

## Irreversible loss of normal beta-cell regulation by glucose in neonatally streptozotocin diabetic rats

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**Summary** Animals with NIDDM display abnormal glucose regulation of insulin secretion and biosynthesis. We tested reversibility of abnormal regulation by normoglycaemia using an islet transplantation technique. Inbred non-diabetic and neonatally STZ diabetic rats (n-STZ) were used. Transplantations insufficient to normalize the blood glucose levels (200 islets under kidney capsule) were performed from diabetic to normal (D-N) and from diabetic to diabetic (D-D), as well as from normal to normal (N-N) and from normal to diabetic (N-D) rats. Four weeks after transplantation, graft bearing kidneys were isolated and perfused with Krebs-Henseleit bicarbonate buffer to measure insulin secretion in response to 27.8 mmol/l glucose and 10 mmol/l arginine. Four weeks of normoglycaemia failed to restore glucose-induced insulin

secretion from n-STZ islets (glucose induced increment:  $-1.7 \pm 2.5$  fmol/min in D-N,  $1.2 \pm 7.1$  fmol/min in D-D). In contrast to normal islets, normoglycaemia reduced insulin mRNA contents ( $60 \pm 24$  in D-N,  $496 \pm 119$  in D-D; O.D.-arbitrary units). However, arginine-induced secretion was markedly enhanced by diabetic environment in both normal and n-STZ islet grafts. These results indicate that selected aspects of glucose recognition are irreversibly damaged by a long-term diabetic state or, alternatively, by a lasting effect of STZ administration. [Diabetologia (1994) 37: 351–357]

**Key words** Animal models NIDDM, insulin secretion, insulin mRNA, cytochrome b mRNA, islets of Langerhans.

The neonatally STZ diabetic rat (n-STZ) is an animal model of NIDDM [review 1, 2]. In n-STZ insulin secretion is markedly abnormal at adult age despite only mild or moderate hyperglycaemia [1, 2]. In particular, the insulin response to glucose is reduced out of proportion to the decrease in beta-cell mass. Responses to non-metabolised secretagogues, such as L-arginine or phosphodiesterase inhibitors, are however relatively preserved. This pattern of abnormalities bears resem-

blance to secretory abnormalities in human NIDDM. Regarding mechanisms behind secretory abnormalities of n-STZ previous studies have suggested that deficient glucose oxidation, possibly linked to a decreased activity of the glycerol phosphate shuttle, could underlie the lack of sensitivity to the hexose [3, 4].

In elucidating the primary causes behind the secretory abnormalities in n-STZ, one needs to know whether these are reversible upon normalization of the diabetic milieu. On this point available evidence is contradictory. Normalization of an insulin response to glucose was previously reported after 5 days of insulin treatment in vivo [5], or after 2 weeks of tissue culture [6]. On the other hand, Leahy et al. [7] showed that insulin treatment restored a normal glucose potentiation of responses to arginine but not a response to glucose per se. The divergent results on reversibility could possibly be due to differences in diabetes induction, with severity of diabetes increasing with injection time

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*Abbreviations:* NIDDM, non-insulin-dependent diabetes mellitus; STZ, streptozotocin; O.D., optical density; IRI, immunoreactive insulin

after birth [8]. This notion is compatible with the report of Serradas et al. [9]. When rats were treated with STZ on day 5 rather than day 0 after birth, the decrease in hyperglycaemia by insulin, phlorizin, or vanadate treatments failed to restore an insulin response to glucose. However, in these and in previous experiments complete normoglycaemia was not obtained.

The further elucidation of possible defects of beta cells in n-STZ would require conclusive evidence whether or not insensitivity is present after long-term normoglycaemia. To achieve such a goal by insulin treatment is a very difficult task since a) insulin treatment rarely produces stable 24 h normoglycaemia b) insulin can reduce beta-cell sensitivity by direct or indirect effects [10]. In the present study we have used a transplantation procedure [11], in which islets of n-STZ were transplanted under the kidney capsule of syngeneic normal or diabetic recipients. Four weeks later we examined the insulin release from the perfused kidney as well as the expression of insulin mRNA from the grafted islets. The results were compared with those obtained in islets isolated from non-diabetic rats and transplanted into syngeneic normal or diabetic recipients.

## Materials and methods

### Animals

Inbred Wistar-Furth rats (B & K, Sollentuna, Sweden) were used. Pregnant rats were delivered at mid-gestation. The day after birth newborn pups were treated with an i. p. injection of 90 mg/kg STZ (kindly supplied by Dr W. E. Dulin, Upjohn, Kalamazoo, Mich., USA) as described [12]. Two days after STZ treatment blood glucose levels were measured by tail snipping. Only the pups with blood glucose levels equal or above 10.0 mmol/l were selected as diabetic animals. Vehicle treated pups served as normal controls. Both normal and diabetic pups were weaned around 28 days of age and allowed free access to water and a standard pelleted diet (B & K) and were kept on a 12 h light–12 h dark cycle.

### Islet isolation and transplantation

Female rats were always used as islet donors, whereas male rats were always used as islet recipients. Before islet isolation, blood glucose levels of donor rats were measured after tail snipping. Islets of Langerhans were isolated by collagenase (Boehringer Mannheim, Mannheim, Germany) essentially as described [13, 14]. After isolation the islets were cultured 1–2 days in RPMI 1640 supplemented with 11.1 mmol/l glucose, 2.1 mmol/l L-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum (Sigma, St Louis, Mo., USA). Around 200 islets were transplanted under the capsule of the left kidney using a capillary tube as described [11]. Four groups of transplantation were performed: from diabetic to normal (D-N) and from diabetic to diabetic (D-D), as well as from normal to normal (N-N) and from normal to diabetic rats (N-D). In some of the islet preparations from normal as well as from diabetic donors, groups of 30 islets were secured before transplantation for measurements of islet insulin and DNA contents [15]. In all recipient rats blood glucose levels (non-fasting) were measured both before and 4 weeks after islet transplantation.

### Kidney perfusion experiments

Four weeks after transplantation, the rats were anaesthetized by i. p. injection of 50 mg/kg pentobarbital sodium. The graft bearing kidneys were isolated and perfused basically by the methods of Korsgren et al. [11]. Kidney perfusion was carried out at a constant flow rate of 2.5 ml/min maintained by a peristaltic pump. The medium used was Krebs-Henseleit bicarbonate buffer [16] supplemented with 20 g/l bovine serum albumin (Sigma), 20 g/l dextran T-70 (Pharmacia, Uppsala, Sweden) and 4.7 mmol/l glucose. The Krebs-Henseleit bicarbonate buffer medium was kept at 37 °C and continuously gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. An equilibration period of 30 min was allowed before the start of perfusion with the test substances. The latter time point was designated as time 0 min. Stimulations with 27.8 mmol/l glucose and 10 mmol/l arginine were carried out with the aid of a side arm infusion pump. Effluents were collected at times indicated in the Figures into prechilled tubes containing aprotinin (Bayer, Leverkusen, Germany) at a final concentration of more than 500 KIE/ml, and stored at –20 °C until insulin assay. At the end of perfusion, the islet grafts were dissected free from kidney parenchyma (but with kidney capsule still adhering), then frozen and stored in liquid nitrogen until extraction of RNA.

### Glucose and insulin assays

Blood glucose determination was carried out by a glucose oxidase method using reagent strips (Boehringer Mannheim) read for absorbance in a reflectance meter (Boehringer Mannheim). IRI was measured by radioimmunoassay [17] using rat insulin as standard, <sup>125</sup>I-labelled insulin as tracer, and our own antibodies raised against porcine insulin.

### RNA extraction and Northern blotting analysis

Total RNA was extracted from the islet grafts by the guanidine isothiocyanate method [18]. After isolation, similar amounts of RNA (0.8–1.0 µg) were applied to 1% agarose gels and electrophoresed. The RNA was then transferred to a nylon membrane (Magna Graph nylon transfer membrane; Micron Separation, Westboro, Mass., USA). The Northern blots were sequentially hybridized to <sup>32</sup>P-labelled cDNA probes coding for rat insulin [PRI-7; 19] and mitochondrial encoded cytochrome b gene [20]. [ $\alpha$ -<sup>32</sup>P]dCTP was obtained from Amersham (Amersham, Bucks., UK), and the probes were labeled using Multiprime DNA Labelling system (Amersham). Hybridization and autoradiography were performed as previously described [21]. Densitometric analysis of the autoradiograms was performed after non-saturating exposures with Quick Scan Jr densitometer (Helena Laboratories, Beaumont, Tx., USA). The values obtained were expressed in arbitrary units of O. D.

### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Integrated insulin responses were calculated as the increment above the secretion rates that were recorded immediately before the administration of test substances, and converted to mean secretion rates by division with certain time periods. Tests of significance were performed using the two-tailed Student's *t*-test for paired or unpaired differences. Multiple comparisons among groups were carried out using one-way analysis of variance and the Scheffe's test.

**Table 1.** Characteristics of the islet donor and recipient rats

Experimental groups		Age (weeks)	Body Weight (g)	Blood Glucose (mmol/l)	
		At transplantation	At transplantation	Before transplantation	4 weeks after transplantation
<i>Donors</i>					
Normal	(15)	11.4 ± 0.5	173 ± 5	5.4 ± 0.1	
Diabetic	(41)	13.0 ± 0.4	168 ± 3	7.3 ± 0.2 <sup>a</sup>	
<i>Recipients</i>					
D-N	(7)	14.9 ± 1.8	309 ± 25	5.5 ± 0.5	4.9 ± 0.3
D-D	(7)	14.7 ± 1.1	259 ± 12	11.1 ± 1.0 <sup>c</sup>	11.4 ± 1.4 <sup>b</sup>
N-N	(9)	15.0 ± 1.2	322 ± 12	5.7 ± 0.3	5.4 ± 0.2
N-D	(10)	15.7 ± 1.1	268 ± 6 <sup>d</sup>	11.4 ± 0.6 <sup>d</sup>	9.7 ± 0.6 <sup>d</sup>

The results are means ± SEM of the number of observations indicated in parentheses. Female rats were used as islet donors, whereas male rats were used as recipients.

D-N, diabetic donor-normal recipient; D-D, diabetic donor-diabetic recipient; N-N, normal donor-normal recipient; N-D, normal donor-diabetic recipient.

<sup>a</sup>  $p < 0.001$  vs normal donors;

<sup>b</sup>  $p < 0.01$ ; <sup>c</sup>  $p < 0.001$  vs D-N;

<sup>d</sup>  $p < 0.001$  vs N-N.

Unpaired Student's *t*-test was used for comparisons

## Results

### Characteristics of islet donor and recipient rats

Body weights of diabetic recipients were significantly lower than those of normal recipients (Table 1). Diabetic recipients showed significantly higher levels of blood glucose than diabetic donors ( $p < 0.001$ ). More severe diabetes in males (= recipients) was expected from previous observations [22].

### Insulin and DNA contents in islets before transplantation

The DNA content of islets from diabetic donor rats was decreased by 70%, compared to normal rats: diabetic,  $5.4 \pm 0.5$  ( $n = 10$ ); normal,  $17.0 \pm 1.7$  (9) ng/islet,  $p < 0.0001$ . Contents of insulin were also markedly reduced: diabetic,  $31 \pm 4$  (10); normal,  $104 \pm 10$  (9) fmol/ng DNA,  $p < 0.0001$ .

### Effects of transplantation on blood glucose and weight gain

Hyperglycaemia in the diabetic recipients persisted after islet transplantation (Table 1). However, the blood glucose levels of N-D recipients decreased significantly after transplantation ( $p < 0.001$ , paired Student's *t*-test), whereas D-D did not show such a decrease. The recipient rats in every group gained body weight to a similar extent after transplantation (results not shown).

### Insulin release from normal islet grafts

Insulin release from the islet grafts of N-N and N-D is shown in Figure 1. During the equilibration period with 4.7 mmol/l of glucose alone, basal insulin secretion of

**Table 2.** Mean insulin secretion rates under basal and stimulative conditions with 27.8 mmol/l glucose or 10 mmol/l arginine

Experimental groups	Basal (fmol/min)	Δ IRI glucose (fmol/min)	Δ IRI arginine (fmol/min)
D-N (7)	23.4 ± 9.1	-1.7 ± 2.5	34.9 ± 10.2
D-D (7)	25.1 ± 8.3	1.2 ± 7.1	95.0 ± 23.7 <sup>a</sup>
N-N (9)	16.2 ± 2.6	96.2 ± 29.0	172.5 ± 42.4
N-D (10)	36.1 ± 5.8 <sup>c</sup>	50.6 ± 18.5	336.1 ± 62.6 <sup>b</sup>

The results are means ± SEM of the number of experiments indicated in parentheses. Experimental groups as in Table 1.

<sup>a</sup>  $p < 0.05$  vs D-N;

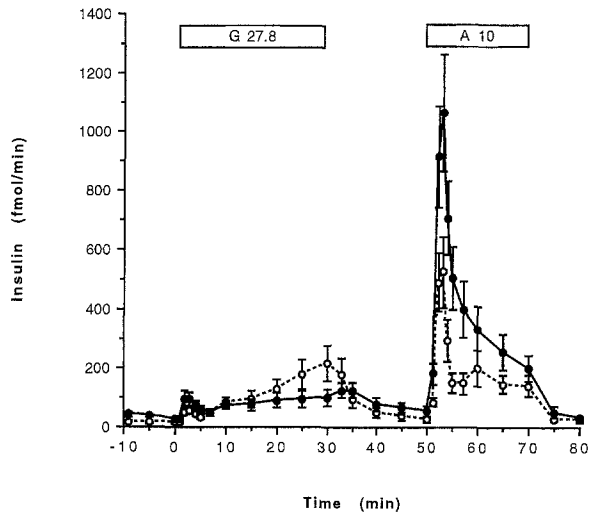
<sup>b</sup>  $p < 0.05$ ; <sup>c</sup>  $p < 0.01$  vs N-N

Unpaired Student's *t*-test was used for comparisons

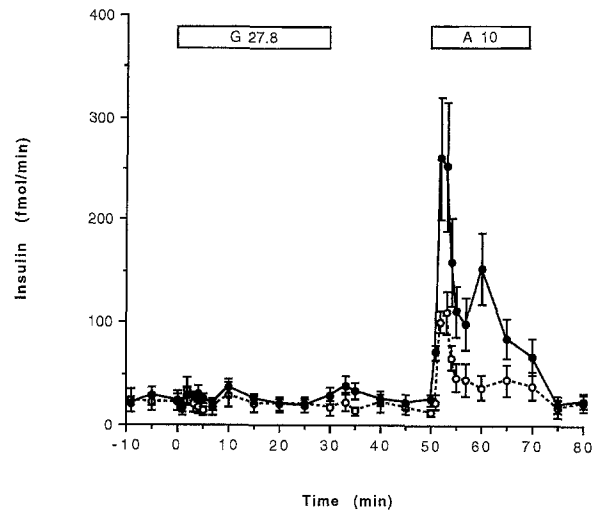
N-D was more than twice that of N-N (Table 2). There was a positive correlation between the blood glucose levels and basal insulin secretion in N-D ( $r = 0.6181$ ) which was however not significant ( $p = 0.0568$ ). Perfused kidneys from N-N showed a biphasic pattern of insulin release in response to 27.8 mmol/l glucose. First phase insulin responses to glucose as assessed from the first 7 min of 27.8 mmol/l glucose did not significantly differ between N-N and N-D. There was a tendency for insulin secretion from N-D to be smaller than that from N-N during the last 10 min of 27.8 mmol/l glucose ( $p = 0.09$ ). After cessation of glucose stimulation a small off-response was noted in N-D (Fig. 1). Thus, the insulin levels of N-D 3 min after cessation of 27.8 mmol/l glucose were significantly higher than those at the last point of 27.8 mmol/l glucose infusion ( $p < 0.01$ , paired Student's *t*-test). Concerning arginine stimulation, the islet grafts from N-D showed a 1.9-fold greater insulin response than the islet grafts from N-N (Fig. 1, Table 2).

### Insulin release from n-STZ islet grafts

In perfused kidneys of D-D rats, there was a complete lack of insulin response to 27.8 mmol/l glucose (Fig. 2, Table 2). Arginine, on the other hand, evoked a



**Fig. 1.** Insulin release from the islet grafts of N-N (○) and N-D (●) in response to 27.8 mmol/l glucose and 10 mmol/l arginine 4 weeks after transplantation. Data are shown as mean  $\pm$  SEM of 9–10 experiments. Glucose concentration in the basal perfusate was 4.7 mmol/l



**Fig. 2.** Insulin release from the islet grafts of D-N (○) and D-D (●) in response to 27.8 mmol/l glucose and 10 mmol/l arginine 4 weeks after transplantation. Data are shown as mean  $\pm$  SEM of seven experiments in each group. Glucose concentration in the basal perfusate was 4.7 mmol/l

marked insulin response in the presence of 4.7 mmol/l glucose (Fig. 2, Table 2).

Four weeks of normoglycaemia failed to restore any insulin response to glucose from transplants of D-N rats (Fig. 2, Table 2). As regards the response to arginine, this was reduced to 37% of the response in D-D rats. This influence of normoglycaemia vis-a-vis hyperglycaemia was not obviously dissimilar to that on normal islet grafts, the response in N-N being 51% of that in N-D (Table 2).

#### Measurements of mRNA

Total RNA contents of the islet grafts (islets plus kidney capsule) did not differ significantly among the four groups (Table 3). In N-D mean insulin mRNA was reduced compared to N-N, but this difference did not reach statistical significance ( $p = 0.16$ ). The results in n-STZ islet grafts were in marked contrast to those of normal ones. Thus the insulin mRNA was markedly and significantly reduced in D-N compared to D-D rats (Table 3). Measurements of cytochrome b mRNA showed no significant differences between N-N and N-D, nor between D-N and D-D. However, the expression of cytochrome b mRNA in islets exposed to n-STZ (groups D-N and D-D) was reduced by 70%, compared to groups N-N or N-D.

#### Discussion

The main aim of the present study was to investigate the long-term outcome of insulin secretion from islets of n-STZ, after removing these islets from their original

**Table 3.** Islet graft contents of total RNA and insulin and cytochrome b mRNA

Experimental groups	Total RNA ( $\mu$ g/graft)	Insulin mRNA (O.D.)	Cytochrome b mRNA (O.D.)
D-N	1.6 $\pm$ 0.6 (7)	60 $\pm$ 24 (7)	413 $\pm$ 147 (5)
D-D	2.4 $\pm$ 0.6 (6)	496 $\pm$ 119 (5) <sup>a</sup>	614 $\pm$ 136 (4)
N-N	2.7 $\pm$ 0.7 (8)	1565 $\pm$ 242 (8)	1863 $\pm$ 431 (4)
N-D	1.5 $\pm$ 0.4 (6)	918 $\pm$ 387 (5)	1487 $\pm$ 182 (5)

The results are means  $\pm$  SEM of the number of experiments indicated in the parenthesis. The contents of insulin and cytochrome b mRNA were evaluated by densitometric scanning of the Northern blots, and the values are expressed in arbitrary units of O.D. Experimental groups as in Table 1.

<sup>a</sup>  $p < 0.01$  vs D-N using unpaired Student's  $t$ -test

and hyperglycaemic environment. To properly evaluate such an influence and to provide information on the validity of the transplantation protocol, we studied in parallel insulin secretion from islets isolated from non-diabetic rats. The transplanted islets from normal to normal rats showed a typical biphasic secretion of insulin in response to glucose, which was very similar to that of perfused pancreata of the Wistar rats [23]. Furthermore, the islet grafts from normal to diabetic rats showed somewhat reduced responses to glucose but enhanced responses to arginine. After cessation of glucose stimulation, off-responses of insulin were also observed. These findings are in good agreement with the pancreas perfusion studies of both spontaneously [24, 25] and experimentally-induced [26] hyperglycaemic rats.

The present study clearly shows that the insulin response to glucose from islets of n-STZ diabetic rats is not restored by long-term normoglycaemia. Indeed

not a trace of glucose-induced insulin secretion could be detected from n-STZ islets after 4 weeks of normoglycaemia. This lack of reversibility is in marked contrast to the situation in 48 h glucose-infused rats, in which secretory abnormalities are similar to those of n-STZ, but are completely reversible within 72 h [26].

Our experiments have also revealed that regulation by blood glucose of insulin mRNA is abnormal in n-STZ. Hence in islets from non-diabetic rats long-term hyperglycaemia tended to decrease insulin mRNA. These observations are in line with previous observations [27]. Conversely in n-STZ islets normoglycaemia markedly decreased the insulin gene expression. This paradoxical effect could be viewed as a second aspect of insensitivity to glucose at normoglycaemia; insensitivity thus encompassing two aspects of glucose regulation: that of insulin secretion and of insulin mRNA expression.

The results on arginine-induced insulin secretion on the other hand were qualitatively similar between non-diabetic and n-STZ islets in showing potentiation of the secretory response by hyperglycaemia. These findings agree with previous ones showing that correction of hyperglycaemia by insulin treatment decreased arginine-induced insulin secretion in n-STZ [7]. The molecular basis for preferential preservation of this aspect of glucose sensitivity is not known. A possibility that comes to mind relates to enhanced deposition of glycogen which is induced by hyperglycaemia [28]. Available energy may be rate-limiting for insulin responses to non-nutrient secretagogues, such as arginine. If so, the breakdown of enlarged glycogen stores could potentiate arginine-induced insulin secretion. Our results indicate that a putative fuel-providing mechanism is distinct from that by which the hexose per se initiates insulin secretion.

What is the cause of the irreversible beta-cell insensitivity in the n-STZ rat? Three different possibilities deserve consideration. First, long-term hyperglycaemia singly or in combination with other factors of an abnormal diabetic state could damage beta cells. Massive glucose infusions have been shown to kill beta cells and induce severe diabetes in dogs [29], but irreversibility of functional aberrations has not to our knowledge been demonstrated. In our n-STZ rats hyperglycaemia was generally mild prior to transplantation, casting some doubt on the impact of this factor for irreversibility. More importantly, the insulin response to glucose and insulin mRNA in islets from non-diabetic rats was at the most only moderately reduced after 4 weeks of hyperglycaemia. However we cannot rule out the possibility that hyperglycaemia occurring early in life, i. e. during the days after the neonatal injection of STZ, could have imparted an irreversible effect on beta-cell function.

Second, one can envisage that STZ has a long-lasting effect on the beta-cell function. A lasting sup-

pressive effect of STZ on glucose-induced insulin secretion has been demonstrated in islets which had been cultured for 7 days after *in vitro* STZ treatment [30]. Such islets also released less insulin following transplantation [31]. It was also reported that islets isolated from n-STZ diabetic rats or treated *in vitro* with STZ showed a marked decrease in contents of mitochondrial DNA and expression of mitochondrial encoded cytochrome b mRNA [30, 32]. These latter findings are compatible with the present observations of a lower content of cytochrome b mRNA in n-STZ than in normal islet grafts, although smaller amounts of islet tissue from the former could also underlie these differences. Since STZ has been reported to alkylate DNA and to induce DNA strand breaks [33], it is conceivable that a long-lasting effect of STZ is transmitted through mutations in DNA sequences. However, n-STZ islets are islets composed of mainly regenerated beta cells [34, 35], and it is unknown whether STZ effects on nuclear or mitochondrial DNA can be transmitted through progenitor cells to regenerated beta cells.

Third, the irreversible loss of a response to glucose at normoglycaemia could be due to selective elimination of beta cells possessing the lowest threshold to glucose. Such a notion is compatible with a documented heterogeneity of responsiveness to glucose among beta cells [36]. Heterogeneity pertains to glucose-induced insulin secretion [36, 37], glucose-induced biosynthesis [38], as well as glucose oxidation [36, 39]. The presence of glucose has been shown to decrease STZ toxicity [40]. It is conceivable that the glucose moiety of the STZ molecule could impart selective uptake of the toxin in glucose-sensitive cells. It is also possible that vulnerability of such cells could be indirectly related to handling of the toxin.

In summary, we have demonstrated that the loss of beta-cell sensitivity to glucose in n-STZ is irreversible during normoglycaemic conditions. Furthermore, n-STZ islet grafts exhibit a paradoxical decline in insulin mRNA upon normalization of blood glucose. The potentiating influence of hyperglycaemia on arginine-induced insulin secretion is however preserved. The cause of the irreversible insensitivity is not clear; however, two basic working hypotheses may be suggested: 1) STZ induces long-lasting genomic damage, especially at the mitochondrial level, rendering beta cells unable to respond to glucose with proper insulin secretion; 2) STZ treatment at neonatal age selectively damages and eliminates a sub-population of glucose-sensitive cells, leaving less sensitive cells as progenitors of regenerating cells.

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