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Glucagon-like peptide 1 receptor signaling influences topography of islet cells in mice

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Abstract Glucagon-like peptide 1 (GLP-1) amplifies glucose-induced insulin release in vivo and in vitro. Activation of GLP-1 receptor (GLP-1R) signaling leads to differentiation of exocrine cells towards a β -cell phenotype in vitro and stimulation of islet cell proliferation in vitro and in vivo, suggesting a potential role for GLP-1 in the modulation of islet growth and differentiation. To determine whether basal levels of GLP-1R signaling are essential for islet development, we examined islet cell composition and topography in GLP-1R $^{-/-}$ mice. Total β -cell volume and number are not altered, but the topography of β cells is markedly different in GLP-1R $^{-/-}$ mice compared with GLP-1R $^{+/+}$ controls. The distribution of β cells is shifted from large to small and medium-sized islets in the absence of GLP-1R signaling (large islets: $50\pm 3\%$ in GLP-1R $^{+/+}$ vs $28\pm 4\%$ in GLP-1R $^{-/-}$, $P<0.01$ and medium islets: $32\pm 2\%$ in GLP-1R $^{+/+}$ vs $48\pm 3\%$ in GLP-1R $^{-/-}$, $P<0.001$). Furthermore, GLP-1R $^{-/-}$ islets exhibit abnormalities in cell topography, with two to threefold more centrally located α cells detected in GLP-1R $^{-/-}$ islets. These alterations in α - and β -cell topography indicate that basal levels of GLP-1 signaling in the normal rodent are involved in the normal cellular organization of the endocrine pancreas.

Keywords GLP-1 receptor · Islet · Morphometry

Introduction

The insulin-releasing effect of glucose varies in amplitude with the degree of cyclic AMP production in the β cells. Among the various peptidergic mediators of β -cell function, glucagon-like peptide 1 (7–36; GLP-1) amide is considered a potentially important regulator of this response in vivo [4, 9]. In order to assess the physiological importance of GLP-1 receptor (GLP-1R) signaling for islet function and metabolic homeostasis in vivo, mice were generated with a targeted disruption of the GLP-1R gene (GLP-1R $^{-/-}$) [13]. GLP-1R $^{-/-}$ mice are viable and develop normally but exhibit moderate fasting hyperglycemia and glucose intolerance following oral or intraperitoneal glucose administration [13].

Although fasting glycemia and glucose-stimulated insulin release is subnormal in GLP-1R $^{-/-}$ mice in vivo, isolated GLP-1R $^{-/-}$ islets exhibit normal insulin release responses to glucose but not to GLP-1 [7]. The abnormalities in glucose tolerance observed in GLP-1R $^{-/-}$ mice reflect the essential role of GLP-1 signaling for glucose homeostasis in vivo. Nevertheless, recent studies suggest that the GLP-1R $^{-/-}$ phenotype reflects a number of upregulated complementary mechanisms that serve to enhance β -cell function in the absence of GLP-1 signaling. For example, GLP-1R $^{-/-}$ mice exhibit increased glucose-dependent insulinotropic polypeptide (GIP) secretion following glucose challenge and increased GIP-stimulated insulin release from the perfused pancreas or isolated islets [8, 10].

Although GLP-1 regulates glucose homeostasis through effects on insulin and glucagon secretion, recent studies imply a potential role for GLP-1 in control of insulin biosynthesis and, possibly, islet proliferation. Administration of GLP-1 to aging Wistar rats reversed age-related defects in glucose tolerance in association with upregulation of the levels of insulin messenger (m)RNA [16]. Administration of GLP-1 to young, lean mice increased islet cell proliferation in a dose-dependent manner [5], and the GLP-1 analogue, exendin-4, promoted β -cell growth in both control and partial pancreatectomized rats [17]. Furthermore,

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GLP-1 and exendin-4 converted pancreatic exocrine tumor cells into glucagon- and insulin-producing cells [18]. Moreover, GLP-1 stimulated DNA synthesis, immediate early gene expression, and β -cell proliferation via activation of a phosphatidylinositol (PI)-3-kinase-dependent pathway in vitro [3, 14]. To address the possibility that the trophic effects observed following exogenously administered GLP-1 are indicative of an essential role for GLP-1 in the regulation of islet development in vivo, we have analyzed islet size and cell distribution in mice with a targeted mutation of the GLP-1R.

Materials and methods

Animals

Control (GLP-1-1R^{+/+}; Iffa Credo, Brussels) and GLP-1R^{-/-} mice, 10-weeks-old [13], were bred under specific pathogen-free conditions and kept according to the Belgian regulations of animal welfare. Control and GLP-1R^{-/-} mice have the same CD1 genetic background. The pancreas was excised and processed for morphometric and hormone content analysis.

Tissue processing and immunocytochemistry

Pancreatic head and tail portions were dissected at the site of connecting pylorus and duodenum, and their weight was determined separately. The tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections (four sets of ten) of 3- μ m thickness were cut from each paraffin block at 100- μ m intervals.

Deparaffinized sections were immunostained for pancreatic hormones using the ABC/HRP (streptavidin-biotin horse-radish peroxidase complex; Dako, K0377, Denmark) method, as described previously [15]. Sections were incubated overnight at 4°C with guinea pig anti-insulin antibody (1:10,000, kindly provided by Dr. Chris Van Schravendijk, Diabetes Research Center, Vrije Universiteit Brussel, Belgium) or with rabbit anti-glucagon antibody (1:5000, kindly provided by Dr. Chris Van Schravendijk). Biotinylated goat anti-guinea pig immunoglobulin (Ig)G (1:1000, Vector Laboratories, Inc., Burlingame, Calif.) and goat anti-rabbit IgG (1:300, Amersham Co., England) were used as second antibodies. After treatment with the ABC/HRP complex, sections were stained with chromogen 3,3'-diaminobenzidine tetrachloride (DAB, Sigma Chemical Co., St. Louis, Mo.) and hematoxylin.

Morphometry

The total α - and β -cell areas in tissue sections were measured according to previously described methods [15]. Sections were scanned with a flatbed scanner (ScanJet IICK, Hewlett Packard, C2500A, Calif.) connected with a Macintosh Quadra 700 computer, and analyzed using National Institutes of Health (NIH) image 1.41. The glucagon and insulin-immunoreactive area were traced manually under a Zeiss Axiophot microscope (final magnification 200 \times), connected with a video camera to an image analysis system (NIH Image 1.41). Total positive area was measured in eight sections at 130- μ m intervals from each pancreas (four in the head and four in the tail portions). The total α - and β -cell volume per pancreas was calculated using the stereological equation: $Ac/Ap = Vc/Vp$, whereby Ac was the total (α or β) cell area per section, Ap was the total pancreatic section area, Vc was the total (α or β) cell volume per pancreas, and Vp was the total volume of the pancreas, which was calculated from pancreatic weight divided by its density. The single cell area was traced manually at a final magnification of 400 \times . Ten cells from two or three islets per section and 80 cells per pancreas were analyzed. The individual cell volume was calculated by the following formula, assuming

the shape of cell approaches a spherical configuration: $volume = 4/3 \pi r^3$, wherein r was the cell radius derived from the formula of spherical surface area: $area = \pi r^2$. The total α - and β -cell number per pancreas was calculated by dividing the total cell volume by the individual cell volume ($N = Vt/Vi$).

Medium (5×10^3 – $2 \times 10^4 \mu m^2$) and large islets ($> 2 \times 10^4 \mu m^2$) were further analyzed for their glucagon-positive α -cell distribution. Glucagon-positive cells located centrally to the three most peripheral cell layers were considered as central α cells. Islets that contained minimally three glucagon-positive cells in the central area were distinguished from the other islets with α cells in the periphery.

Measurement of pancreatic hormone content

Pancreatic insulin and glucagon content was measured as previously described [7]. Briefly, the pancreatic tissue was homogenized in 2 mol/l acetic acid containing 0.25% bovine serum albumin (BSA), and the tissue extracts were analyzed for their insulin and glucagon content by means of radioimmunoassay [7].

Statistical analysis

Data represent mean \pm SEM. The statistical significance of the differences between the different groups was calculated using analysis of variance (ANOVA).

Results

Pancreatic weight and hormone content

GLP-1R^{+/+} and GLP-1R^{-/-} mice did not differ in their pancreatic insulin content as expressed per organ or per milligram of pancreatic tissue (Table 1). Their respective insulin immunoreactivities were distributed over comparable β -cell volumes and numbers (Table 2). The individual β -cell volumes were similar (620–660 μm^3 /cell in all groups).

Pancreatic glucagon content in GLP-1R^{-/-} mice was also comparable to that in GLP-1R^{+/+} mice. This glucagon immunoreactivity in GLP-1R^{-/-} mice appeared distributed over a larger α -cell volume and a larger α -cell number than that in gender-matched control mice, but this difference did not reach statistical significance (Table 2). In both wild-type and null mice, female mice presented a higher pancreatic glucagon content than male mice (Table 1). The α -cell numbers were also higher, but the differences reached only statistical significance in female GLP-1R^{-/-} mice ($P < 0.05$, GLP-1R^{+/+} vs GLP-1R^{-/-} mice; Table 2). Furthermore, the ratios of α - to β -cell volumes and cell numbers were markedly increased in female GLP-1R^{-/-}, with significant differences between the ratios measured in gender-matched control mice and with those in male GLP-1R^{-/-} mice ($P < 0.001$ – 0.05 , GLP-1R^{+/+} vs GLP-1R^{-/-} mice, Table 2).

Tissue distribution of insulin- and glucagon-immunoreactive areas

The insulin immunoreactive area is distributed over islets with varying size (Fig. 1). Size differences in sec-

Table 1 Pancreatic weight and hormone content. Data represent mean±SEM of five animals. Statistical significance of differences was calculated using analysis of variance (ANOVA). *GLP-1R* (glucagon-like peptide 1 receptor)

Experimental group	Pancreatic weight (mg)	Insulin (μg)		Glucagon (μg)		Glucagon/insulin (μg/μg)
		Per organ	Per gram tissue	Per organ	Per gram tissue	
Male						
GLP-1R+/+	366±22	18±2	51±8	0.24±0.02	0.7±0.1	0.02±0.003
GLP-1R-/-	401±24	16±3	43±9	0.29±0.02	0.8±0.1	0.02±0.003
Female						
GLP-1R+/+	357±15	11±2 ^a	30±4 ^a	0.34±0.03 ^b	1.0±0.1 ^b	0.04±0.005 ^c
GLP-1R-/-	361±18	14±2	40±5	0.38±0.02 ^a	1.1±0.1 ^a	0.03±0.002

^a Female vs male: $P<0.05$ ^b Female vs male: $P<0.01$ ^c Female vs male: $P<0.001$ **Table 2** Pancreatic β-cell and α-cell content. Cell volume and number were expressed as cubic millimeters and 10⁶ cells per pancreas, respectively. Data represent mean±SEM of five animals. Statistical significance of differences was calculated using analysis of variance (ANOVA)

Experimental group	β-Cell content		α-Cell content		α/β-Cell content	
	Volume	Number	Volume	Number	Volume	Number
Male						
GLP-1R+/+	1.3±0.3	2.1±0.5	0.07±0.02	0.21±0.05	0.06±0.01	0.10±0.01
GLP-1R-/-	1.4±0.2	2.2±0.3	0.11±0.02	0.27±0.04	0.08±0.01	0.13±0.02
Female						
GLP-1R+/+	1.9±0.2	2.8±0.3	0.12±0.02	0.32±0.03	0.06±0.01	0.11±0.01
GLP-1R-/-	1.2±0.3	1.9±0.5	0.17±0.02	0.46±0.08 ^b	0.15±0.02 ^{ac}	0.26±0.04 ^{ac}

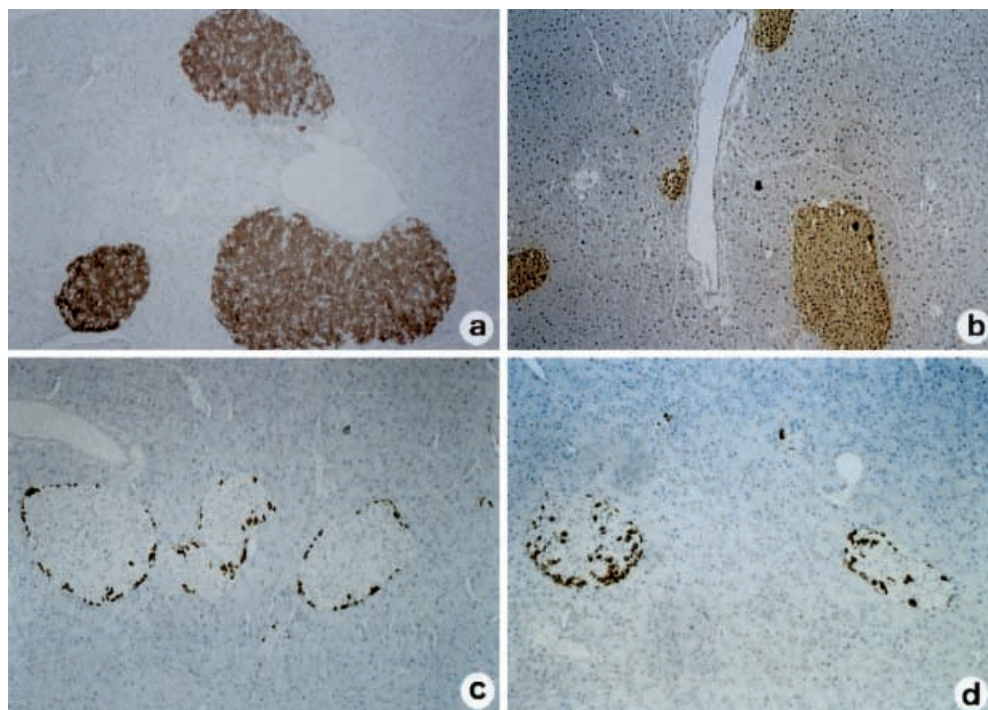
^a GLP-1R-/- vs GLP-1R+/+: $P<0.001$ ^b Female vs male: $P<0.05$ ^c Female vs male: $P<0.001$ **Fig. 1** Immunostaining for insulin (a, b) and glucagon (c, d) on pancreatic sections of glucagon-like peptide 1 receptor (GLP-1R)+/+ (a, c) and GLP-1R-/- (b, d) mice. Islets of GLP-1R-/- were generally smaller than those of the GLP-1R+/+ animals (see Fig. 2 for quantitative analysis). Glucagon-positive cells appeared almost exclusively in the periphery of the islets in GLP-1R+/+ mice (c), whereas they were distributed in both the periphery and the center of the islets in GLP-1R-/- mice (d; see Table 3 for quantitative analysis). Magnification ×330

Fig. 2 Distribution of islet sizes in glucagon-like peptide 1 receptor (GLP-1R)^{+/+} (□) and GLP-1R^{-/-} (■) mice. Size differences: single <300 μm^2 ; small 300–5000 μm^2 ; medium 5000–20,000 μm^2 ; and large >20,000 μm^2 . The upper panels show the percentage of islets that were of a particular size. The lower panels show the contribution of these islets to the total islet volume. Data represent mean \pm SEM of five animals. GLP-1R^{+/+} vs GLP-1R^{-/-}: ‡ $P<0.05$, * $P<0.01$, † $P<0.001$

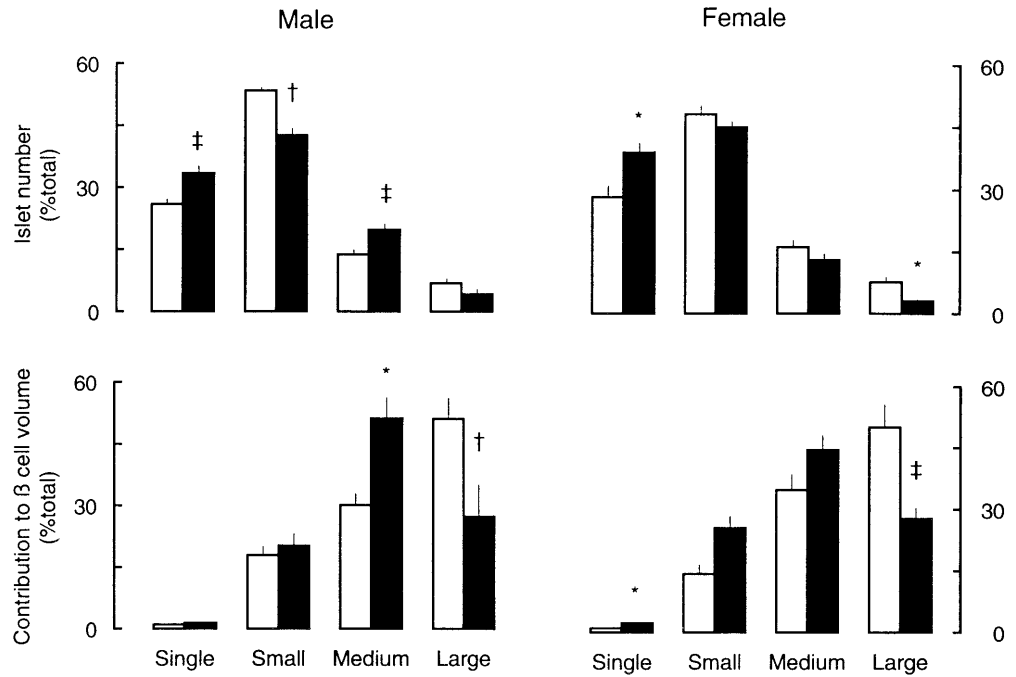


Table 3 Distribution of α cells. α -Cell number is expressed as 10^6 cells per pancreas. Data represent mean \pm SEM of five animals. Statistical significance of differences was calculated using analysis of variance (ANOVA)

Experimental group	α -Cell number			Percentage of islets with α cells in the center		
	Peripheral	Center	Center % total	Medium	Large	Total
Male						
GLP-1R ^{+/+}	0.19 \pm 0.04	0.02 \pm 0.004	11 \pm 1	10 \pm 5	36 \pm 4	17 \pm 4
GLP-1R ^{-/-}	0.21 \pm 0.02	0.06 \pm 0.01 ^a	22 \pm 2 ^c	40 \pm 4 ^b	73 \pm 11 ^a	46 \pm 5 ^c
Female						
GLP-1R ^{+/+}	0.27 \pm 0.03	0.05 \pm 0.01	15 \pm 1	10 \pm 2	34 \pm 7	19 \pm 3
GLP-1R ^{-/-}	0.37 \pm 0.06	0.10 \pm 0.02 ^a	22 \pm 1 ^b	48 \pm 6 ^b	79 \pm 7 ^c	54 \pm 6 ^b

^a GLP-1R^{-/-} vs GLP-1R^{+/+}: $P<0.05$

^b Female vs male: $P<0.01$

^c Female vs male: $P<0.001$

tions are used to classify the islets as single (<300 μm^2), small (300–5000 μm^2), medium (5000–20,000 μm^2) or large (>20,000 μm^2). In each experimental group, 1200–1300 islets were analyzed. In control mice, the single β -cell units and small islets accounted for 75–80% of total islet number but represented only 15% of the total β -cell volume (Fig. 2). Fifteen percent of islets were classified as medium and contributed to 30%–50% of the total β -cell volume. Five to ten percent of islets corresponded to large size but contributed to 50% of the total volume (Fig. 2). In contrast, significant differences in distribution of islet size were observed in GLP-1R^{-/-} mice, where the main β -cell volume contribution came from the medium-sized islets (48 \pm 3% in GLP-1R^{-/-} vs 32 \pm 2% in GLP-1R^{+/+} mice; $P<0.001$), while the large islets accounted for only 28 \pm 4% vs 50 \pm 3% in GLP-1R^{+/+} mice ($P<0.01$, Fig. 2).

We next examined the distribution of the total glucagon immunoreactive area, with reference to the relative location

of α cells within the islet. All medium and large islets on sections (250–300 islets per experimental group) were analyzed. In control mice, only 11% (male) and 15% (female) of the glucagon-positive cells are located in the center area of the islets, whereas 22% of glucagon-positive α cells are centrally located in GLP-1R^{-/-} mice ($P<0.01$ – 0.05 for male and female mice; Table 3 and Fig. 1). The increased proportion (twofold) of centrally located α cells is not simply a reflection of a lower number of peripherally located α cells, since this number was similar in $+/+$ and $-/-$ mice (Table 3). The significantly increased occurrence of centrally located α cells was a feature of both medium and large-sized islets (Table 3); they were found in 40–79% of GLP-1R^{-/-} male and female islets relative to 10–36% of GLP-1R^{+/+} islets ($P<0.01$ – 0.001 , Table 3).

Discussion

The results of the present study demonstrate that disruption of GLP-1R signaling is not associated with a marked alteration in the number of islet β cells, which is consistent with the finding that pancreatic insulin content is not significantly perturbed in GLP-1R $^{-/-}$ adult mice [7, 10]. However, when the β -cell number is expressed relative to the α -cell number, the ratio of α - over β -cell numbers is twofold higher in female mice lacking the GLP-1R. We therefore compared the topography of the glucagon-containing α cells with respect to the β cells in female mice. It was found that both male and female knockout mice exhibited more α cells in the center of pancreatic islets, while no significant differences were noticed in the number of α cells at the periphery of the islets.

Whether this alteration in topography has functional consequences is unknown. In most normal rodent islets, blood flows from the central core of β cells to the peripheral mantle where α cells are located [1]; this raises the potential for β -cell secretory products to modify α -cell functions [1]. Similarly, centrally located α cells might exert effects on the more peripheral β cells; this form of regulation might normally occur in a minority (<20%) of control $+/+$ islets but in 50% of GLP-1R $^{-/-}$ islets. Because glucagon amplifies glucose-induced insulin release [11], this topographical alteration may help compensate for the lack of GLP-1-potentiated insulin release. Such effects are difficult to identify *in vitro*, since isolated islets do not mimic the flow-dependent interstitial glucagon concentrations of the pancreas *in situ*. It should be further clarified whether the increased α -cell number and their topographic alteration in female GLP-1R $^{-/-}$ mice could account for the better glucose control following oral glucose challenge in these animals than that of the male null mice [13].

In addition to the altered distribution of α cells, we also observed an altered distribution of β cells in GLP-1R knockout mice. While islets of over $2 \times 10^4 \mu\text{m}^2$ contributed to over 50% of the total β -cell volume in control mice, they contributed to less than 30% of β -cell volume in GLP-1R $^{-/-}$ mice. The absence of GLP-1R signaling is thus associated with the formation of a lower number of large β -cell clusters. In the preliminary experiments, we also compared the islet size and cell distribution in neonatal and 2-week-old animals. It was found that both in control and in GLP-1R $^{-/-}$ mice, islets smaller than $2 \times 10^4 \mu\text{m}^2$ contributed to the major part of β -cell volume, and most islets contained central α cells (unpublished). Previous studies have shown that the shift to large β -cell clusters is a postnatal, age-dependent process [15], but its underlying mechanisms remain unknown.

Segregation of islet β and non- β cells generally continues postnatally, reaching completion in 5-week-old rodents [6]. Thus, GLP-1 seems to influence postnatal development of islet topography. This role is certainly not preponderant, because no major disturbances in islet

configuration were seen. Studies in rat and in young obese mice have shown that GLP-1 can increase islet growth [5, 17]; this observation is compatible with the present finding that GLP-1R $^{-/-}$ mice exhibit a lower number of large β -cell clusters.

Intriguingly, the disturbances in islet topography in GLP-1R $^{-/-}$ mice partially resemble findings observed in mice harboring a disruption of the neural cell adhesion molecule (NCAM) gene [6]. NCAM $^{-/-}$ mice lose the normal peripheral distribution of α cells and may exhibit compensatory upregulation of cadherin-mediated cell adhesion [6]. Whether the changes observed in islet topography in NCAM $^{-/-}$ or GLP-1R $^{-/-}$ mice are functionally relevant for islet cell function is difficult to determine. Glucose tolerance was reported to be normal in NCAM $^{-/-}$ mice [6] and is only modestly perturbed in GLP-1R $^{-/-}$ mice [13]. Nevertheless, the identification of cell adhesion molecules and signaling pathways essential for topographical organization of the endocrine pancreas increases our understanding of the mechanisms underlying the structural and functional determinants of islet cell function *in vivo*. Whether signaling of the GLP-1R could influence expression of one or more cell adhesion molecules functionally important for α -cell localization in the endocrine pancreas should be further clarified. Moreover, future studies directed at elucidating the possible downstream effectors responsible for the abnormal islet cell phenotype observed in GLP-1R $^{-/-}$ mice is clearly indicated.

In conclusion, our observations demonstrate that the absence of GLP-1Rs does not alter the number of α and β cells in adult mice but alters their topography. The occurrence of more islets with centrally located α cells and the reduced formation of large β -cell clusters suggest that GLP-1 plays a role in the postnatal organization of endocrine islet cells. Whether these findings reflect a direct interaction of GLP-1R signaling with signal transduction pathways upstream of molecules regulating cell proliferation and migration is not yet clear. The present findings indicate, however, that even in the absence of exogenous GLP-1 administration in the setting of experimental diabetes and severe hyperglycemia, the GLP-1R plays a role in the structural organization of the adult endocrine pancreas. Hence, whether dysregulated GLP-1R signaling contributes to the development of abnormalities in islet mass observed in other knockout models exhibiting changes in the islet cell topography [2, 12] should also be considered in future studies.

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