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Pancreatic β cells require NeuroD to achieve and maintain functional maturity

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Summary

NeuroD, an insulin transactivator, is critical for development of the endocrine pancreas, and NeuroD mutations cause MODY6 in humans. To investigate the role of NeuroD in differentiated β cells, we generated mice in which *neuroD* is deleted in insulin-expressing cells. These mice exhibit severe glucose intolerance. Islets lacking NeuroD respond poorly to glucose and display a glucose metabolic profile similar to immature β cells, featuring increased expression of glycolytic genes and LDH-A, elevated basal insulin secretion and O_2 consumption, and overexpression of NPY. Moreover, the mutant islets appear to have defective K_{ATP} channel-mediated insulin secretion. Unexpectedly, virtually all insulin in the mutant mice is derived from *ins2*, whereas *ins1* expression is almost extinguished. Overall, these results indicate that NeuroD is required for β cell maturation and demonstrate the importance of NeuroD in the acquisition and maintenance of fully functional glucose responsive β cells.

Introduction

Diabetes is a metabolic disease that involves the death or dysfunction of the insulin-secreting β cells of the pancreas. Although diabetes can be managed with insulin and other drugs, physiological glucose homeostasis is difficult to achieve by these means, and hyperglycemia is largely responsible for the co-morbidities associated with diabetes. Consequently, much research on diabetes is aimed at understanding the molecular and cellular basis for pancreatic β cell development, survival, and regulated insulin secretion in order to discover ways to restore β cells or their functions in diabetic patients.

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NeuroD is a basic helix-loop-helix (bHLH) transcription factor that is crucial for development of the pancreas (Chae et al., 2004; Chao et al., 2007; Huang et al., 2002; Malecki et al., 1999; Naya et al., 1997; Naya et al., 1995). *NeuroD*-null mice die of severe diabetes shortly after birth; their α and β cells are poorly differentiated, islets fail to form, and the majority of β cells are lost (Naya et al., 1997). Although the spatiotemporal expression pattern of NeuroD during pancreatic development has been characterized (Chae et al., 2004), its molecular, cellular, and physiological roles are still unknown. NeuroD has been shown to be critical for insulin gene expression *in vitro* (Naya et al., 1995; Qiu et al., 2002); however, *neuroD*-null pancreata contain 10–15% as much insulin as controls, an amount that has been shown to be sufficient to support viability in mice (Bonner-Weir et al., 1983). Therefore, the reduced amount of insulin in *neuroD*-null mice is unlikely to be the sole reason for their severe hyperglycemia and neonatal death.

In humans, mutations in *neuroD* can predispose individuals to develop maturity onset diabetes of the young (MODY6) (Malecki et al., 1999), suggesting a critical role for NeuroD in mature β cells. To separate out the β cell function of NeuroD, we generated mice in which *neuroD* is deleted in the cells that express insulin (NeuroD$^{loxP/-}$; RIP:Cre mice, hereafter referred to as *neuroD* β-CKO mice). In parallel, we also generated mice in which *neuroD* is deleted in mature β cells in an inducible manner (tamoxifen-injected NeuroD$^{loxP/-}$; Pdx1:CreER^{TM} adult mice, hereafter referred to as *neuroD* PE-CKO mice). Unlike *neuroD*-null mice, *neuroD* β-CKO and *neuroD* PE-CKO mice have no impairment of pancreatic islet formation and survive to adulthood. However, they are mildly hyperglycemic and contain half the normal amount of insulin. Surprisingly, in each NeuroD mutant model tested, almost all insulin is derived from the expression of *ins2*, whereas little or no expression of *ins1* is detected. Although differential regulation of the two rodent *insulin* genes has been described previously (Deltour et al., 1993; Giddings et al., 1991; Ling et al., 1998), no transcription factor has been linked to this phenomenon *in vivo*.

Although the amount of insulin found in *neuroD* β-CKO mice should be sufficient to maintain normoglycemia, these mice are severely glucose intolerant and display greatly reduced insulin secretion. Isolated islets from *neuroD* β-CKO mice respond poorly to high glucose and other fuel secretagogues, but are fully capable of insulin secretion following global membrane depolarization with exogenous KCl. Further physiological analysis of *neuroD* β-CKO islets suggests that they display many characteristics of neonatal islets, which respond poorly to glucose. For example, *neuroD* β-CKO islets have elevated levels of lactate dehydrogenase (LDH-A) and basal oxygen consumption, and overexpress Neuropeptide Y, all of which are features associated with fetal and neonatal beta cells (S. Bonner-Weir, personal comm.; (Asplund and Hellerstrom, 1972; Boschero et al., 1990; Freinkel et al., 1984; Rozzo et al., 2009)). Deletion of NeuroD in adult β cells (*neuroD* PE-CKO mice) similarly causes glucose intolerance and reversion to immature β cell characteristics. One of the key features of postnatal pancreatic maturation is an acquisition of glucose-stimulated insulin secretion (GSIS), which is critical for β cell function. The lack of this feature in *neuroD* β-CKO islets demonstrates for the first time that NeuroD is essential for the maintenance of β cell maturation and function, which could explain the role of NeuroD in MODY6 (Malecki et al., 1999).

Results

Impaired glucose tolerance and failure to secrete insulin in *neuroD* β-CKO mice

To study the role of NeuroD in mature β cells, we generated conditional knockout mice in which the RIP:Cre transgene (Herrera, 2000) was used to delete *neuroD* in approximately 90% of differentiated insulin-producing cells (*neuroD* β-CKO; Fig. S1A–C). Although NeuroD is known as a critical transcriptional activator of the *insulin* genes (Naya et al., 1995; Qiu et al., 2002), both male and female *neuroD* β-CKO mice survive and are indistinguishable from

control littermates in their appearance and body weight (data not shown). In neonatal *neuroD* β-CKO mice (P1.5), the blood glucose concentration was higher and more variable than in the control mice. Periodic measurements of blood glucose during maturation (1–8 weeks) and adulthood (10–24 weeks) showed that the mutant mice fed *ad libitum* were mildly hyperglycemic (Fig. 1A) with greater variability in their blood glucose levels: 11% of readings were ≥ 250 mg/dL for mutant mice versus 0% for control mice (n=148–149 per genotype). These episodes of hyperglycemia occurred in mice that were normoglycemic at other times, indicating that the hyperglycemic episodes were not an inherent property of a few individual mice.

To determine whether *neuroD* β-CKO mice are glucose intolerant, young adult mice (1–3 months) were fasted during the day for 5 hours or overnight for 16 hours, followed by either feeding *ad libitum*, or intraperitoneal injection of glucose (2g/kg body weight). In both conditions, mutant mice had significantly higher fasting blood glucose levels. Following feeding or glucose injection, their blood glucose rose to levels twice as high as those in sibling control mice and took longer to return to homeostatic levels (Fig. 1B, C and Fig. S2A).

The glucose intolerance exhibited by the *neuroD* β-CKO mice is in sharp contrast to the phenotype of *neuroD*-null mice, which die shortly after birth with severe and sustained hyperglycemia (> 500 mg/dl). This difference indicates that NeuroD has a distinct function in committed β cells that is different from its earlier developmental function. To confirm that the phenotype of *neuroD* β-CKO mice is independent of a developmental defect, we generated tamoxifen-inducible mice by crossing *neuroD*$^{loxP/loxP}$ mice with Pdx:CreER^{TM}; *neuroD* $^{+/-}$ mice that express the inducible Cre recombinase (CreER^{TM}) under the control of the Pdx-1 promoter (Gu et al., 2002). Injection of tamoxifen in adult mice (*neuroD*$^{loxP-}$; Pdx-1:CreER^{TM}) resulted in a 94% reduction in *neuroD* mRNA in fully developed β cells, and these mice (*neuroD* PE-CKO) were glucose intolerant by three weeks after treatment (Fig. 1D). These results indicate that glucose intolerance is a characteristic of mice that lack NeuroD in their β cells, regardless of their age at the time of deletion of *neuroD*.

Glucose intolerance can occur because of a lack of glucose-stimulated insulin secretion (GSIS), decreased action of insulin in the peripheral tissues, or both. To distinguish between these possibilities, we measured plasma insulin in *neuroD* β-CKO and control mice (Fig. 1E). In fasted animals, the plasma insulin level in control mice ranged between 0.29–0.63 ng/ml, while that of *neuroD* β-CKO was significantly lower at 0.18–0.32 ng/ml ($p<0.001$). Although mean plasma insulin was not significantly different in control and mutant mice fed *ad libitum*, it ranged from 0.47–2.64 ng/ml in controls, whereas it never exceeded 1.20 ng/ml in *neuroD* β-CKO mice. More dramatic differences were observed following intraperitoneal glucose injection, as the *neuroD* β-CKO mice were poor at mounting an insulin secretion response when challenged with glucose (Fig. 1F). Both the first and second phase responses were smaller and developed more slowly in the mutant mice. Thus, *neuroD* β-CKO mice are severely deficient in GSIS, resulting in glucose intolerance.

Mice were also tested for their ability to take up glucose in their peripheral tissues in response to exogenous insulin. Although the mutant mice had a higher fasting level of blood glucose, their ability to take up glucose was not significantly different from controls (Fig. S2B). This result indicates that *neuroD* β-CKO mice are not insulin-resistant, further supporting the hypothesis that they are glucose intolerant owing to defective insulin secretion.

Because glucose homeostasis partly depends on the balance of glucose storage and release, we investigated whether aberrant levels of glucagon or perturbation of hepatic gluconeogenesis contributed to the phenotype of *neuroD* β-CKO mice. Plasma glucagon levels were not significantly different in *neuroD* β-CKO mice versus control mice regardless of whether they

were fed *ad libitum*, fasted for 5 hours, or fasted overnight for 16 hours (Fig. 1G). These results imply that aberrations in glucagon secretion do not account for the modest hyperglycemia of *neuroD* β-CKO mice. We also investigated whether the mice differed in their regulation of hepatic glucose-6-phosphatase (G6Pase), an indicator of gluconeogenesis. Mutant and control mice had a similar amount of hepatic G6Pase mRNA when fasted overnight, suggesting that gluconeogenesis was equally stimulated in both cases (Fig. 1H). However, at 90 minutes after glucose injection, G6Pase mRNA fails to decrease in the mutant mice. Since insulin is a powerful inhibitor of G6Pase expression (Onuma et al., 2009), the failure to downregulate G6Pase mRNA in *neuroD* β-CKO mice is likely due to their severe insulin secretion defect. Therefore, in *neuroD* β-CKO, sustained gluconeogenesis may exacerbate hyperglycemia during glucose challenge.

Islet morphology and β cell phenotype in the *neuroD* β-CKO pancreas

Because *neuroD*-null mice fail to form mature islets (Naya et al., 1997), we investigated whether *neuroD* β-CKO mice are glucose intolerant due to defective islet formation and/or maintenance. Pancreata from perinatal and 7 week-old mice were stained with antibodies to glucagon, insulin, somatostatin and pancreatic polypeptide, as markers for α, β, δ and PP cells, respectively. Although *neuroD* β-CKO mice formed islets similar in size to those of control mice, their α, δ and PP cells were intermingled in the β cell core instead of residing at the periphery (Fig. 2A–B, E–F; Fig. S3A–B; Fig. S5A–B; data not shown). Interestingly, the *neuroD* PE-CKO mice did not display disrupted islet architecture, suggesting this phenotype is associated with a developmental defect (Fig. 2C–D; Fig. S5C–D). The number of cells co-stained for insulin and somatostatin was increased in both the *neuroD* β-CKO and *neuroD* PE-CKO mice. The cells co-expressing insulin and somatostatin were elevated 60-fold in the islets of *neuroD* β-CKO mice: 0.77% of insulin-positive cells co-expressed somatostatin vs. 0.013% in controls (Fig. 2E–H). However, the total number of cells involved is small and thus may not contribute to the overall physiology of *neuroD* β-significantly different in *neuroD* β-CKO and control mice, indicating that other β cell characteristics are intact in the mutant β cells (Fig. S3C–D). To determine whether the failure to respond to glucose occurs at the level of glucose transport across the membrane, we examined the level of Glut-2 in *neuroD* β-CKO mice and found that the mutant β cells have only half as much Glut-2 protein as controls, as determined by quantification of immunostaining (Fig. 2I–J). However, *neuroD* PE-CKO mice, which are also glucose intolerant, have normal amounts of Glut2 in their islets at both 3 weeks and 2 months after tamoxifen treatment was initiated (Fig. 2K–L; Fig. S5E–F), indicating that reduction of Glut-2 is not responsible for glucose intolerance observed in both strains. Importantly, Pdx-1, Nkx6.1 and MafA, all of which are β cell transcription factors that are involved in the activation of insulin transcription and the regulation of insulin secretion (Ohlsson et al., 1993; Schisler et al., 2005; Wang et al., 2007), are unaffected by the absence of NeuroD (Fig. S3E–J, L). These results indicate that the defective GSIS observed in *neuroD* β-CKO mice is not a secondary consequence of altered regulation of these transcription factors.

Because apoptosis is increased 10-fold in *neuroD*-null mice (Naya et al., 1997), we investigated whether apoptosis is also increased in *neuroD* β-CKO mice. We measured apoptosis by immunostaining for activated caspase 3; and also examined compensatory β cell proliferation by immunostaining for PCNA and Ki67. Overall, only a small fraction of the β cells were engaged in either proliferation (≤2%) or apoptosis (≤2%) in *neuroD* β-CKO and control islets, indicating that deletion of NeuroD in differentiated β cells does not cause increased apoptosis or proliferation, which is consistent with the observed normal islet area in these mice.

Expression of *insulin 2*, but not *insulin 1*, in *neuroD* β-CKO mice

Detection of a significant amount of insulin staining in the β cells of *neuroD* β-CKO islets was surprising because previous studies had shown that NeuroD is a critical factor for insulin gene transcription (Naya et al., 1995; Qiu et al., 2002). Morphometric quantification of >500 islets indicated that on average the intensity of insulin staining in *neuroD* β-CKO islets was half that of control islets. Moreover, although we observed no significant difference in either islet size or islet number between control and *neuroD* β-CKO mice, *neuroD* β-CKO pancreata contain 53% as much insulin as control pancreata (15.3 ± 4.2 ug/mg vs. 29.0 ± 7.8 ug/mg protein, n=7–13, $p < 0.001$). Taken together, these data indicate that *neuroD* β-CKO islets contain half the normal amount of insulin due to a corresponding reduction in insulin content per cell rather than a reduction in cell number.

Because rodents express two closely related insulin genes, *ins1* and *ins2* (Davies et al., 1994), we measured the level of expression of each insulin gene to determine the source of the remaining insulin in *neuroD* β-CKO mice. In pancreatic sections costained for insulin and C-peptide 1, >90% of the β cells in *neuroD* β-CKO pancreata lacked C-peptide 1 staining, suggesting that they fail to express *ins1*. In striking contrast, C-peptide 2 was present in all of the mutant β cells, implying that *ins2* expression is relatively unaffected (Fig. 3A–D). Consistently, *ins1* transcripts are also reduced by 95% in *neuroD* β-CKO islets, while *ins2* transcripts are present at a level comparable to controls (Fig. 3I). These data indicate that *neuroD* is not required for *ins2* expression *in vivo*, but is necessary for *ins1* expression. Deletion of *neuroD* in adult β cells using the *neuroD* PE-CKO mice also resulted in the loss of *ins1*, but not *ins2*, expression (Fig. 3E–H, I). The differential regulation of *ins1* and *ins2* does not appear to be an artifact associated with the *neuroD* conditional allele because we also detect a dramatic decrease in immunostaining for C-peptide 1, but not C-peptide 2, in the few remaining β cells in e18.5 *neuroD*-null pancreata (Fig. S4A–D). Furthermore, in the *neuroD*-null embryos, there is a gradual decline in the ratio of *ins1/ins2* mRNA between e14.5 and P0, (Fig. S4E). A similar phenomenon can be observed in the postnatal *neuroD* β-CKO mice, where the ratio of *ins1/ins2* mRNA declines from 80% of control at e18.5 to <10% of control at 2 months (Fig. S4F).

Previous studies have shown that mice can lack either one of the *insulin* genes and remain glucose tolerant (Leroux et al., 2001). We confirmed this result independently in *ins1*-null mice (data not shown). Moreover, studies of pancreatectomized animals have indicated that half the normal amount of insulin is more than sufficient to maintain glucose homeostasis (Bonner-Weir et al., 1983). Hence, neither the loss of insulin 1 *per se*, nor the reduction in total insulin, accounts for the defective insulin secretion and glucose intolerance of *neuroD* β-CKO mice.

Formation of Dense Core Granules in *neuroD β-CKO* mice

Insulin secretion is poor in some diabetic mouse models because of a paucity of insulin dense core granules (DCGs) in the β cells (Bruin et al., 2008; Like and Rossini, 1976; Pechhold et al., 2009; Piaggi et al., 2007). In addition, fewer DCGs are present in the underdeveloped β cells of *neuroD*-null mice (data not shown). We analyzed β cells of *neuroD* β-CKO mice using electron microscopy to determine whether they have normal DCG's. The mutant β cells contain a large number of DCG's, similar to those of control mice, implying that neither defective formation of DCG's, nor depletion thereof, is the reason for defective insulin secretion in *neuroD* β-CKO mice (Fig. S4H–I).

Defects in stimulus-secretion coupling in isolated islets from *neuroD* β-CKO mice

To understand the physiological basis of defective GSIS observed in the NeuroD mutant mice, we stimulated islets isolated from control and *neuroD* β-CKO mice with a variety of insulin secretagogues (Fig. 4A). The amount of secreted insulin was normalized to total insulin content to take into account the reduction of insulin in the *neuroD* β-CKO islets. We found that the

mutant islets secrete a larger percentage of their insulin (0.13 ± 0.01% vs. 0.05 ± 0.01% for controls) under basal conditions (2.8 mM glucose). However, during 1 hour of static incubation in 16.7mM glucose, the control islets secreted 1.0% of their insulin, whereas the *neuroD* β-CKO islets secreted only 0.26% of their insulin (Fig. 4A). The mutant islets also responded poorly to leucine (Fig. 4A). These secretion defects do not appear to be associated with a lack of readily releasable insulin granules because exposure of *neuroD* β-CKO islets to 30mM KCl induced robust insulin secretion that was not significantly different from control islets (Fig. 4A).

Extensive *in vitro* and *in vivo* studies of the gene products associated with MODY have suggested that they play a predominant role in glucose sensing-insulin secretion coupling (Giuffrida and Reis, 2005; Mitchell and Frayling, 2002). Because Sur1, a regulatory subunit of the pancreatic K_{ATP} channel, has been shown to be a transcriptional target of NeuroD *in vitro* (Keller et al., 2007; Kim et al., 2002), we assessed whether *neuroD* β-CKO mice display defective K_{ATP} channel function. Consistent with a defect in K_{ATP} channel function, *neuroD* β-CKO islets respond poorly to glipizide, a sulfonylurea drug, by secreting only 0.22% of their insulin content vs. 0.79% for control islets (Fig. 4A). Interestingly, there is no difference in the mRNA expression of the K_{ATP} channel gene (Kir6.2, *kcnj11*), or its regulatory subunit (Sur1, *abcc8*) between mutant and control islets (Fig. 4B), indicating that NeuroD does not regulate these genes at the level of transcription *in vivo*.

It remains possible that NeuroD affects other aspects of K_{ATP} channel activity. The *neuroD* β-CKO islets respond poorly to methyl pyruvate (Dufer et al., 2002)(Fig. 4A) and are deficient in molecules that link the activity of K_{ATP} channels to other parts of the machinery necessary for regulated insulin exocytosis. The expression of Piccolo (*pclo*) and Noc2 (*rph3al*) are both decreased in the β-CKO islets (Fig. 4B). *Pclo* encodes a scaffold protein that is necessary for assembly of insulin secretion complexes that link K_{ATP} channels, L-type calcium channels and insulin granules into functional units (Shibasaki et al., 2004), and Noc2 is a Rab effector that is required for GSIS through its interaction with small monomeric GTPases (Cheviet et al., 2004).

We also investigated whether there are defects in the steps that precede closure of the K_{ATP} channel in *neuroD* β-CKO islets. We measured O_2 consumption in islets cultured in low and high glucose to measure the degree of oxidative metabolism that leads to ATP production. Compared to the controls, the mutant islets had a significantly greater rate of O_2 consumption under the basal conditions (Fig. 4C). When challenged with high glucose, O_2 consumption increased to a lesser extent in mutant than control islets, but achieved a similar rate overall. Virtually all of the O_2 consumption in mutant and control islets was inhibited by treatment with the ATP synthase inhibitor Oligomycin A, implying that it is coupled to ATP production (Fig. 4C). Interestingly, these characteristics of respiration in *neuroD* β-CKO islets resemble those of GSIS-deficient neonatal islets (Boschero et al., 1990).

Defects in the amplification of GSIS in *neuroD* β-CKO islets

Although global membrane depolarization by KCl releases a normal amount of insulin from *neuroD* β-CKO islets, their reduced secretion of insulin in response to glucose, other fuel secretagogues, and glipizide implies that they are defective in the initial phase of GSIS upstream of Ca^{2+} influx. Therefore, to determine whether the mutant islets are capable of amplifying their insulin secretion following Ca^{2+} influx, we treated them with BayK8644, a drug that specifically opens L-type voltage sensitive calcium channels (VDCCs), together with 16.7mM glucose. The mutant islets secreted 2.6% of their total insulin, which is 10 times greater than their response to high glucose alone, but is still less than the 4.7% of insulin secreted by the control islets (Fig. 5A). Similar results were obtained using 20 mM L-arginine to enhance plasma membrane depolarization in the mutant and control islets. In addition, an agent (cAMP)

that enhances the amplifying phase of GSIS (Doyle and Egan, 2003) also partially rescued insulin secretion in the mutant islets (Fig. 5A). Thus, neither type of treatment alone fully rescued insulin secretion in *neuroD* β-CKO islets, suggesting that the mutant islets have defects in both the initial and amplifying phases of GSIS.

Adenyl cyclase activity, which is necessary for the conversion of ATP to cAMP, is inhibited by Neuropeptide Y (NPY), a hormone whose expression is normally decreased in islets after birth (Fig. S6A–J; (Motulsky and Michel, 1988)). We found that NPY mRNA is significantly increased in the *neuroD* β-CKO and *neuroD* PE-CKO islets, and that the immunostaining of NPY is clearly increased in >40% of the mutant β cells of each strain (Fig. 5B–J). The upregulation of NPY in the β cells of *neuroD* β-CKO and *neuroD* PE-CKO mice may contribute to their observed GSIS defects by decreasing the level of cAMP.

Increased glycolytic gene expression in the *neuroD* β-CKO islets

Impaired GSIS, as well as elevated rates of O_2 consumption and insulin secretion in basal conditions, are properties shared by *neuroD* β-CKO islets and fetal beta cells (Freinkel et al., 1984; Hughes, 1994; Rozzo et al., 2009; Tu and Tuch, 1996). To investigate whether *neuroD* β-CKO islets display an expression profile similar to functionally immature β cells, we performed unbiased global gene expression analyses of adult islets isolated from the *neuroD* β-CKO pancreata and their littermate controls. Sixty-eight genes were significantly affected by the deletion of *neuroD* in β cells (Table S1–2). These results indicated that expression of lactate dehydrogenase A (LDHA) was significantly altered in the adult *neuroD* β-CKO islets. Normally, mature β cells are different from most mammalian cell types in that they have an unusually low amount of lactate dehydrogenase (LDH) (Schuit et al., 1997; Sekine et al., 1994). In contrast, fetal and neonatal β cells have elevated amounts of LDHA and an increased rate of glycolysis (Fig. S6L; S. Bonner-Weir, personal comm.; (Boschero et al., 1990)). Surprisingly, LDHA (*ldha*) mRNA and protein are increased dramatically in *neuroD* β-CKO islets in both low glucose and high glucose (Fig. 6A–E, J). Accordingly, the mutant islets exhibit a 3.5-fold increase in LDHA activity, a 2-fold increase in lactate production, and a >2-fold increase in LDHA immunostaining (Fig. 6K–L). Furthermore, *neuroD* β-CKO islets have elevated expression of several other glycolytic genes, including aldolase B, phosphofructokinase, liver form (PFKL), triose phosphate isomerase (TPI), enolase 1 (ENO1) and pyruvate kinase, liver and RBC form (PKLR) (Fig. 6A). These changes in gene expression suggest that glycolysis is enhanced in *neuroD* β-CKO islets. In contrast, there is no significant difference in the expression of key genes whose products participate in pyruvate metabolism and oxidative phosphorylation in mitochondria, such as pyruvate dehydrogenase A1 (Pdha-1) and its regulatory protein pyruvate dehydrogenase kinase 1 (PDK1), succinate dehydrogenase C (SDHC), and ATP synthase (ATP6) (Fig. 6A). Overall, the pattern of gene expression in *neuroD* β-CKO islets is consistent with the increase in glycolysis that is characteristic of neonatal β cells (Boschero et al., 1990). Comparable increases in LDHA mRNA and immunostaining, and glycolytic gene expression, also occur in *neuroD* PE-CKO islets, indicating that adult β cells require NeuroD to maintain their mature metabolic phenotype (Fig. 6A, F–I).

Discussion

NeuroD is known to be important for β cell development and insulin transcription, however which aspects of β cell development and mature β cell function require NeuroD is not clearly understood. To determine the specific role of NeuroD in mature β cells, we generated *neuroD* β-CKO mice, in which NeuroD is deleted in the insulin-producing cell population at the onset of their formation. These mice survive and form islets that contain half the normal amount of insulin, and yet they are severely glucose intolerant. To determine whether continued

function of NeuroD is required in mature adult β cells, we also deleted *neuroD de novo* in adult mice (*neuroD* PE-CKO) using inducible expression of Cre recombinase and found that these mice largely phenocopy the *neuroD* β-CKO mice. We performed extensive molecular, cellular and physiological analyses on both mouse models and found: 1) Despite the widely accepted belief that NeuroD is a critical transcription factor for insulin gene transcription, NeuroD is dispensable for *ins2* gene expression in mice; 2) Although β cells lacking NeuroD produce sufficient insulin to support glucose homeostasis, the mutant mice are glucose intolerant, indicating that NeuroD regulates other aspects of β cell function that are unrelated to *insulin* transcription; 3) Continued activity of NeuroD is required for the proper function of β cells, providing the molecular and physiological basis for MODY6; and 4) β cells lacking NeuroD have a striking resemblance to immature β cells, indicating that NeuroD plays an important role in achieving and maintaining maturity of β cells.

NeuroD and activation of insulin transcription

NeuroD β-CKO mice retain half the normal amount of insulin because activation of the *ins2* gene is unaffected *in vivo* both during development and in adult mice. Although differential expression of *ins1* and *ins2* has been detected under certain physiological conditions (Deltour et al., 1993; Giddings et al., 1991; Ling et al., 1998), this is the first time that a transcription factor has been associated with their differential regulation *in vivo*. This result was surprising because several prior *in vitro* studies have shown that NeuroD is capable of activating both *ins1* and *ins2* through conserved E-box elements (Clark and Docherty, 1993; German et al., 1991; Naya et al., 1995). Therefore, it was anticipated that expression of both genes would be affected in the absence of NeuroD. On the other hand, each of the E-box elements in the rat and human insulin genes has been shown to contribute variably to the regulation of *ins* expression (Crowe and Tsai, 1989; Karlsson et al., 1987). Our results demonstrate that such differential regulation of two rodent *ins* genes is mediated through NeuroD and that NeuroD is dispensable for *ins2* gene expression in its native *in vivo* environment. To resolve the divergent mechanisms by which the insulin genes are regulated, it will be necessary to perform complementary *in vitro* and *in vivo* studies to understand fully the direct regulation of *ins1* and *ins2* by NeuroD and perhaps other E-box binding factors.

Mild hyperglycemia and severe glucose intolerance

Because *neuroD* β-CKO mice exhibit severe glucose intolerance, it seems paradoxical that they are only mildly hyperglycemic when fed *ad libitum*. It is unlikely that induced changes in hepatic glucose output can explain this phenotype since *neuroD* β-CKO mice fasted for 5 hours or 16 hours have similar amounts of plasma glucagon compared to control mice. It is also possible that insulin secretion is increased under *ad libitum* feeding conditions owing to mechanisms that do not depend on GSIS. The Sur1-knockout mice are also glucose intolerant and yet normoglycemic when fed *ad libitum* (Seghers et al., 2000), and it is believed that they secrete nearly normal amounts of insulin in response to feeding due to cholinergic stimulation of insulin secretion (Shiota et al., 2002). Similar compensatory mechanisms may regulate glucose homeostasis in *neuroD* β-CKO mice fed *ad libitum*.

NeuroD regulates β cell maturation

Isolated islets from *neuroD* β-CKO mice have a metabolic profile that resembles that of immature pancreatic β cells, which are found in late fetal or neonatal islets. Both the mutant β cells and immature β cells have insulin secretory granules, but lack GSIS. Compared to mature β cells, immature β cells have a higher rate of O_2 consumption, produce more lactate, and secrete more insulin under basal conditions (Boschero et al., 1990; Rozzo et al., 2009), and these properties also characterize *neuroD* β-CKO islets. When challenged with high glucose, immature β cells fail to increase their oxidative metabolism as dramatically as do

mature β cells, and consequently fail to secrete as much insulin (Hughes, 1994; Rozzo et al., 2009; Tu and Tuch, 1996). Glycolysis predominates in fetal and neonatal islets resulting in impaired GSIS, which relies on oxidative metabolism of glucose. Likewise, we found that expression of a number of glycolytic genes, including lactate dehydrogenase A (LDHA), was increased in *neuroD* β-CKO and *neuroD* PE-CKO islets. LDHA is of particular importance because it converts pyruvate to lactate in the cytosol, thereby preventing its oxidative metabolism in mitochondria. LDHA is expressed at high levels in the embryonic and neonatal pancreas, but becomes downregulated in the adult islets (Fig. S6L). Accordingly, the reduction of LDHA in mature β cells is believed to be critical for their ability to couple glucose metabolism to insulin secretion (Schuit et al., 1997; Sekine et al., 1994; Zehetner et al., 2008; Zhao and Rutter, 1998). Thus, it is likely that NeuroD plays a major role in β cell maturation by downregulating LDHA and other glycolytic genes because these changes are necessary for efficient oxidative metabolism of glucose and GSIS in mature β cells. Interestingly, mice with a β cell-specific knockout of the von Hippel-Lindau gene (*vhlh*) also displayed increased insulin secretion in low glucose and decreased GSIS owing to overexpression of LDHA and other glycolytic genes (Zehetner et al., 2008). The phenotype of the *vhlh*-deficient islets results from activation of Hif1α (Zehetner et al., 2008); however, it is unlikely that this pathway is operating in *neuroD* β-CKO islets because other genes that are strongly upregulated by Hif1α, including *vegfa* and *pdk1* are unaffected in *neuroD* β-CKO islets (data not shown). Currently, we do not have evidence that LDHA or the glycolytic genes that we examined are direct targets for NeuroD (Keller et al., 2007). There is a canonical NeuroD E-box (CATCTG) at position -430 in the LDHA promoter of mice and rats, but this site is not conserved in the human gene.

NeuroD β-CKO mice have defects in the mechanism for GSIS beyond their immature oxidative metabolism of glucose. In particular, *neuroD* β-CKO islets secrete insulin poorly in response to glipizide-mediated closure of their K_{ATP} channels. Physiological induction of insulin secretion by closure of K_{ATP} channels involves the elevation of cytosolic [Ca^{2+}] in a microenvironment around docked and primed insulin granules (Bokvist et al., 1995). This process is facilitated by the formation of an insulin secretion complex that links the ATP sensor (Kir6.1 and Sur1), cAMP sensor (cAMPGEFII), VDCCs and insulin granules into a functional unit (Shibasaki et al., 2004). Because NeuroD-deficient β cells have the basic components necessary for the first phase of GSIS, i.e., K_{ATP} channels, VDCCs, readily releasable insulin granules and ATP-coupled O_2 consumption, we hypothesize that they are deficient in GSIS because they lack the structure necessary for those components to function together. In support of this hypothesis, we found that *neuroD* β-CKO islets have decreased expression of piccolo, a large scaffold protein and possible Ca^{2+} sensor that helps form this complex, and Noc2, a Rab effector that is associated with the insulin secretory granules and is required for efficient GSIS (Cheviet et al., 2004). NeuroD may also be important for the expression of key exocytotic proteins, such as SNAP25 and syntaxin1A (Ishizuka et al., 2007). Given the apparent immaturity of β cells that lack NeuroD, our hypothesis suggests further that formation of the insulin secretion complex may be a key step in β cell maturation.

Early precursor cells in the endocrine pancreas coexpress insulin and neuropeptide Y (NPY), but as development proceeds, NPY is decreased in β cells (Fig. S6A–J; (Teitelman et al., 1993)). NPY inhibits adenyl cyclase and cAMP production (Motulsky and Michel, 1988), which is required for efficient GSIS. The inhibitory role of NPY in GSIS has been demonstrated in several rodent models and in isolated islets (Imai et al., 2007; Myrsen-Axcrona et al., 1997; Myrsen et al., 1995; Wang et al., 1994). Consistent with the immature state and impaired GSIS of β cells that lack NeuroD, NPY mRNA and protein are greatly increased in both *neuroD* β-CKO and *neuroD* PE-CKO islets. Thus, it appears that NeuroD plays a global role in both activating β cell maturation-specific genes and down-regulating immature β cell-specific genes. A recent study has demonstrated that neonatal islets display a molecular profile

that is distinct from the adult islets and identified a number of markers displaying transient expression in the perinatal period (Aye et al.). In support of our hypothesis, the gene encoding one of the markers of neonatal β cells, CK19, is highly upregulated in the *neuroD* β-CKO islets (Fig. S6M). *NeuroD* mRNA levels also gradually increase during this early postnatal maturation period (Fig. S6K); however, since NeuroD activity also depends on posttranscriptional and posttranslational regulation we need to further determine which of these gene expression changes are due to direct or indirect regulation by NeuroD.

Importance of NeuroD for creation of β cells suitable for therapy

The creation of functional β cells suitable for transplantation into patients with diabetes is a major goal of research on therapies for diabetes. Although many studies have attempted to produce β cells from many different cell sources, they have only been partially successful in inducing β cell differentiation (D'Amour et al., 2006; Jiang et al., 2007). Meanwhile, generation of mature β cells with tight control of insulin secretion in low glucose and a robust GSIS response in high glucose *in vitro* has not been successful. Our study of *neuroD* β-CKO and *neuroD* PE-CKO mice has shown that NeuroD is required for the transition of β cells from an immature to mature state during development and that NeuroD is essential for the maintenance of the mature β cell state in the adult. Thus, understanding the role of NeuroD in promoting β cell maturation could help point the way toward achieving β cell maturation *in vitro*.

Methods

Immunostaining and morphometric analysis of islets

Pancreata were fixed with 4% paraformaldehyde, cryosectioned, postfixed in methanol: acetone (1:1) for 5 min at −20°C and stained with the primary and secondary antibodies listed in Table S3. Antigen retrieval was used prior to staining for nuclear factors. Morphometric analysis was carried out using Slidebook 4.1 software (Intelligent Imaging Innovations) to quantify the area, intensity and overlap of staining for each antigen. The fraction of NPY-positive β cells was confirmed by counting immunostained cells in 3 islets of each genotype.

LDH activity and lactate assay

LDH activity was measured with an LDH assay kit (Cayman) and lactate production was analyzed with a lactate assay kit (Biovision). Additional details are provided in Supplemental Data.

Measurement of O_2 consumption

O_2 consumption was measured using a 96 well BD Oxygen Biosensor plate (BD). Equilibrated islets were added the BD Oxygen Biosensor plate at ~50 islets/well, and O_2 consumption was measured at 1 min intervals in a fluorometric plate reader (Bio-Tek) at 37°C. DNA content of the islets was determined with Picogreen dye (Invitrogen).

Insulin secretion assay

Equilibrated islets were placed in wells of a 24-well plate at 10–15 islets/well with 400ul Kreb's buffer containing 2.8mM glucose, incubated for 1 hour, and the supernatant was collected to measure basal insulin secretion. The islets were transferred to Kreb's buffer containing different insulin secretagogues (see results) and incubated for 1 hour before the supernatant was collected. The islets were sonicated in 500 ul lysis buffer (10 mM Tris pH7.5, 200mM NaCl and 1mM EDTA), and insulin was extracted by acid ethanol. Insulin in supernatants and islet lysates was measured with a rat insulin RIA kit (Millipore). Methyl pyruvate, dibutyryl cAMP and Bay K8644 were purchased from Sigma.

Microarray

Total RNA was extracted from adult islets and islet purity was assessed and matched for 4 pairs of control and mutant RNA samples. 25 ng of each RNA sample was amplified using the Ovation™ RNA Amplification system V2 (Nugen, Inc.). The mouse PancChip 6.0 was used for microarray analysis (Kaestner et al., 2003). Genes that exhibited a fold change > 1.5, with a FDR ≤5% are shown in Tables S1 and S2. The full dataset is available at ArrayExpress under experiment accession number E-MTAB-152.

Statistics

Results are expressed as means ± S.E.M., and significance was determined by Student's *t*-test for two-tailed unpaired groups. $P \leq 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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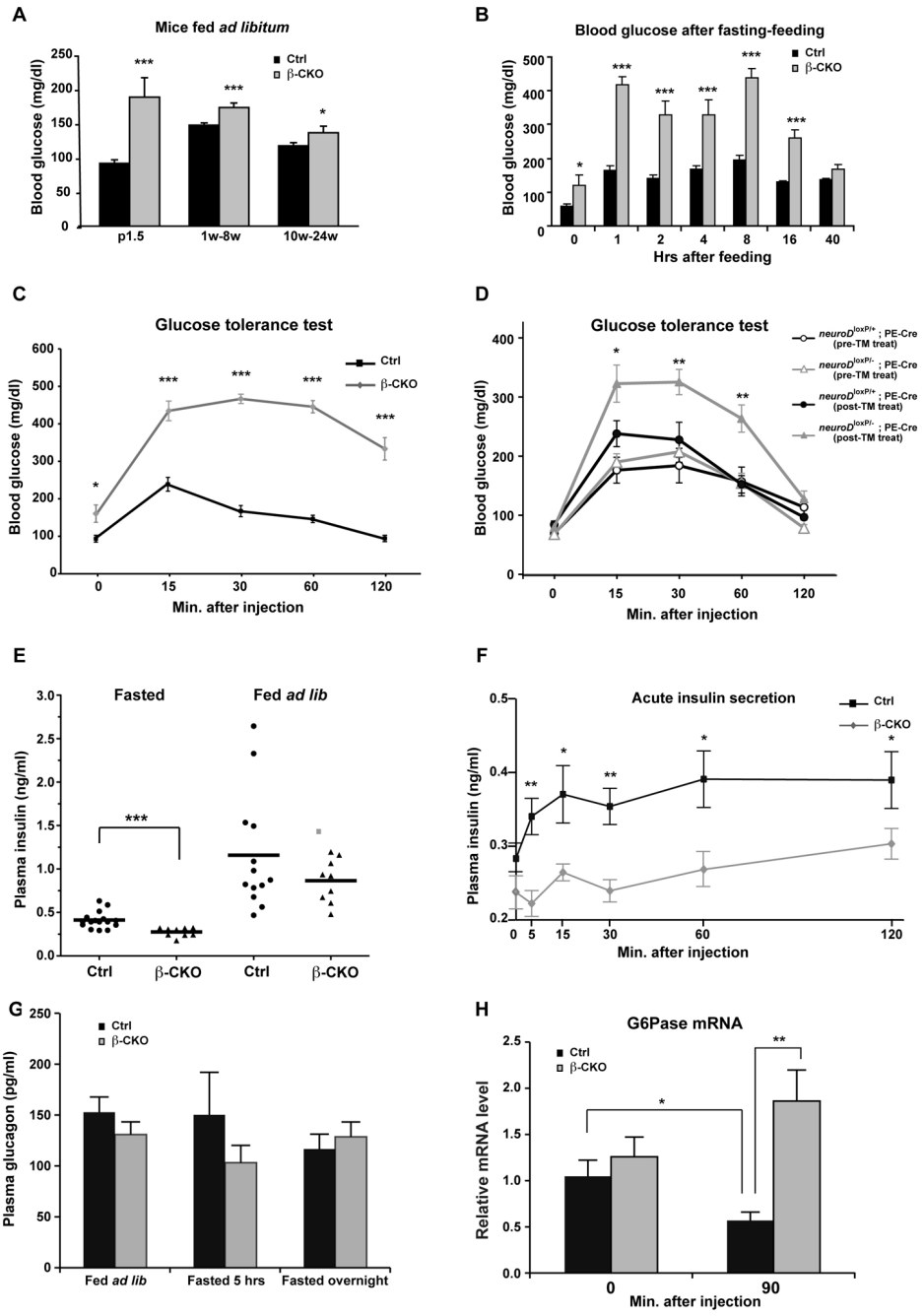

Figure 1. Physiological effects of β cell-specific ablation of *neuroD*

(A) Blood glucose levels of *neuroD* β-CKO and control mice fed *ad libitum*: P1.5 (n=9–21), 1–8 weeks (n=114–115) and 10–24 weeks (n=34–35). (B) Blood glucose levels of *neuroD* β-CKO and control mice fasted for 16 h and fed mouse chow (n=9 per genotype). (C) Glucose tolerance test for *neuroD* β-CKO and control mice (n=10–11 per genotype). (D) Glucose tolerance test for *neuroD*$^{loxP/-}$; PE-Cre and *neuroD*$^{loxP/+}$; PE-Cre pre-tamoxifen injection and post-tamoxifen injection (n=8–9 each type). The *neuroD*$^{loxP/-}$; PE-Cre mice with tamoxifen injection are considered as *neuroD* PE-CKO. (E) Plasma insulin levels after fasting (16 hours) or fed *ad libitum* (n=9–16 per genotype). (F) Plasma insulin levels after glucose injection (n=5–7 per genotype).. (G) Plasma glucagon levels fed ad libitum, fasted (5 hours) or fasted (16hours)

(n=10–17 per genotype). (H) The expression of G6Pase in control and *neuroD* β-CKO after fasted for 16 hours (0) and 90 minutes after glucose injection (90′). Values were normalized to *β2-microglobin* mRNA and expressed as relative to control (n=5 per genotype). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

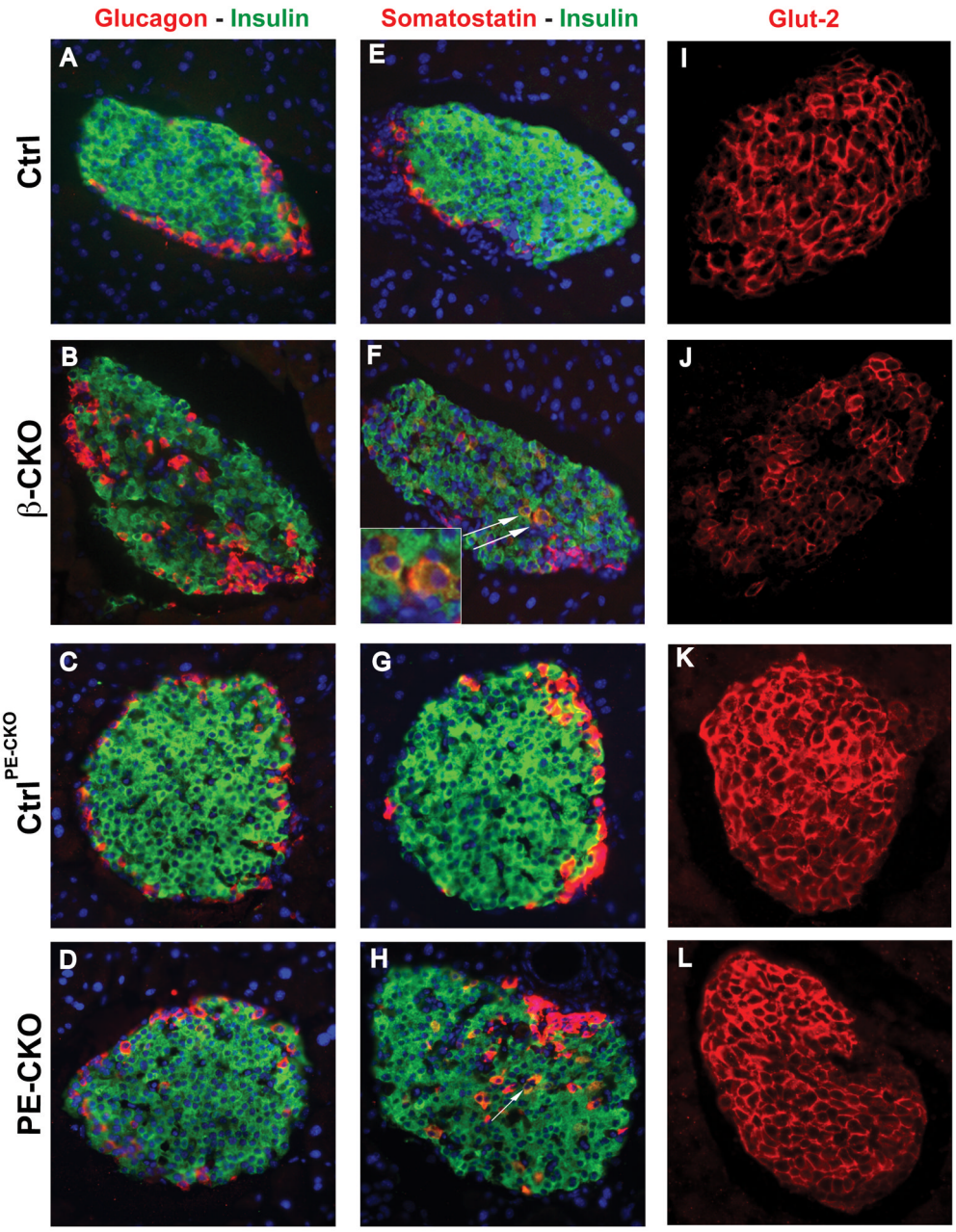

Figure 2. Effect of β cell-specific ablation of *neuroD* on islet characteristics

(A–D) Pancreatic sections from control, *neuroD* β-CKO and *neuroD* PE-CKO mice were immunostained for insulin (green) and for either glucagon (red, A–D) or somatostatin (red, E–H). Nuclei are stained with DAPI (blue). The white arrows in (F) and (H) indicate insulin and somatostatin co-stained cells. Original magnification was 200x. (I–L) The expression of Glut-2 (red) in the control, *neuroD* β-CKO and *neuroD* PE-CKO pancreas. Original magnification was 200x.

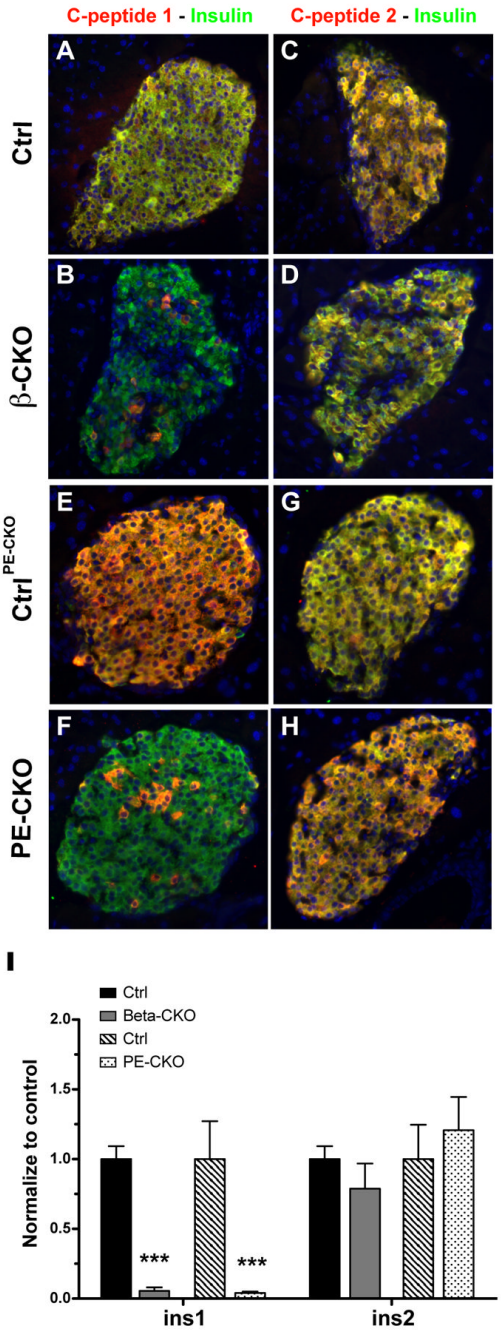

Figure 3. Deletion of *neuroD* results in loss of insulin 1

(A–D) Pancreatic sections from control, *neuroD* β-CKO and *neuroD* PE-CKO mice were co-stained with antibodies to insulin (green) and C-peptide 1 (red) (A–B, E–F) or C-peptide 2 (C–D, G–H). (K) Quantitative RT-PCR of *ins1* and *ins2* mRNA levels in control, *neuroD* β-CKO and *neuroD* PE-CKO islets. Values were normalized to *β-actin* mRNA and expressed as relative to the respective controls (n=4–6 per genotype). *** P≤0.001.

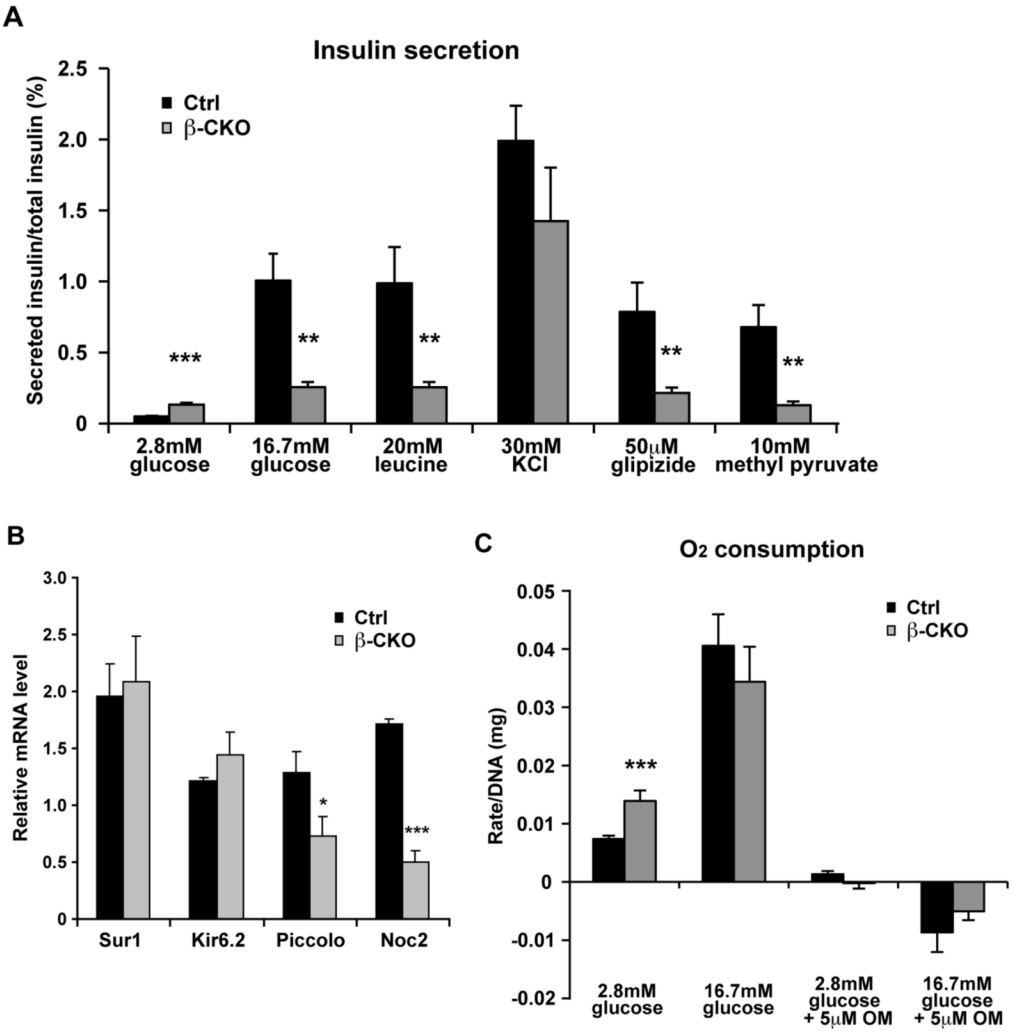

Figure 4. Insulin secretion profiles in *neuroD* β-CKO islets

(A) Isolated islets from control and *neuroD* β-CKO mice treated with different insulin secretagogues (2.8mM glucose, 16.7mM glucose, 20mM leucine, 30mM KCl, 50uM glipizide and 10 mM methyl pyruvate). Secreted insulin was normalized to total insulin in the islets (n= 6–10 per genotype). (B) Quantitative RT-PCR of *Kir6.2*, *Sur1*, *piccolo* and *Noc2* mRNA in control and *neuroD* β-CKO islets. The data were normalized to *β-actin* mRNA (n=3–8 per genotype). (C) Respiration rate of control and *neuroD* β-CKO mice islets incubated with glucose and oligomycin (OM) (n=6–17 per genotype). * P≤0.05, ** P≤0.01 and *** P≤0.001.

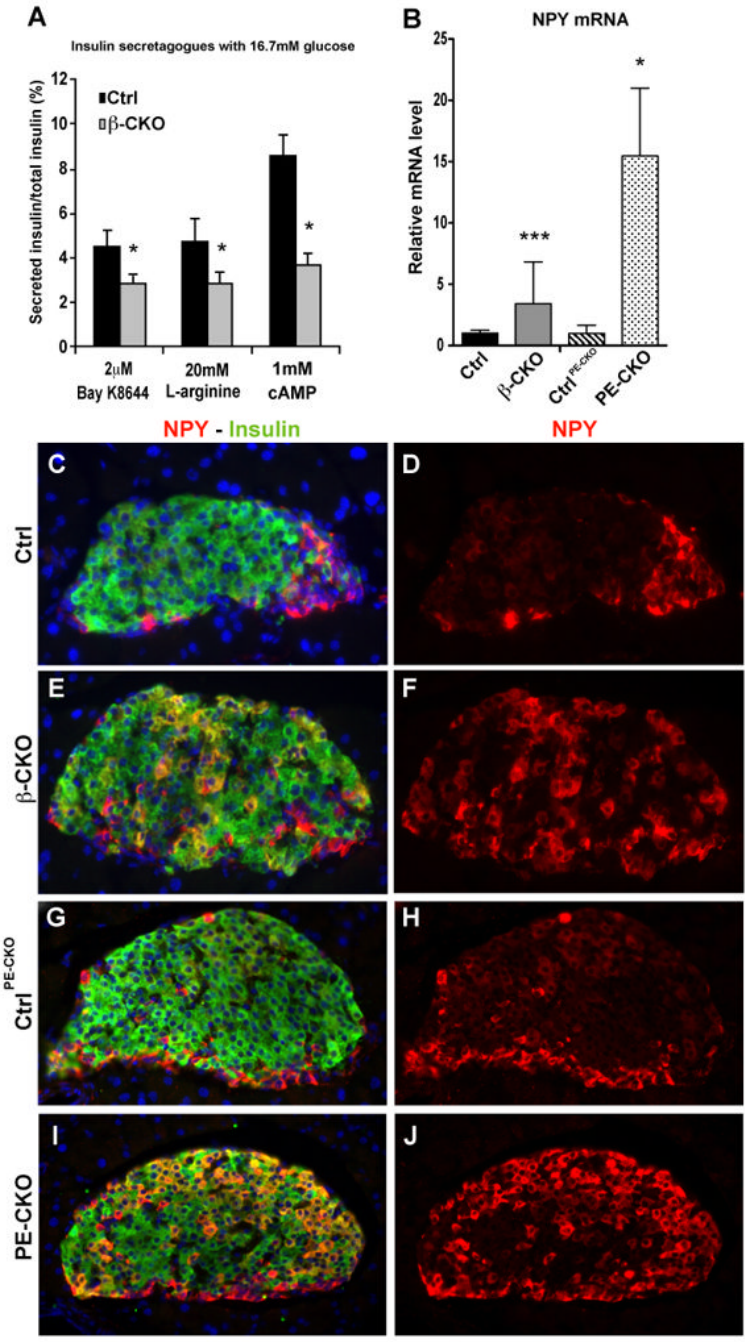

Figure 5. Deletion of *neuroD* leads to increased NPY expression

(A) Isolated islets from control and *neuroD* β-CKO mice treated with different insulin secretagogues (2uM BayK8644, 20mM L-arginine and 1mM dibutyryl cAMP) in the presence of 16.7mM glucose. Secreted insulin was normalized to total insulin in the islets (n= 6–9 per genotype). (B) Quantitative RT-PCR of NPY mRNA in control, *neuroD* β-CKO and *neuroD* PE-CKO islets. The data were normalized to *β-actin* mRNA (n=4–8 per genotype) and presented as relative to the respective controls. (C–J) Coimmunostaining of neuropeptide Y (red) and insulin (green) in the pancreatic sections of control for *neuroD* β-CKO (C–D), *neuroD* β-CKO (E–F), control for *neuroD* PE-CKO (G–H) and *neuroD* PE-CKO (I–J). Nuclei

are stained with DAPI (blue). Original magnification was 200x. * P≤0.05, ** P≤0.01 and *** P≤0.001.

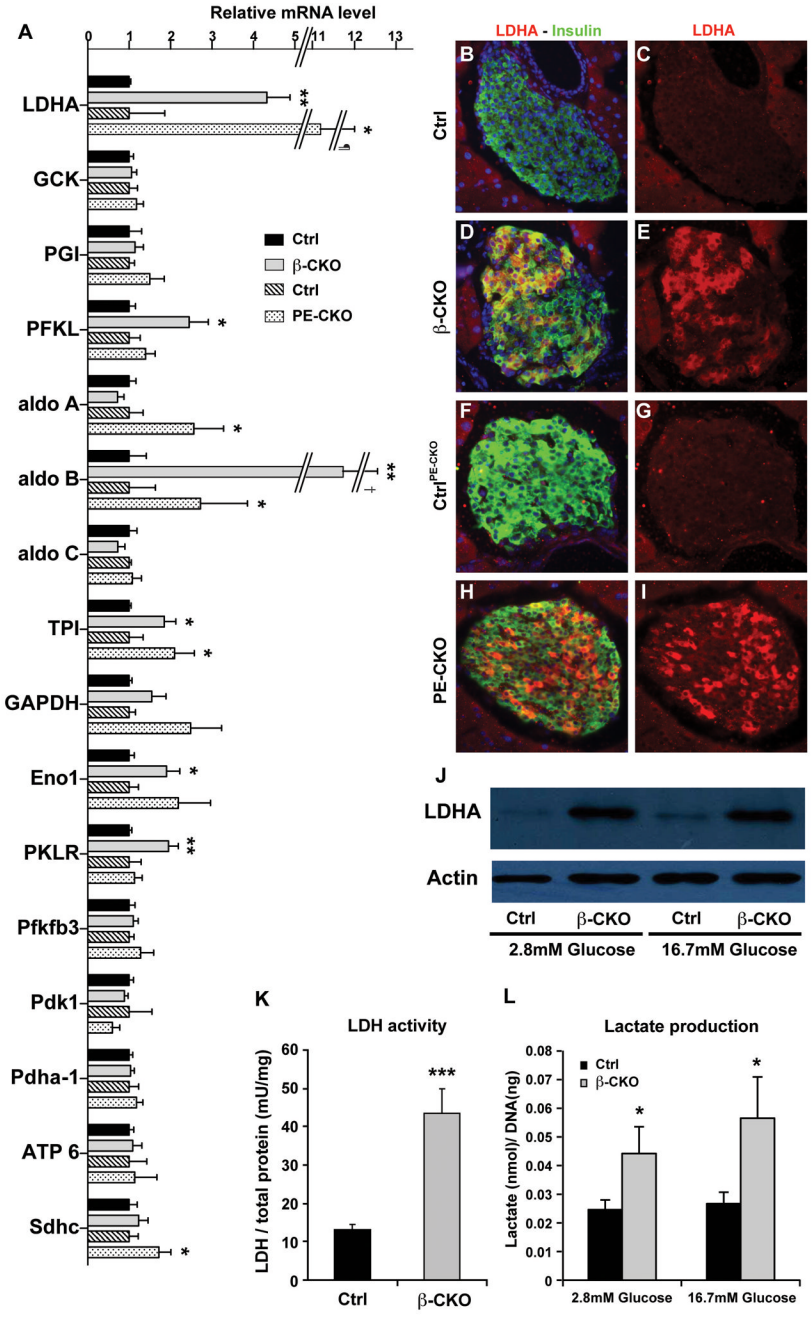

Figure 6. Increased expression of glycolytic genes

(A) Quantitative RT-PCR of genes involved in glycolysis and mitochondrial function (n=4–8 per genotype). ¶: Error bar ±1.85; †: Error bar ±3.78. (B–I) Coimmunostaining of LDHA (red) and insulin (green) in the pancreatic sections of control for *neuroD* β-CKO (B–C), *neuroD* β-CKO (DE), control for *neuroD* PE-CKO (F–G) and *neuroD* PE-CKO (H–I). Nuclei are stained with DAPI (blue). Original magnification was 200x. (J) Increased LDHA protein in both low glucose (2.8mM) and high glucose (16.7mM) in the *neuroD* β-CKO islets. (K–L) Increased LDHA activity and increased production of lactate in *neuroD* β-CKO islets (n=8–11). * P≤0.05, ** P≤0.01 and *** P≤0.001.