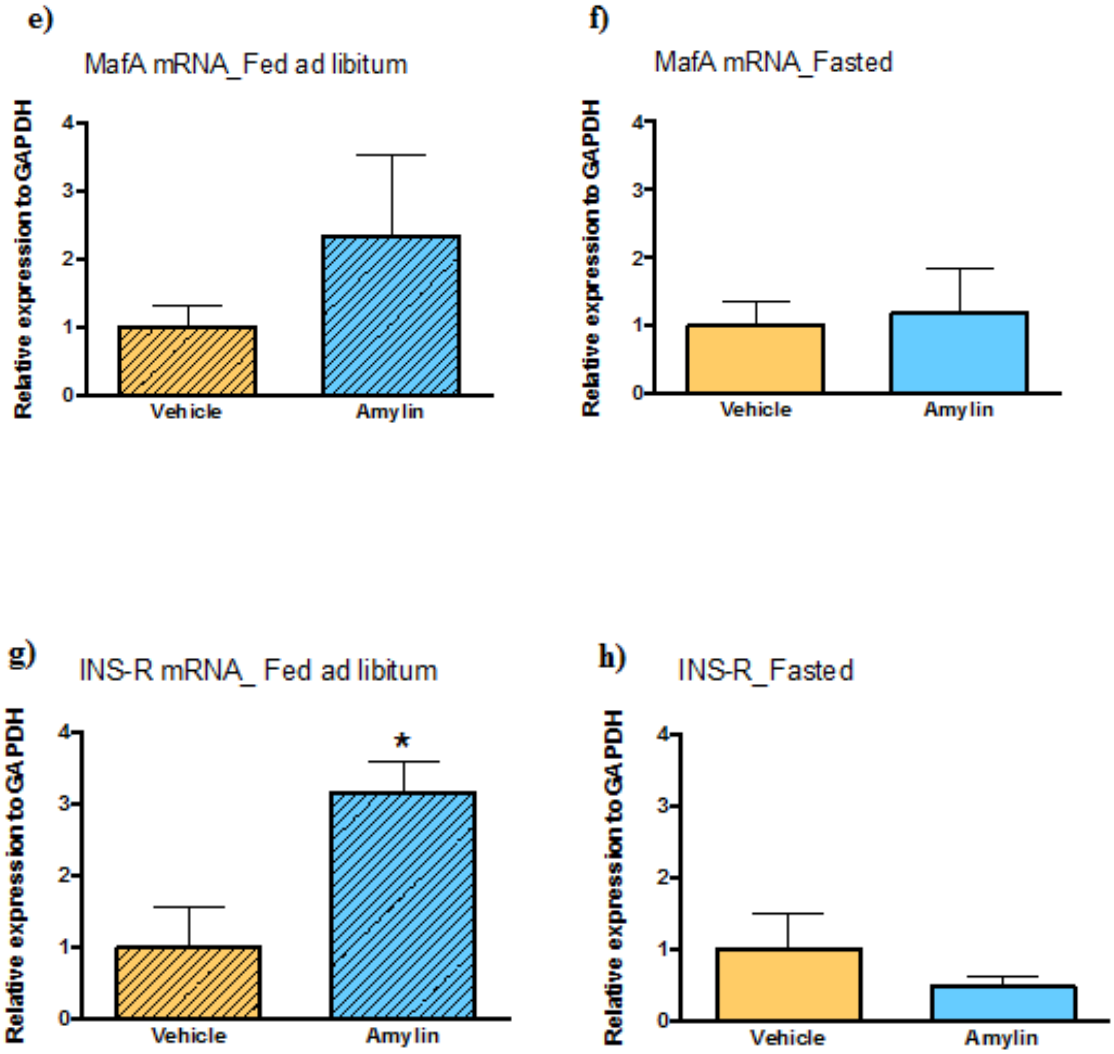


Figure 14. *Effects of amylin on NeuroD1, PDX-1, MafA and INS-R gene expression in the pancreas.*

Mice (n=5/group) were fed ad libitum or fasted for four hours and intraperitoneally injected with either amylin (50 µg/kg) or vehicle (0.9% NaCl; 1ml/kg). mRNA expression level of NeuroD1 at (a) fed or (b) fasted condition. mRNA expression levels of PDX-1 at (c) fed or (d) fasted condition. mRNA expression levels of MafA at (e) fed or (f) fasted condition. mRNA expression levels of INS-R at (g) fed or (h) fasted condition. Data is represented as mean ± SEM. Statistical significance was consider as $p< 0.05$.

3.3 Pancreatic islet gene expression after culture at low- and high-glucose concentrations

To confirm the expression of PDX 1 and INS-R in pancreatic islets and to investigate the effect of glucose on their transcriptional levels, we cultured isolated mouse islets in standard media (well number: 6; 2.8mmol/L glucose) or exposed them to high glucose (well number: 6; 16.7mmol/L glucose) for 24h.

As expected, exposure to high glucose increased the insulin secretion index (calculated as the fold stimulation of insulin release induced by 1-h incubation in 16.7 versus 2.8 mmol/L glucose) up to times basal (data not shown).

After 24 h of culture in high (16.7mmol/L) glucose, the mRNA expression of both PDX-1 and INS-R was markedly downregulated compared to levels measured in control islets cultured at basal (2.8mmol/L glucose) condition, although differences were not statistically significant. Based on the findings of study 1, GAPDH was used as housekeeping gene in this study. However, we observed that the mean of raw Ct values of GAPDH was significantly decreased after the exposure to high glucose compared to basal values (p = 0.003; data not shown).

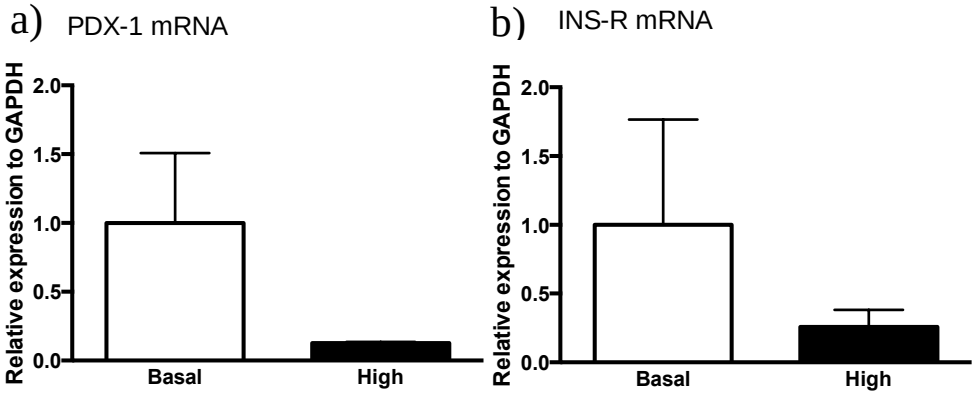


Figure 15. *The mRNA expression of PDX-1 and INS-R in the islets of Langerhans after culture at low- and high-glucose concentrations.* The mRNA expression of (a) PDX-1 and (b) INS-R was confirmed at basal condition (2.8mmol/L glucose). After exposure to high concentrations of glucose (16.7mmol/L glucose), the transcriptional levels of both targets showed a trend for a down-regulation. Results are mean ± S.E.M; Mice n=8; well number 6.

4. DISCUSSION

The neuroendocrine system governs essential survival and homeostatic functions mainly through glucose-sensing mechanisms. Glucose sensing is modulated by the hormonal milieu, which reflects peripheral energy homeostasis. The pancreatic hormone insulin is known to reduce eating by central action and plays a pivotal role in the regulation of glucose to maintain energy balance (Routh V.H. et al.; 2014). In addition, insulin actions in the brain contribute to the control of nutrient homeostasis, reproduction, cognition, and memory, as well as to neurotrophic, neuromodulatory, and neuroprotective effects (Blàzquez E. et al.; 2014). The control of food intake is also mediated by the peptide hormone amylin, which is co-secreted with insulin by pancreatic β-cells in response to nutrient stimuli and elevated blood glucose levels (Kahn S.E. et al., 1990; Johnson K.H. et al., 1988).

Amylin acts as a satiation signal by activating specific amylin-sensitive neurons in the AP (Lutz T.A et al. 2010; Potes C.S. and Lutz T.A., 2010). Further, central insulin administration enhances amylin action to decrease food intake (Osto M. et al.; 2007).

Recently more attention has been focused on the possible functional cross-talk between bHLH factors and neuropeptides in the control of energy balance. For instance, the adipocyte secreted hormone leptin which plays a major role in energy homeostasis and weight balance (Zhang Y. et al.; 1994), was recently found to exert an effect on Nhlh-2 (neurological basic-helix-loop-helix 2) and NeuroD1 expression regulation in the paraventricular nucleus of the hypothalamus (Nilaweera K.N. et al.; 2002). Interestingly, our preliminary results indicated that NeuroD1 is strongly up-regulated (15 fold change) in the AP after amylin treatment in food-deprived rats and that this effect can be blunted by blocking the amylin receptor with the AC187 treatment. Moreover, this response is positively correlated with an increase (2 fold change) in the expression of insulin receptors (INS-R).

Finally, it is known that in pancreatic β-cells the canonical Wnt/β-catenin pathway results in the activation of three main transcriptional factors: NeuroD1, MafA and PDX-1, that in turns act together to regulate insulin transcription (Lee J. et al., 2016; Wilson M.E., 2003). Whether a similar mechanism also regulates amylin transcription in the pancreas is not known.

However, our preliminary data suggest that amylin might use the same signalling pathway to activate the transcription of NeuroD1 in the AP.

4.1 The role of acute amylin treatment on the genetic regulation of NeuroD1, PDX-1, MafA and INS-R in the pancreas and in the AP of mice.

Our main study here confirms previous result obtained in our laboratory and provides new insight on the role of amylin on the transcriptional regulation of NeuroD1, MafA, PDX-1 in both the AP and the pancreas. It is known that insulin transcription in the pancreas is driven by the activation of the canonical Wnt-β catenin pathway through the activation of NeuroD1, MafA, PDX-1 (Lee J. et al., 2016; Wilson M.E., 2003). However, no evidence of the pancreatic hormone amylin using the same mechanism is present. Therefore we hypothesize that amylin might act through the activation of NeuroD1, MafA, PDX-1 both in the pancreas and in the AP. Since our analysis aimed to investigate the transcriptional effect of amylin on our target genes, first we first selected the most appropriate housekeeping gene to be used in our experimental conditions.

Our results clearly demonstrated that GADPH, compared to S18 and Beta-actin, was the most appropriate internal control for our experimental conditions. Thus, confirming previous findings reporting GAPDH use as housekeeping gene in both brain and peripheral tissues (Liberini et al., 2016; Livak K.J. and Schmittgen T.D., 2001; Selvey S. et al., 2001; Vandesompele J. et al., 2002).

Next, we investigated a potential amylin effect on the transcriptional regulation of NeuroD1, PDX-1, MafA and INS-R mRNAs both in the pancreas and in the AP of mice. Since food itself might have an effect on gene transcription, we challenged our animals with fasting.

Our results describe a scenario in which acute amylin treatment decreased the AP-expression of NeuroD1, PDX-1 and MafA mRNA in mice fed ad libitum, whereas amylin administration after fasting (4h food-deprivation) did not exert any significant effect on the transcriptional expression of NeuroD1, PDX-1 and MafA. However, an inverse trend compared to fed status can be observed.

Specifically, NeuroD1 mRNA appeared to be increased by amylin in fasted mice, thus confirming what we reported previously in rats (See Figure 9a). Interestingly, the mRNA expression of the INS-R, in both fed and fasted conditions, was not measurable in the AP of mice.

A potential explanation is that the expression of insulin and INS-R mRNAs is quite low in the brain and the total RNA extracted from the AP of a mouse is less than 10ng, thus making low-expressed target of difficult detection.

In the pancreas, our results indicate that acute amylin treatment potentially, but not significantly, up-regulated the expression of NeuroD1, PDX-1 and MafA transcripts in mice fed ad libitum, whereas amylin administration in the fasted status (4h) did not exert any significant effect on gene transcription.

Interestingly, the pancreatic INS-R mRNA expression was significantly up-regulated by amylin during fed condition (3 fold change), while in the fasted status amylin seemed to down-regulate the INS-R mRNA levels. Thus, suggesting that amylin might potentially activate the NeuroD1/MafA/PDX-1 complex to trigger the transcriptional activation of the INSR gene and therefore facilitate insulin binding to its own receptor.

All together, our result suggested that amylin as the potential to differentially modulate NeuroD1, PDX-1, MafA transcripts in the AP and, the same genes plus the INS-R, in the pancreas. The transcriptional expression of NeuroD1, PDX-1 and MafA in pancreas and AP displayed an opposite pattern: NeuroD1, PDX-1 and MafA mRNA levels were slightly downregulated in the AP, whereas they seemed to be up-regulated in the pancreas, in mice fed ad libitum.

However, food-depriving the animals resulted in an up-regulation of our gene targets in the AP, whereas in the pancreas this trend seemed to be reversed. This suggests that the assumption of a meal could influence the transcriptional regulation of NeuroD1, PDX-1, MafA and INS-R in the pancreas, via an upregulation of the transcriptional factors; which in turn results in an increased expression of INSR mRNA.

Potentially, the availability of INSR in the pancreas of mice fed ad libitum, would prepare the pancreatic cells to receive more insulin when glucose level drop (e.g intrameal interval, fasting). Further studies investigating the regulation of INS-R in the islets are still required.

Moreover, our previous data in rat (see Figure 9) and our work in mice, clearly demonstrated that amylin increases the expression of NeuroD1 mRNA in the AP, thus suggesting that NeuroD1 is a new interesting candidate in the amylin-signalling pathway. Further studies to deepen the role of amylin on the transcriptional regulation of NeuroD1 are still required.

To assess a direct role of endogenous amylin, by treating rodents with AC187 per se, would clarify whether exogenous and endogenous amylin results in the increase in NeuroD1 transcripts.

4.2 Investigate the presence of PDX-1 and INS-R mRNAs in pancreatic islets

The endocrine part of the pancreas consists in a cluster of cells called islets of Langerhans that work in a co-operative manner to maintain normoglycemia. (Edlund H., 2001; Naya F. et al., 1997; Slack J.M., 1995; Wilson M.E. et al., 2003).

The β-cells secrete both amylin and insulin hormones, and these secretion is activated by the action of several transcription factors (i.e. NeuroD1, PDX1 and MafA) (Andrali et al., 2008; Hay C.W. and Docherty K., 2006; Zhuo F. et al., 2013). Moreover, the release of insulin is characterized by a specific binding with its receptor, the INS-R, that plays a key role in the regulation of glucose homeostasis (Chiu S.L. et al., 2008; Ward C.W. and Lawrence M.C., 2009).

In our experiment, we investigated the functionality of murine islets to release insulin upon glucose stimulation by the GSIS assay in half of the wells (Sauter et al.; 2015; Piro S. et al.; 2002). The mRNA expression of PDX-1 and INS-R were confirmed and the transcriptional levels of both target showed a potential downregulation after an exposure to high concentrations of glucose (See figure 15). These preliminary results revealed that the architecture of β-cells fails during a strong treatment that simulates a hyperglycaemic condition. In addition, the release of insulin by β-cells is strictly compromised. Therefore, our results described a potential role of PDX-1 and INS-R in the main process of the insulin expression.

Because NeuroD1 acts in a synergistic manner with PDX-1 and MafA in the release of insulin, to better clarify our study it will be interesting to elucidate the cooperation of these transcription factors, comparing them with the INS-R.

It has been reported that expression of NeuroD1, MafA or PDX-1 alone led to a modest activation of the insulin promoter, whereas an high activation level was found when NeuroD1 interacted with PDX-1 (Hui H. and Perfetti R.; 2002).

Despite this, is important to consider the fact that the insulin expression might changes when more transcription factors are expressed together, when one of them is over expressed or silenced.

Moreover, it will be interesting to understand if their mRNA expression would change after high exposure to glucose. Further studies are still required to better elucidate whether changes in transcriptional regulation are affected by glucose levels.

5. CONCLUSION AND PERSPECTIVES

The central action of metabolic hormones as amylin and insulin extend well beyond their role in regulating metabolic function. Clinical and laboratory evidence that these hormones play a role in neuronal development and in the regulation of glucose sensing mechanism have been extensively reported.

Insulin transcription in the pancreas is driven by the activation of the canonical Wnt-B catenin pathway through the activation of the three main transcription factors NeuroD1, PDX-1 and MafA, respectively. However, no evidence of amylin using the same mechanism in the pancreas is present. Furthermore, NeuroD1 represents an interesting new candidate for its key role in the insulin release and for the influence of amylin on the NeuroD1 modulation in the AP.

Our results will help to elucidate the effect of amylin on the regulation of energy balance through a new NeuroD1- insulin based pathway.

Thus, we investigate whether amylin upregulates NeuroD1 mRNA expression in the AP of mice during fasting, and we compared our finding with our previous data in rats. Our results demonstrate that mice respond in a similar manner as rats to acute amylin treatment. Although our results did not reach the statistical significance (due to the small number of experimental animals), a clear trend of up-regulation of NeuroD1 is present in the AP and also in the pancreas. Furthermore, the up-regulation of NeuroD1 was positively correlated with an increase in the transcriptional expression of the INS-R in the rats' AP. Interestingly, our results in mice failed to detect INS-R expression in the AP, whereas in the pancreas an up-regulation of the INSR is present during fed status. However, further studies to deepen the knowledge on the amylin signalling pathway are still required.

Understanding how amylin acts in a co-operated manner with insulin and how β-cells specific transcription factor might play critical roles during the insulin gene expression, could allow to better control the use of stem/progenitor cells to create new β-cells or diabetes treatment and improve the expression of insulin. However, studies are still required to clarify the role of amylin in the pancreas regard its correlation with the insulin hormone.

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