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Original Research

Protein Restriction during Early Life in Rats Alters Pancreatic $GABA_A$ Receptor Subunit Expression and Glucagon Secretion in Adulthood

Michelle A. Durst MSc [a,c], Victoria A. Lux-Lantos PhD [b], Daniel B. Hardy PhD [c,d], David J. Hill DPhil [a,c,d,e,*], Edith J. Arany MD, PhD [a,d,f]

[a] *Lawson Health Research Institute, St. Joseph's Health Care, London, Ontario, Canada*
[b] *Instituto de Biologia y Medicina Experimental, Conicet, Buenos Aires, Argentina*
[c] *Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada*
[d] *Department of Medicine, University of Western Ontario, London, Ontario, Canada*
[e] *Department of Paediatrics, University of Western Ontario, London, Ontario, Canada*
[f] *Department of Pathology, University of Western Ontario, London, Ontario, Canada*

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ABSTRACT

Objective: Gamma-aminobutyric acid (GABA) is present in the endocrine pancreas and contributes to insulin and glucagon secretion. Protein restriction during pregnancy and lactation causes impaired glucose homeostasis in adulthood. We investigated the effect of low protein (LP) diet on the GABAergic system within islets and associated changes in hormone release.
Methods: Wistar rats were fed control (C) or LP diet throughout gestation and lactation; all offspring received C diet from weaning (day 21). $GABA_A$ receptor presence in islets was examined by Western blot, and mRNA expression for glutamic acid decarboxylase (GAD) 65 and $GABA_A$ and $GABA_B$ receptor isoforms by real-time polymerase chain reaction at 130 days. Insulin and glucagon secretion were measured in vivo after L-arginine stimulation.
Results: L-glutamate, the precursor of GABA, was reduced in pancreas from offspring of LP-fed rats at birth. At day 21, animals receiving LP diet had an elevated $GABA_A$ presence within islets, lower fasting plasma insulin, and decreased islet insulin and glucagon content. By day 130, $GABA_A$ protein did not differ with diet, but the expression of $GABA_{A\beta3}$ subunit mRNA was decreased in the LP group. Glucagon secretion and gene expression were increased in LP-fed rats. Expression of $GABA_B$ and GAD within islets was unaltered.
Conclusions: LP diet caused changes to $GABA_A$ receptor expression within islets with implications for glucagon secretion and glucose homeostasis.

Mots clés:
programmation fœtale
GABA (GABA, *gamma-aminobutyric acid*, pour « acide gamma-aminobutyrique »)
régime pauvre en protéines
développement du pancréas
diabète de type 2

RÉSUMÉ

Objectif : L'acide gamma-aminobutyrique (GABA : *gamma-aminobutyric acid*) est présent dans le pancréas endocrine et contribue à la sécrétion d'insuline et de glucagon. La restriction en protéines durant la grossesse et la lactation cause une altération de l'homéostasie du glucose chez l'adulte. Nous avons examiné l'effet d'un régime pauvre en protéines (PP) sur le système GABAergique des îlots et associé les changements à la libération des hormones.
Méthodes : Les rates Wistar étaient nourries selon la diète témoin (T) ou selon la diète PP durant toute la gestation et la lactation; toute la progéniture recevait la diète T dès le sevrage (21^e jour). La présence du récepteur $GABA_A$ dans les îlots a été étudiée par la méthode *western-blot*, et l'expression de mARN de l'acide glutamique décarboxylase (AGD) 65 et les isoformes du récepteur $GABA_A$ et $GABA_B$, par la réaction en chaîne par polymérase en temps réel au 130^e jour. La sécrétion d'insuline et de glucagon était mesurée *in vivo* après la stimulation de la L-arginine.
Résultats : Le L-glutamate, le précurseur du GABA, était réduit dans le pancréas de la progéniture des rates nourrie selon le régime PP à la naissance. Au 21^e jour, les animaux observant un régime PP avaient une présence élevée de $GABA_A$ dans les îlots, une plus faible insuline plasmatique à jeun et une diminution du contenu d'insuline et de glucagon des îlots. Au 130^e jour, la protéine $GABA_A$ ne différait pas

* Address for correspondence: David J. Hill, 268 Grosvenor Street, London, Ontario, Canada N6A 4V2.
E-mail address: david.hill@lhrionhealth.ca (D.J. Hill).

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selon le régime, mais l'expression du mARN sous-unité $GABA_{A\beta3}$ était diminuée dans le groupe PP. La sécrétion du glucagon et l'expression du gène étaient augmentées chez les rats nourris selon le régime PP. L'expression du $GABA_B$ et AGD des îlots n'était pas altérée.

Conclusions : Le régime PP causait des changements dans l'expression du récepteur $GABA_A$ des îlots ayant des implications dans la sécrétion du glucagon et dans l'homéostasie du glucose.

Introduction

Epidemiological studies demonstrated a link between impaired growth in utero and increased risk of chronic diseases in adulthood, such as type 2 diabetes mellitus (1). We, and others, have previously demonstrated that dietary protein restriction in rats administered during pregnancy and lactation (2–4) has long-term effects on the development of the endocrine pancreas and contributes to impaired glucose homeostasis in adulthood (5). Furthermore, maternal low protein (LP) diet resulted in changes to the pancreatic islet morphology and a reduction of beta cell mass in the neonates (4,6) through a decrease in beta cell proliferation, increased apoptosis and diminished intra-islet vascularity, although this is corrected by adulthood (4,7,8).

Gamma-aminobutyric acid (GABA) is present within the endocrine pancreas (9) and is synthesized by glutamic acid decarboxylase (GAD) through the decarboxylation of L-glutamate (10). The enzyme GABA-transaminase (GABA-T) that is present in beta cells (11) is responsible for GABA metabolism. GABA regulates both insulin and glucagon release within the islets (12). Most nonmetabolized GABA is rapidly released into the extracellular medium where it can act as an autocrine or paracrine regulator of islet hormone secretion (12–16). GABA has been proposed to have an inhibitory effect on insulin secretion through an autocrine negative feedback at high glucose concentrations (14). However, GABA can directly stimulate insulin secretion from beta cells at low or physiological concentrations of glucose (17). Braun et al. (18) demonstrated that the majority of GABA was co-released with insulin by the exocytosis of large dense core vesicles from beta cells in response to glucose. Recently treatment with GABA was shown to prevent beta cell loss, increase circulating insulin while reducing glucagon and to normalize glucose tolerance in mice treated with multiple low dose streptozotocin (STZ) (19). The same study demonstrated that GABA reduced insulitis, prevented beta cell apoptosis and restored beta cell mass in the non-obese diabetic (NOD) mouse model of type 1 diabetes (19). The actions of GABA were mediated through a depolarizing effect on the beta cell, and the activation of the phosphoinositol 3 kinase/Akt signalling pathway (19).

The GABA receptors comprise of 2 main species $GABA_A$ and $GABA_B$. The $GABA_A$ receptor is a pentamer whose 5 subunits arising from 7 subunit families that contain multiple subtype isoforms. The subunits that have been demonstrated in rodent pancreas include: $\alpha1$, $\alpha4$, $\beta1$, $\beta2$, $\beta3$, and $\gamma3$ and are localized predominantly in alpha cells (20-23). Insulin can enhance $GABA_A$ receptor activity on alpha cells by promoting translocation of receptors to the plasma membrane (24) via an Akt kinase-dependent pathway. $GABA_A$ receptor–GABA interaction causes membrane hyperpolarization and subsequent suppression of glucagon secretion (23).

$GABA_B$ receptors mediate a slower GABA response and are composed of 2 heptahelical subunits, $GABA_B$ receptor subunit 1 and 2, each a G-protein-coupled receptor linked by their C-termini (25–27). These receptors are mainly localized on beta cells in rat islets (28). Administration of the $GABA_B$ receptor agonist, baclofen, inhibited glucose-stimulated insulin secretion in rat islets (28) and the clonal beta cell line, MIN6-cells (14). Activation of $GABA_B$ receptors has been demonstrated to inhibit insulin secretion, suppressing exocytosis of insulin and GABA (28). Utilization of $GABA_{B1}$ knockout mice (29) demonstrated that functional $GABA_B$ receptors are essential for maintaining insulin content and secretion as well as glucose homeostasis. The $GABA_{B1}$ knockout mice had increased beta cell insulin content and secretion and also demonstrated insulin resistance (29). Surprisingly, the ability of GABA to promote beta cell survival in animals that received STZ, and to delay the onset of diabetes in the NOD mouse was mediated by $GABA_A$ receptors on the beta cell, although these are less abundant than the $GABA_B$ forms (19).

Although a complete GABA response system has been characterized within the endocrine pancreas (30), the impact of LP diet during early development on the GABA axis has not yet been described. Because exposure to LP diet in utero results in impaired glucose-stimulated insulin release in the offspring, it is possible that the normal signalling by GABA to regulate the balance between glucagon and insulin secretion could be altered in the short- or long-term. We hypothesized that impaired glucose homeostasis in adulthood arising from nutritional imbalance in utero, involves persistent changes in GABA receptor subunit expression and presence, leading to altered glucagon release.

Methods

Animals

All procedures were carried out with the approval of the Animal Care Committee of the University of Western Ontario in accordance with the guidelines given by the Canadian Council of Animal Care. Virgin female Wistar rats (Charles River Laboratories, Montreal, QC) were maintained at the Lawson Health Research Institute (London, ON). Rats were housed individually and maintained at 22°C on a 12:12 hour light/dark cycle with food and water available ad lib. Males were mated with nulliparous rats weighing 250 to 300 g at the onset of proestrous. Pregnancy was confirmed by the presence of sperm in the vaginal smear and this was noted as day 1 of pregnancy. Immediately, dams were randomly allocated to 1 of 2 experimental conditions, control diet (20% w/w casein) or isocaloric LP diet (8% w/w casein) (Bioserv, Frenchtown, NJ), and maintained on these diets through gestation and lactation. The LP-fed offspring were weaned onto a control diet. The detailed composition of the diets has been published previously (31). At birth, litter size was restricted to 8 pups with body weight closest to the litter mean (4 each of males and females). Pups were weaned at postnatal day 21 and both groups were fed control diet ad lib until day 130. At least 8 separate litters were used per dietary treatment and studies were carried out at postnatal days 21 and 130.

Islet isolation

Pancreatic islets were isolated on 21 and 130 days as described previously (32) with minor modifications. Briefly, on day 21 animals were anaesthetized and approximately 5 mL of collagenase XI (0.23 mg/mL, Sigma-Aldrich, St. Louis, MO) was injected into the pancreatic duct before the pancreas was removed and digested at 37°C for 15 minutes. The digestion was stopped with ice-cold RPMI 1640 medium supplemented with 7% (v/v) bovine serum, 10 mM HEPES, and 2.8 mM glucose. Islets were isolated from the exocrine pancreas by gradient centrifugation using Histopaque 1077. Gradients were then centrifuged at 4°C at 1500 × *g* for 23 minutes and islets

were recovered at the Histopaque-RPMI interface. Islets were removed and washed in RPMI containing 7% (v/v) bovine serum. Pancreata of offspring at 130 days of age were digested via the common biliary duct with approximately 20 mL of collagenase XI (0.23 mg/mL) solution, removed and incubated in a shaking water bath for 20 minutes at 37°C and islets separated by Histopaque 1077 gradients.

Quantification of amino acid neurotransmitters

Amino acid neurotransmitters (glutamate, aspartate and taurine) and GABA were determined in hypothalamus (bordered by the optic chiasm, hypothalamic fissures, mammary bodies and subthalamic sulcus; including the preoptic suprachiasmatic area), and pancreata of 3 to 11 day 1 and day 130 rats of both control and LP groups. Amino acids were determined by high-performance liquid chromatography (HPLC) as previously described, with minor modifications (33). In brief, an HPLC system, including a Programmable Solvent Module (Beckman Instruments, model 125, Fullerton, CA), a 5 μm, 25 cm × 4.6 mm Ultrasphere ODS reversed-phase column (Beckman), a Programmable UV Detector Module (254 nm) (Beckman Instruments, model 166) and System Gold software (Beckman) was used. The mobile phase was a mixture of water/acetonitrile (82:18, v/v) containing 0.15% phosphoric acid. Samples and standards were treated as follows: the dansyl derivatives were placed in 1.5 mL microfuge tubes and evaporated in a Savant Speed-Vac Concentrator (Savant Instruments, Holbrook, NY). Dried residues were then resuspended in 200 μL of mobile phase, mixed, placed in an ultrasonic bath, and centrifuged at 13 000 × *g* for 3 minutes to remove particulate matter. Twenty microlitres of supernatants were then injected into the HPLC system for analysis.

Quantification of islet insulin and glucagon content by radioimmunoassay

Between 200 and 500 islets were isolated from each separate animal for each experimental group, washed twice in Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and added to 200 to 400 μL of ice-cold extraction buffer (5% formic acid, 1% trifluoroacetic acid [TFA], 1% sodium chloride, 1N hydrochloric acid), dispersed by sonication and stored at −80°C. Proteins were extracted by Sep-Pak C18 reverse-phase chromatography as previously described (34), and insulin and glucagon content were measured by radioimmunoassay (RIA). Rat insulin and glucagon RIA kits (Millipore, Billerica, MA) were used as per manufacturer's instructions. The rat insulin RIA had a limit of sensitivity of 0.1 ng/mL. The within-assay coefficient of variation was <4.6% and between-assay variation <9.4%. The rat glucagon RIA had a sensitivity of 10 pg/mL and within and between-assay variations of <6.8% and <13.5%, respectively.

Protein quantification by Western blot analysis

Western blot analysis for the $GABA_{A\beta 2/3}$ receptor subunit was carried out in control and LP islet preparations, obtained as described above. After islet isolation, approximately 200 islets were placed in 200 μL of lysis buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 1 mM phenylmethanesulfonylfluoride [PMSF], 0.1% Igepal, Tryasylol, Complete Mini tablet [Roche Diagnostics, Indianapolis, IN]). Samples were sonicated and stored at −80°C. Total protein content was determined using a MICRO BCATM Protein assay reagent Kit (Thermo Scientific Pierce Protein Products Rockford, IL). Approximately 10 μg of total protein was loaded and subjected to 10% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Blots were blocked for 90 minutes in Tris buffer saline (TBS), 0.05% Tween 20 and 20% to 5% nonfat milk and incubated overnight at 4°C with a mouse anti-$GABA_{A\beta 2/3}$ clone 62-3G (antibody (1:5000, Millipore, Billerica, MA). The secondary antibody was horseradish peroxidase (HRP) coupled anti-mouse-IgG (1:2000, Santa Cruz, Santa Cruz, CA). Blots were stripped by incubating the membranes in stripping buffer (100 mM beta-mercaptoethanol, 2% SDS, 6.25 mM Tris-HCL, pH 6.7) at 37°C and reprobed for beta-actin (1:5000, Abcam, Cambridge, MA) with anti-rabbit-IgG-HRP (1:10 000, Santa Cruz). Final detection was carried out using chemiluminescence (picoLucent TM PLUS; g-Biosciences 786-02, A Geno Technology, MO) and protein bands were measured by densitometry (GeneTools, Syngene, Frederick, MD).

Quantification of mRNA by quantitative polymerase chain reaction

After islet isolation, approximately 100 to 200 fresh islets from each of at least 8 animals in each experimental group were stored in 300 μL of RNAProtect (Qiagen, Mississauga, ON) at −80°C until RNA extraction. RNA was isolated using Qiagen QIAshredders and RNeasy Plus Micro and Mini kits (Qiagen) according to the manufacturer's specifications. Samples were eluted with either 14 μL or 30 μL of RNase-free water for the micro or mini kits, respectively, and stored at −80°C. Integrity and concentration of RNA was determined using Agilent RNA nanodrop kit. RNA was reverse transcribed into cDNA using a SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA).

Quantitative analysis of the abundance of $GABA_A$ receptor subunits (α1, α4, β1, β2, β3, and γ3), $GABA_B$ receptor subunits (1 and 2) and GAD65 were carried out using a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) sequence detection system. Quantitative polymerase chain reaction (qPCR) detection used iQ SYBR Green Supermix (Bio-Rad Laboratories) qPCR probe technologies. Hypoxanthine-guanine phosphoribosyl-transferase (HPRT) was used as the internal standard gene as it is unaffected by diet or age (35). Gene-specific primers for genes of interest were designed using GenBank sequences (Table 1). Arbitrary values were calculated by converting cycle differences to fold differences relative to HPRT, by multiplying by 2 to the power of "n" (n=CT value) and then multiplying by −1.

Table 1
Gene-specific primers used for the measurement of GABA axis mRNAs

Gene of interest		Primers (5'–3')	Expected product size	Tm	GC content (%)
GABA-Aα1	F	AAAGTGCGACCATAGAACCG	87	60.1	50.0
NM_183326	R	CGATTTTGCTGACGCTGTTA		60.0	45.0
GABA-Aα4	F	TCTGCCCTCTCCTCGCACCC	125	60.0	70.0
NM_080587.3	R	CGGAGCACAGCACGATCGCA		60.1	65.0
GABA-Aβ1	F	ACAAAGTCCAGGTTGATGCC	131	60.0	50.0
NM_012956.1	R	GGCGCTGTCGTATGAGTACA		59.9	55.0
GABA-Aβ2	F	GGGATTTGGTCATTTCCCTT	89	60.0	45.0
NM_012957	R	ACCGTCTCTTTAACCAGCGA		60.0	50.0
GABA-Aβ3	F	GGTCTTCGCAGCTCAAAATC	86	60.0	50.0
NM_017065	R	GGTGAATGGAAACACGATCC		60.2	50.0
GABA-Aγ3	F	TCACCACACCCAACCAGCTCCTTA	122	59.5	54.2
NM_024370.3	R	GGCATGCGCATCCATGGGGAA		60.1	61.9
GABA-B1	F	CTAACCAAGCGGCTGAAAAG	84	60.0	50.0
NM_031028	R	CAAGGCCCAGATAGCATCAT		60.1	50.0
GABA-B2	F	ATCCTGAAGCTCCTGAAGCA	102	60.1	50.0
NM_031802	R	CAGAACCCCAGTCAGGTCAT		60.0	55.0
GAD65	F	GAGGGCCAACTCTGTGACAT	95	60.1	55.0
M72422	R	TCTGCATCAGTCCCTCCTCT		59.9	55.0
HPRT	F	TTGCTCGAGATGTCATGAAGGA	91	53.6	45.5
NM_012583.2	R	AGCAGGTCAGCAAAGAACTTATAG		52.9	41.7

GABA, gamma-aminobutyric acid; *GAD*, glutamic acid decarboxylase; *GC*, guanine-cytosine; *HPRT*, hypoxanthine-guanine phosphoribosyl-transferase; *Tm*, melting temperature.

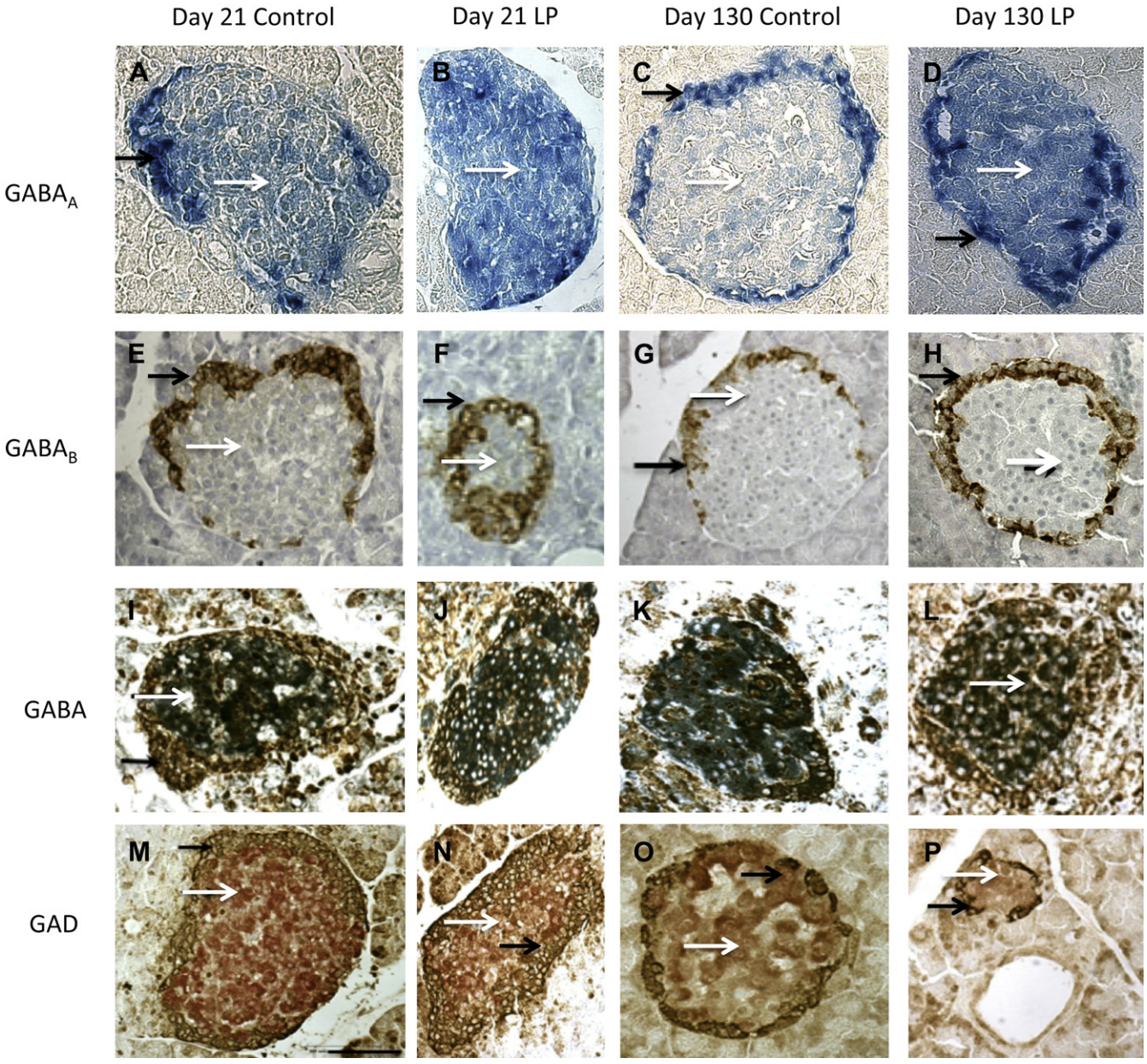

Figure 1. Representative micrographs of pancreatic islets at age 21 and 130 days from animals exposed previously to control or low protein (LP) diet, and stained for $GABA_{A\beta2/3}$ (A–D [blue]), $GABA_{B2}$ (E–H [grey] and counter-stained for glucagon [brown]), GABA (I–L [black] and counter-stained for glucagon [brown], and GAD (M–P [red] and counter-stained for glucagon [brown]. $GABA_{A\beta2/3}$ was predominantly localized to the alpha cells in the periphery of the islet (black arrows) with less intense staining in the beta-cell-rich islet core (white arrows). $GABA_B$ was localized to the islet core, as was GABA and GAD. No differences in distribution were noted with diet or age. Magnification bar = 100 μm.

Intraperitoneal L-arginine challenge

At 130 days of age, 3 to 9 animals from separate litters of each group were subjected to an L-arginine test after a 14-hour fast to assess islet function in vivo. Animals received a single bolus of 0.5 g/kg L-arginine intraperitoneally (36). Blood glucose estimation was determined using a handheld glucose monitor (Ascensia Breeze 2, Bayer HealthCare, Indianapolis, IN) taken from the tail vain at 0, 2, 5, 15, 30, 60, 90 and 120 minutes postinjection (37). Blood was also collected at each time point in chilled lithium heparin tubes for plasma insulin and glucagon determination. Plasma was separated by centrifugation at 4°C at 300 × *g* for 5 minutes and then frozen at −20°C until further analysis.

Immunohistochemistry

Pancreata from 21- and 130-day-old animals were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were cut and mounted on SuperFrost Plus glass slides (Fischer Scientific, Toronto, ON). Immunohistochemistry was carried out to verify receptor location, as per previous reports, using avidin–biotin peroxidase and alkaline phosphatase methods, as we have described previously (6). Antibodies against $GABA_{A\beta2/3}$ (monoclonal mouse anti-$GABA_{A\beta2/3}$ 1:100 dilution; Millipore), $GABA_{B2}$ (monoclonal guinea pig anti-$GABA_{B2}$ 1:500 dilution; Millipore), GAD 65/67 (monoclonal rabbit anti-GAD65/67 1:500 dilution; Chemicon International, Temecula, CA), and glucagon (monoclonal mouse anti-glucagon 1:2000 dilution; Sigma-Aldrich) were used. To establish the

Table 2
Levels of amino acid neurotransmitters within the pancreas or hypothalamus (nmol/mg) of offspring from control or LP diet-fed mice at postnatal days 1 and 130

	Control	LP
Pancreas		
Day 1	32.8 ± 5.0	6.3 ± 1.1†
Taurine	21.6 ± 3.3	6.4 ± 0.8*
Aspartate	66.4 ± 9.9	15.3 ± 1.4†
Glutamate		
Day 130		
Taurine	13.6 ± 1.9	10.9 ± 1.8
Aspartate	18.8 ± 3.1	14.3 ± 2.2
Glutamate	43.7 ± 5.9	34.9 ± 5.2
Hypothalamus		
Day 1		
Taurine	115.2 ± 7.5	113.8 ± 7.1
Aspartate	21.8 ± 4.1	23.0 ± 2.4
Glutamate	50.1 ± 11.4	38.1 ± 3.6
Day 130		
Taurine	12.7 ± 0.6	13.0 ± 1.3
Aspartate	47.6 ± 9.2	60.4 ± 3.8
Glutamate	21.7 ± 3.8	28.2 ± 3.4

HPLC, high-performance liquid chromatography; *LP*, low protein.
Amino acid neurotransmitters were isolated by HPLC from hypothalamus or pancreas, and taurine, aspartate and glutamate levels were quantified. Values represent mean ± SEM derived from 3 to 11 animals per group.
* $p<0.01$ vs. control diet (ANOVA with Bonferroni's posttest).
† $p<0.001$ vs. control diet (ANOVA with Bonferroni's posttest).

specificity of the antibodies, the primary antibodies and secondary antibodies were substituted by antibody diluting solution.

Statistical analysis

Data is presented as mean ± standard error of the mean from at least 3 separate litters for each experiment. Differences between mean values for variables within individual experiments were compared statistically using a 2-way analysis of variance (ANOVA) to analyze diet and age followed by a Bonferroni's posttest. Differences were considered statistically significant at $p<0.05$.

Results

GABA and GABA receptor localization

Pancreata were analyzed by immunocytochemistry for the presence of the $GABA_A$ receptor to verify localization as previously reported (38). The receptor was localized predominantly in the alpha-cell-rich area of the islet mantle (Figs. 1A–1D), whereas less intense staining was seen in the region of beta cells. Conversely, $GABA_B$ was located predominantly in the beta-cell-rich core of the islets, as was GABA and GAD 65/67 (Figs. 1I–1P). No differences in the cellular distribution of the $GABA_B$ receptors, GABA or GAD were observed between 1 and 130 days of age, or in animals exposed to LP diet. The intensity of immunostaining for $GABA_A$ receptor appeared stronger within the beta-cell-rich islet core in animals that received LP diet at both 21 and 130 days, compared to control diet.

GABA and GABA receptor abundance

We attempted to measure GABA content within pancreata of offspring from LP or control diet-fed mice at postnatal days 1 and 130, but levels of recoverable GABA were extremely low. However, we could quantify the levels of the immediate substrate of GABA, L-glutamate and compare these with 2 other amino acid neurotransmitters, taurine and aspartate (Table 2). Levels of all 3 amino acids were lower in pancreata from offspring of LP-fed rats on day 1, but not at day 130. To determine if these differences were common to multiple tissues we also examined hypothalamus from the same animal. No differences in amino acid content were observed in hypothalamus with diet.

Table 3
Messenger RNA expression measured with qPCR for $GABA_A$ α1, α4, β1, β2, β3 and γ3 subunits, $GABA_{B1}$, $GABA_{B2}$, and GAD65 in islets isolated at 130 days of age from rats that had previously received control of LP diet

Subunit	Control	LP
$GABA_{A\alpha1}$	5.6 ± 1.1	9.3 ± 1.2*
$GABA_{A\alpha4}$	2.6 ± 0.3	3.4 ± 0.4
$GABA_{A\beta1}$	2.5 ± 0.1	4.6 ± 0.2†
$GABA_{A\beta2}$	3.5 ± 0.2	6.1 ± 1.2*
$GABA_{A\beta3}$	12.4 ± 2.3	3.8 ± 0.3*
$GABA_{A\gamma3}$	4.0 ± 0.2	5.4 ± 0.3*
$GABA_{B1}$	3.8 ± 0.3	4.4 ± 0.5
$GABA_{B2}$	1.6 ± 0.1	1.7 ± 0.1
GAD65	4.3 ± 0.5	5.9 ± 0.6

GABA, gamma-aminobutyric acid; *GAD*, glutamic acid decarboxylase; *HPRT*, hypoxanthine-guanine phosphoribosyl-transferase; *LP*, low protein; *qPCR*, quantitative polymerase chain reaction.
Values represent mean ± SEM, relative to the reference gene HPRT and derived from 7 to 8 animals per group.
* $p<0.05$ vs. control.
† $p<0.001$ vs. control.

Expression levels of $GABA_A$ α1, β1, β2 and γ3 subunit mRNAs were all slightly increased in animals given LP diet, whereas the $GABA_{A\alpha4}$ subunit was not changed (Table 3). However, the mRNA abundance for $GABA_{A\beta3}$ was decreased by approximately 70% in LP vs. control-fed rats in adulthood. This substantial reduction in $GABA_{A\beta3}$ receptor subunit expression could have impaired the ability of GABA to mediate its inhibitory effect on glucagon release or the trophic effect on beta cell mass (19). There was no effect of previous dietary regimen on $GABA_{B1}$, $GABA_{B2}$ or GAD65 mRNA expression within islets (Table 3), hence the long-term effects of LP diet on the GABA receptor system were restricted to $GABA_A$.

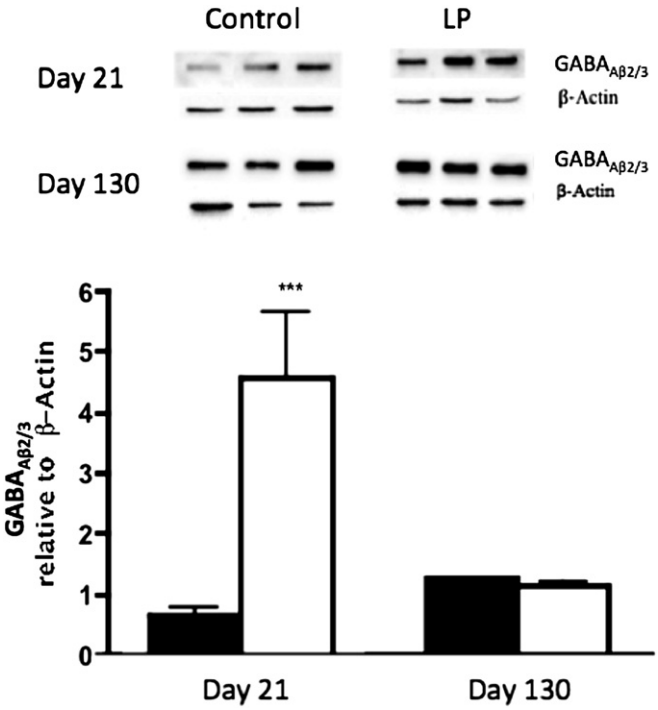

Figure 2. Western blot analysis of $GABA_{A\beta2/3}$ subunits from isolated islets at 21 or 130 days of age after feeding either low protein (LP) or control diet, relative to beta-actin. Triplicate lanes are shown representing islets from different animals. Values show the mean ± standard error of the mean (SEM) of the relative density of proteins for 3 to 5 animals per group (control diet, filled bars; LP diet, open bars). ***$p<0.001$.

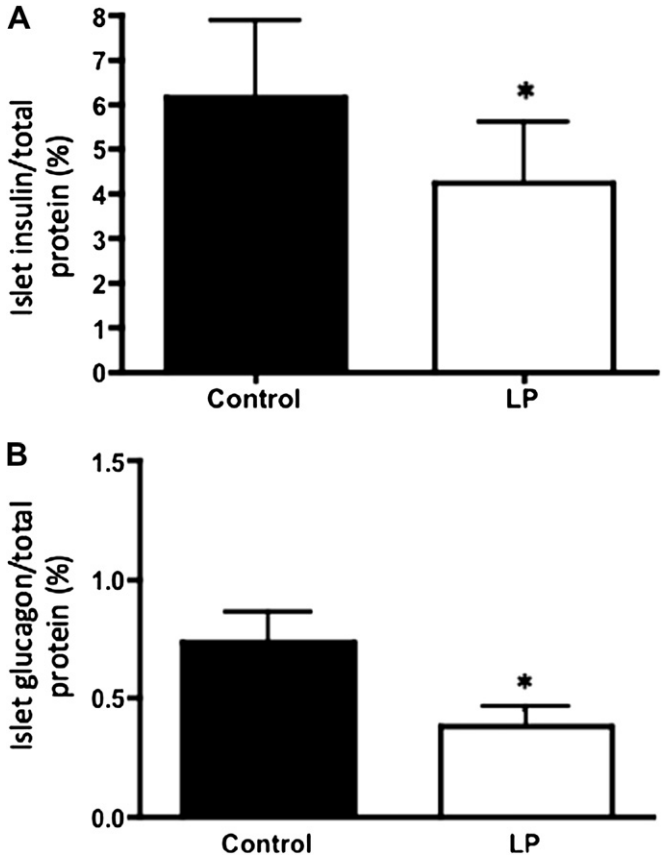

Figure 3. Insulin (A) and glucagon (B) content in isolated islets from 21-day-old animals expressed as a percentage of total islet protein. Animals received either control (filled bars) or low protein (LP) (open bars) diet. Values represent mean ± SEM from 6 to 9 animals per group. *p<0.05 vs. control.

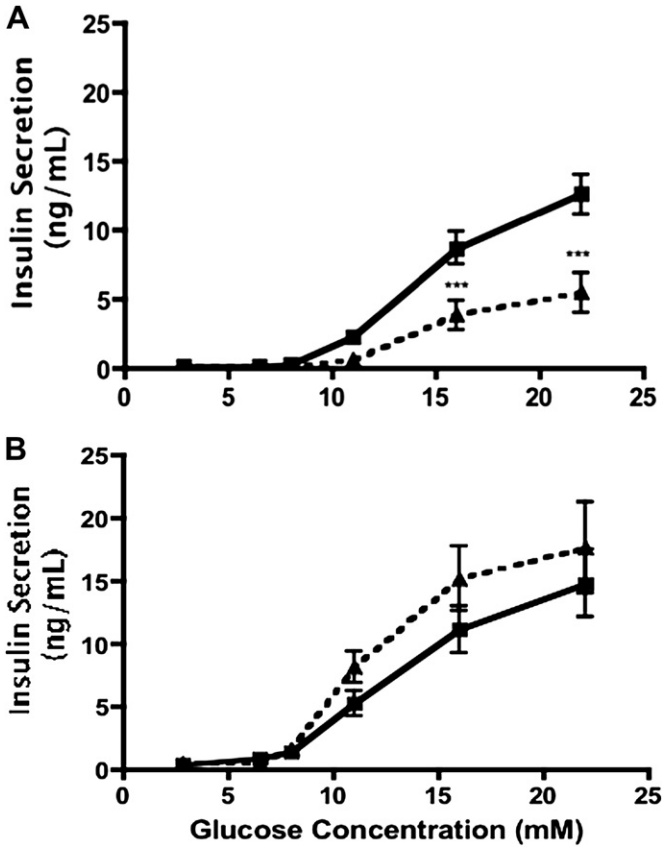

Figure 4. Release of insulin in response to increasing concentrations of glucose from islets isolated from animals at 21 days (A) or 130 days (B) of age exposed previously to control (solid line) or low protein (LP) diet (broken line). Values represent mean ± SEM from 6 to 9 animals per group. ***p<0.001 vs. control.

When the presence of $GABA_{A\beta 2/3}$ was assessed in isolated islets by Western immunoblot using an antibody that detected both subunits, the net protein content was significantly increased in LP-fed rats at 21 days of age (Fig. 2). To determine the relationship of this to islet hormone content and release, insulin and glucagon content were measured in isolated islets from LP or control-fed animals. At day 21, both insulin and glucagon content (Fig. 3) were reduced in LP-fed animals compared with controls. Fasting plasma insulin values were also significantly reduced in animals that had received LP diet (0.18 ± 0.04 ng/mL, n=13) compared with controls at day 21 (1.25 ± 0.27 ng/mL, n=14, p<0.01). Thus, at 21 days of age, prior exposure to LP diet was associated with an increased abundance of $GABA_A$ receptor subunits, which would be expected to result in a decrease in glucagon secretion from the alpha cells but increased beta cell growth and survival. However, this was accompanied by reduced pancreatic glucagon and insulin content and lower circulating insulin during fasting.

The presence of $GABA_{A\beta 2/3}$ was also assessed in isolated islets by Western immunoblot at 130 days, at which time no difference in protein abundance was seen between previously LP-fed and control-fed rats (Fig. 2). At 130 days, insulin and glucagon content in isolated islets from offspring of LP-fed animals were comparable to controls when expressed as a percent of total islet protein (insulin: control 35.7 ± 8.2%, n=9; and LP 37.7 ± 6.0%, n=12; glucagon: control 0.93 ± 0.07%, n=9; and LP 1.11 ± 0.16%, n=12). However, proglucagon mRNA within islets was significantly elevated in islets from LP-fed animals (control 27 ± 4-fold increase relative to HPRT, LP 51 ± 5-fold increase; p<0.01, n=6–8). Fasting plasma insulin levels were still lower in offspring of LP-fed rats (0.40 ± 0.11 ng/mL, n=21) than in those that received control diet (0.92 ± 0.35 ng/mL, n=14, p<0.05).

Islet function

Glucose-stimulated insulin release was measured in isolated islets (Fig. 4). Islets from animals exposed previously to LP-diet demonstrated a lower insulin release than islets from offspring from control-fed mothers at postnatal day 21. However, no such difference was found at day 130. After an L-arginine challenge to mobilize both insulin and glucagon secretion, a significantly lower insulin release was found in the LP group over 120 minutes (p<0.01) (Fig. 5A), with a reduced area under the curve. However, in the same samples, LP-treated animals had higher circulating levels of glucagon compared to controls (p<0.001) (Fig. 5B) and a significantly increased area under the curve.

Discussion

Previous studies have shown (12,38) that the pancreatic GABAergic system contributes to the paracrine and autocrine regulation of intra-islet insulin and glucagon secretion. We found here, as expected, that GABA, GAD and $GABA_B$ receptors were all localized predominantly to beta cells, whereas the $GABA_A$ receptors were found on both alpha and beta cells. Glucose indirectly alters $GABA_A$ receptor gene expression within the alpha cells (39) by inhibiting the voltage-dependent calcium channels and preventing a rise in intracellular calcium after receptor stimulation. Low calcium concentrations remove the negative regulation of $GABA_A$

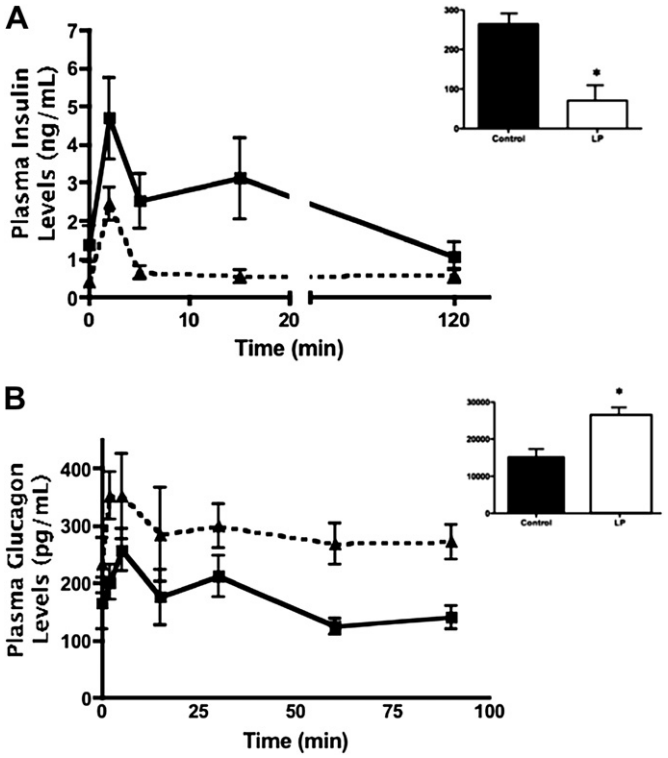

Figure 5. Changes in plasma insulin (A) and glucagon (B) over 120 minutes after L-arginine injection, and the area under the curve for each. Animals received either control (solid line) or low protein (LP) diet (broken line) Values represent mean ± SEM from 4 to 11 animals per group. $p<0.01$ for effect of diet in (A), and $p<0.001$ in (B). *$p<0.01$ vs. control diet.

receptor gene expression (39) resulting in increased overall expression. Under normal glucose homeostasis, this increase in $GABA_A$ receptor expression would likely lead to increased receptor trafficking to the alpha cell plasma membrane and ultimately the inhibition of glucagon secretion. The translocation of the $GABA_A$ receptor to the plasma membrane is also controlled directly by insulin through the Akt pathway (23,24). However, GABA acting via $GABA_A$ receptors on beta cells was able to promote beta cell replication and cell survival when exposed to cytotoxic cytokines, also via the Akt pathway (19). A potential disruption of the GABA axis after nutritional insult early in life would therefore be expected to alter islet hormone release and glucose homeostasis.

In young offspring of LP-fed rats the level of $GABA_{A\beta2/3}$ protein within islets was significantly increased, and immunostaining more intense, consistent with less glucagon release. This happened despite a reduced fasting blood insulin level, which would be expected to slow receptor translocation to the alpha cell membrane, and tend to increase glucagon secretion. Also, the increased abundance of $GABA_{A\beta2/3}$ did not result in greater trophic effects of GABA on the beta cells because beta cell mass is reduced at 21 days compared to control-fed animals (4). Glucose-stimulated insulin release from isolated islets was impaired, and insulin content and circulating levels were reduced in the present studies after exposure to LP diet. We were not able to directly compare the pancreatic content of GABA in pancreata from day-old rats previously fed control or LP diet, but we were able to measure the presence of the direct GABA precursor, L-glutamate, that was significantly lower in offspring of LP-fed animals at birth, as were other amino acids that serve as neurotransmitters. The glutamate representative was not seen in another representative tissue, the hypothalamus. These results suggest that prior exposure to LP diet has a wide impact on the development and function of the islets, not simply an altered beta cell mass (4), and we suggest that this includes a change in GABA levels and $GABA_A$ receptor expression.

When LP-fed animals were weaned onto a control diet there was no longer a difference in $GABA_{A\beta2/3}$ protein presence within islets compared to controls when the rats reached adulthood, pancreatic L-glutamate levels were comparable to controls, and glucose-stimulated insulin release from isolated islets was similar between diets. However, there was clear evidence of abnormal glucagon secretion with an increase in pro-glucagon mRNA and much increased glucagon release after an L-arginine challenge. Analysis of the expression levels of $GABA_A$ receptor subunit mRNAs showed modest increases in expression of $GABA_{A\alpha1}$, $GABA_{A\beta1}$, and $GABA_{A\beta2}$ in LP-fed animals, but a substantial reduction in the expression of $GABA_{A\beta3}$. Immunohistological localization of $GABA_A$ receptor showed a greater intensity of staining after LP diet. Alpha subunits are instrumental in receptor translocation from the cytosolic compartments to plasma membrane (23). Therefore, a major reduction in $GABA_{A\beta3}$ would reduce receptor translocation to the cell membrane, resulting in increased glucagon secretion from alpha cells in response to GABA without any trophic effect on beta cells. The reduced insulin secretion after L-arginine challenge in LP-fed animals at 130 days would also lead to increased pro-glucagon gene transcription, and greater glucagon release. No changes in $GABA_{B1}$, $GABA_{B2}$ receptor subunit mRNA or the GABA-synthesizing enzyme GAD65 were found between LP and control groups at 130 days of age, all of which are predominantly localized to the beta cells. Our results therefore suggest that long-term changes to the GABAergic system occur within islets after exposure to LP diet in early life, predominantly through the $GABA_A$ receptor. This may contribute to the impaired glucose tolerance that develops in offspring of LP-fed mothers during adulthood (5).

Individuals with type 2 diabetes have been shown to lack insulin-induced glucagon suppression in hyperglycemic conditions (40). Therefore, insulin co-release with GABA may play an important role in intra-islet communication and regulation of blood glucose through the functioning of the $GABA_A$ receptor system. We recognize that GABA is not the only regulator of glucagon release and it is possible that other paracrine factors such as zinc (41) or somatostatin (41) may also contribute. However, this study suggests that the changes seen in the pancreatic islet $GABA_A$ receptor expression after protein restriction in early life may alter the intra-islet signalling and change the balance of glucagon to insulin secretion.

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Author Disclosures

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Author Contributions

The experimental work was done by MAD as part of her MSc studies at the University of Western Ontario. The experiments were planned by MAD with the assistance of DJH, EJA and VAL-L. DBH assisted with the design of RT-PCR studies. DJH and EJA contributed to the writing of the manuscript and with the analysis of the results.

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