1   **Disruption of glucagon receptor signaling causes hyperaminoacidemia exposing a possible**

2   **liver - alpha-cell axis**

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20   **Running head:** Disruption of glucagon signaling causes hyperaminoacidemia

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24

Disruption of glucagon signaling causes hyperaminoacidemia

Abstract

25

26 Glucagon secreted from the pancreatic alpha-cells is essential for regulation of blood glucose levels.

27 However, glucagon may play an equally important role in the regulation of amino acid metabolism

28 by promoting ureagenesis. We hypothesized that disruption of glucagon receptor signaling would

29 lead to an increased plasma concentration of amino acids, which in a feedback manner stimulates

30 the secretion of glucagon, eventually associated with compensatory proliferation of the pancreatic

31 alpha-cells.

32 To address this, we performed plasma profiling of glucagon receptor knockout ($Gcgr^{-/-}$) mice and

33 wild-type (WT) littermates using liquid chromatography mass spectrometry (LC-MS)-based

34 metabolomics, and tissue biopsies from the pancreas were analyzed for islet hormones and by

35 histology. A principal component analysis of the plasma metabolome from $Gcgr^{-/-}$ and WT

36 littermates indicated amino acids as the primary metabolic component distinguishing the two groups

37 of mice. Apart from their hyperaminoacidemia, $Gcgr^{-/-}$ mice display hyperglucagonemia, increased

38 pancreatic content of glucagon and somatostatin (but not insulin), and alpha-cell hyperplasia and

39 hypertrophy compared to WT littermates. Incubating cultured α-TC1.9 cells with a mixture of

40 amino acids (Vamin 1%) for 30 minutes and for up to 48 hours led to increased glucagon

41 concentrations (~six-fold) in the media and cell proliferation (~two-fold), respectively. In

42 anesthetized mice, a glucagon receptor specific antagonist (Novo Nordisk 25-2648, 100 mg/kg)

43 reduced amino acid clearance. Our data supports the notion that glucagon secretion and hepatic

44 amino acid metabolism are linked in a close feedback loop, which operates independently of normal

45 variations in glucose metabolism.

46 **Keywords:** Alpha-cell, Amino Acids, Glucagon, Glucagon Receptor, Hyperglucagonemia.

Introduction

Glucagon is a peptide-hormone of 29 amino acids processed from the prohormone, proglucagon, by pro-hormone convertase 2 (PC-2) in pancreatic alpha-cells (39). Activation of the hepatic glucagon receptor (GR) increases hepatic glycogenolysis and gluconeogenesis (10), and the physiological role of glucagon has been coupled to glucose metabolism with opposing actions to insulin (1, 28, 43). However, several studies have suggested that glucagon may play an equally important role in the regulation of hepatic amino acid metabolism (2, 5, 7, 13, 21, 41). Increased fasting and postprandial plasma concentrations of glucagon have been reported in clinical conditions including non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (33) raising the question whether this is related to amino acid metabolism.

Knockout of the GR in mice has been associated with disturbed metabolism of amino acids (14, 50) and alpha-cell hyperplasia that appears to be mediated by (a) humoral factor(s) secreted from the liver (29, 32), and recent studies suggest that this factor may be increased plasma concentrations of amino acids (12, 24, 41). We hypothesized that a feedback circuitry may exist by which glucagon increases amino acid turnover while amino acids conversely stimulate secretion of glucagon from the pancreas.

We therefore initially performed metabolomics analysis of plasma, histology of the pancreas, and protein expression profiles in liver and pancreas tissue from glucagon receptor knockout ($Gcgr^{-/-}$) mice in order to dissect the principal components of GR disruption. In addition, we investigated the hepatic clearance of amino acids *in vivo* after prior pharmacological or genetically induced GR blockage. Finally, amino acids were administered to the alpha-cell line αTC1.9 to monitor glucagon secretion and alpha-cell proliferation.

Materials and methods

Animal studies

Animal studies were conducted with permission from the Danish Animal Experiments Inspectorate, Ministry of Environment and Food of Denmark, permit 2013−15−2934−00833, and in accordance with the EU Directive 2010/63/EU and guidelines of Danish legislation governing animal experimentation (1987), and the National Institutes of Health (publication number 85-23). All studies were approved by the local ethical committee.

Female C57BL/6JRj mice (12 weeks of age) were obtained from Janvier Labs, Saint-Berthevin Cedex, France. Mice were housed in groups of six to eight in individually ventilated cages and followed a light cycle of 12 hours (lights on 6 am to 6 pm) with ad libitum access to standard chow (catalog no. 1319, Altromin Spezialfutter GmbH & Co, Lage, Germany) and water. Glucagon receptor knockout ($Gcgr^{-/-}$) mice C57BL/6^{Gcgrtm1Mjch} were previously described (14). Male and female homozygotes and wild-type (WT) littermates, age 9-29, weeks were used. During all $Gcgr^{-/-}$ and littermate studies, the investigator was blinded to the genotype of the mice in order to avoid bias.

Biochemical and morphometric characterization of $Gcgr^{-/-}$ mice

Eleven $Gcgr^{-/-}$ mice (six females 18-28 weeks of age and five males 15-25 weeks of age) and 15 WT littermates (eight females 18-25 weeks of age and seven males 15-22 weeks of age) were fasted for four hours with free access to water. A 50 µL blood sample was collected from the tail vein, and subsequently the mice were anesthetized with isoflurane (Baxter A/S, Søborg, Denmark). When the mice were sufficiently sedated (absence of reflexes) the abdominal cavity was opened with a midline incision. Tissue samples from the pancreas and liver were harvested and either fixed in formaldehyde 4 % and methanol 1-2 % (Hounisen Laboratory Equipment, Skanderborg, Denmark)

93   or snap frozen on dry ice. Finally, the mice were subjected to a total blood collection from the

94   inferior vena cava. The blood was immediately transferred to pre-chilled EDTA-coated Eppendorf

95   tubes and stored on ice until centrifuged (1,650 x g, 15 min, 4°C). Plasma was collected and stored

96   in pre-chilled Eppendorf tubes at -20°C until further analysis.

97   **Amino acid clearance in *Gcgr*⁻/⁻ mice**

98   Seven *Gcgr*⁻/⁻ mice (six females 9-21 weeks of age and one male 28 weeks of age), and seven WT

99   littermates (six females 9-29 weeks of age and one male 20 weeks of age) were fasted and

100   anesthetized as above and the inferior caval vein exposed. At time 0 min the mice received an

101   injection of 1 µmol/g body weight amino acid mixture, Vamin (Vamin® 14 g/l electrolyte free,

102   catalog no. B05ABA01; Fresenius Kabi, Copenhagen, Denmark, composition of amino acids is

103   shown in table 1), diluted with sterilized phosphate-buffered saline (PBS) to a total volume of 100

104   µL in the caval vein. At time 12 min, the mice were subjected to a total blood collection from the

105   inferior vena cava. Blood samples were handled as described above. Plasma samples were later

106   analyzed for total L-amino acid concentration.

107   **Amino acid clearance after pharmacological disruption of glucagon receptor signaling in mice**

108   Female C57BL/6JRj mice (12 weeks of age) received a glucagon receptor antagonist (GRA, 25–

109   2648, a gift from Novo Nordisk A/S (25)) 180 min prior to the amino acid stimulation experiment.

110   GRA was dissolved in 5% ethanol, 20% propyleneglycol, 10% 2-hydroxypropyl-β-cyclodextrin

111   (vol./vol.) and phosphate buffer at pH 7.5–8.0 to a concentration of 10 mg/mL and administered by

112   oral gavage (100 µL) as a suspension in a dose of 100 mg/kg body weight (42). A control group

113   received 100 µL vehicle. At time 0 min, the tip of the tail was cut and blood glucose concentrations

114   were measured. The mice were then anesthetized with isoflurane, and 1 µmol/g body weight Vamin

115   diluted in sterilized PBS (100 µL) was injected into the inferior vena cava. Control groups received

116   100 µL sterilized PBS. At times 0, 2, 4, 12, or 20 min, the mice were subjected to total blood

117 collection from the inferior vena cava, and blood glucose concentrations were measured. Blood

118 samples were handled as described above and analyzed for total L-amino acid, insulin, and

119 glucagon concentrations. Clearance was defined as the incremental area under the curve ($_iAUC_{0-20}$

120 $_{min}$).

Amino acid clearance after ligation of the kidneys

122 Female C57BL/6JRj mice (12 weeks of age) were anesthetized with isoflurane and subsequently

123 subjected to kidney ligation (n=4); four mice were sham operated and served as controls. At time 0

124 min, 1 µmol/g body weight Vamin diluted in sterilized PBS (100 µL) was injected into the caval

125 vein. At time 12 min, the mice were subjected to total blood collection from the inferior vena cava.

126 Blood samples were handled as described above and analyzed for total L-amino acid

127 concentrations.

Biochemical analysis

129 Plasma concentrations of total L-amino acids were quantified using an enzyme-linked

130 immunosorbent assay (ELISA) (catalog no. ab65347; Abcam, Cambridge, UK). This kit determines

131 concentrations of free L-amino acids, but neither protein bound nor D-amino acids. The assay was

132 evaluated by recovery experiments using pooled (n=4) mouse plasma with added known amounts of

133 amino acids (product no. A6282; Sigma Aldrich, Copenhagen, Denmark). Recoveries of amino

134 acids were on average 79±9% in mouse plasma.

135 Plasma concentrations of glucagon were measured using a validated (52) two-site enzyme

136 immunoassay (catalog no.10-1281-01; Mercodia, Upsala, Sweden) according to the manufacturer´s

137 protocol.

138 Plasma concentrations of ammonia/ammonium, and bile acids were quantified using enzymatic

139 assays (catalog no. ab83360; Abcam, Cambridge, UK and catalog no. STA-361; Cell Biolabs Inc.,

140 San Diego, USA, resp.).

141 Plasma concentrations of corticosterone and insulin were quantified using ELISAs (catalog no.

142 ADI-900-097; Enzo, AH diagnostics, Aarhus, Denmark, and catalog no. 10-1247-10; Mercodia AB,

143 Uppsala, Sweden, respectively).

144 In order to avoid bias when performing biochemical analysis all samples were assigned a number,

145 so that genotype and treatment were unknown at the time of analysis.

146 **Plasma Metabolomics**

147 Metabolomic analysis was performed on plasma samples from 11 *Gcgr*$^{-/-}$ (six females 18-28 weeks

148 of age and five males 15-25 weeks of age), and 11 WT littermates (six females 18-25 weeks of age

149 and five males 18-22 weeks of age), also used for biochemical and morphometric characterization.

150 Using liquid chromatographic-triple quadrupole mass spectrometric (LC-MS/MS) measurements

151 and the Absolute*IDQ*^{TM} p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria), 188

152 metabolites were quantified out of 10 µL plasma, including free carnitine, 39 acylcarnitines, 21

153 amino acids, 21 biogenic amines, hexoses, 90 glycerophospholipids (14 lysophosphatidylcholines

154 and 76 phosphatidylcholines), and 15 sphingolipids. The assay procedures of the Absolute*IDQ*^{TM}

155 p180 Kit as well as the metabolite nomenclature have been described in detail previously (40, 54).

156 Sample handling was performed by a Hamilton Microlab STAR^{TM} robot (Hamilton Bonaduz AG,

157 Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.),

158 besides standard laboratory equipment. Mass spectrometric analyses were done on an API 4000

159 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200

160 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL

161 auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data

162 evaluation for quantification of metabolite concentrations and quality assessment were performed

163 with the software MultiQuant (Sciex) and the Met*IDQ*™ software package, which is an integral

164   part of the Absolute*IDQ*™ Kit. Metabolite concentrations were calculated using internal standards

165   and reported in μmol/L.

166   All data has been deposited at Figshare at DOI:10.6084/m9.figshare.5364082.v1
167

168   **Histology and immunohistochemistry**

169   Pancreas and liver tissue samples from the ten *Gcgr*^{-/-} mice (five females 18-25 weeks of age and

170   five males 15-25 weeks of age), and eleven WT littermates (six females 18-25 weeks of age and

171   five males 18-22 weeks of age), also used for plasma metabolomics analysis were fixed for 24

172   hours, and then transferred to 70% ethanol. The tissue samples were embedded in paraffin at the

173   Finsen Laboratory (Rigshospitalet, Copenhagen Biocenter, Copenhagen, Denmark).

174   Tissue sections were stained for insulin (in-house developed guinea pig anti-insulin antibody 2006,

175   1:10000) and glucagon (in-house developed rabbit anti-glucagon 4304, 1:2000). For antigen

176   retrieval, sections were pretreated by boiling in Triethylene glycol (TEG) buffer, pH 9, for 15 min.

177   The sections were then incubated overnight at 4°C with primary antibodies, washed with PBS

178   buffer, pH 7.4, and subsequently incubated with a mixture of Alexa568 labeled donkey-anti rabbit

179   antibody (red, 1:500, Abcam, Cambridge, UK) and Alexa488-labeled goat-anti guinea pig antibody

180   (green, 1:500, Life technologies, Carlsbad, CA, USA). The slides were mounted with DAKO

181   fluorescence mounting medium (Agilent, Santa Clara, CA, USA), and examined using an

182   Axioscope 2 plus microscope (Zeiss, Jena, Germany). Images were taken using CoolSNAP camera

183   (Photometrics, Tucson, AZ, USA).

184   For composition and size, 30 islets from each mouse (two-three sections separated by 200-300 μm

185   evaluated per mouse) were photographed and the relevant areas (insulin, glucagon, and total islet

186   area) were measured using Image-Pro 7 software (Media Cybernetics, Rockville MD, USA) as

187   previously described (31). To measure the mean size of alpha-cells and beta-cells in the two groups

188   of mice, 20 glucagon positive and 20 insulin positive cells were measured for each mouse using

189   Image-Pro 7 software.

190   The pancreas sections were also double-stained for the glucagon-like-peptide-1 receptor (GLP-1R)

191   (mouse GLP-1 receptor antibody 7F38, 1: 200, generous gift from Charles Pyke, Novo Nordisk,

192   Måløv, Denmark) and insulin, glucagon, and somatostatin (in-house developed rabbit somatostatin

193   antibody 1759, 1:4000), respectively. The sections were pretreated with pronase for 10 min (0.1%,

194   firma) at $37^{o}C$ and incubated overnight with the GLP-1R antibody, and after washing in PBS buffer

195   pH 7.4 subsequently incubated with biotin-anti mouse antibody, 1:200, Vectastain-complex

196   according to the manufacturer´s instruction (Vector laboratories, Burlingame, CA, USA), stained

197   with DAB and counterstained lightly with haematoxylin. After this staining was completed, the

198   sections were incubated overnight with either glucagon, insulin or somatostatin antibody and after a

199   wash in PBS, incubated with either Alexa 568 (1:500), or Alexa 488. The slides were then mounted

200   with DAKO fluorescence mounting medium, the sections were examined using Axioscope 2

201   microscope, and images were taken using CoolSNAP camera.

202   **Pancreatic protein extraction and measurements of pancreatic concentrations of glucagon,**

203   **GLP-1, insulin, and somatostatin**

204   Snap-frozen pancreatic tissues from six WT mice (two females 22 and 25 weeks of age and four

205   males 15-20 weeks of age), and five $Gcgr^{-/-}$ mice (three females 22-25 weeks of age and two males

206   22 and 25 weeks of age) were subject to peptide extraction carried out as described previously (51).

207   The dipetidyl peptidase-4 (DPP-4) inhibitor, valine-pyrrolidide, was added to all samples and

208   standards (final concentration 0.01 mmol/L) to prevent N-terminal degradation of GLP-1 during the

209   assay incubation. Total amidated GLP-1 concentrations (the sum of 1-36 NH_2, 7-36NH_2, and 9-

210   36NH_2) were quantified using an RIA (codename 89390) specific for the amidated C-terminal of

211   the GLP-1 molecule (35). Active GLP-1 concentrations (7-36 NH_2) were measured using a well-

212   established immunoassay specific for the N-terminus (47). Glucagon concentrations were measured

213   using an RIA (codename 4305) (18) validated by ELISA (48). Insulin concentrations were

214   measured using an RIA (codename 2006-3) (34). Somatostatin concentrations were measured using

215   an RIA (codename 1758-5) (4).

216   **Stimulation of the alpha-cell line, alphaTC1.9, with amino acids**

217   The alpha-cell line, alphaTC1 Clone 9 (ATCC® CRL-2350™) was used (16). The cells were

218   seeded in 24-well plates (Nunc™, thermo scientific) at a cell density of 4×10^4 per well. Cells 80%

219   confluent were incubated for two hours with PBS (controls) or 1% Vamin added to the media. The

220   cell media were subsequently harvested and centrifuged (1,500 G, 15 min, 4°C,) to remove any

221   cells or debris and kept at -80°C until analysis. For long-term incubation (48 hours) cells were

222   treated with Vamin or PBS as described above and also treated with 5-Bromo-2-DeoxyUridine

223   (BrdU) to assess cell proliferation (catalog no. C10337; Invitrogen, Carslsbad, USA) according to

224   the manufacturer´s protocol. Concentrations of glucagon were normalized to total protein content

225   assessed with a BCA kit from Thermo Fisher Scientific (catalog no. 23225).

226   **Statistics**

227   All bioinformatic analyses were done with the Perseus software of the MaxQuant computional

228   platform. For the principal component analysis we included the dataset derived from mass

229   spectrometry and, in addition, all available biochemical data obtained in the current study. A false

230   discovery rate of < 0.05 after Benjamini-Hochberg correction was used in order to correct for

231   multiple testing. When more than two groups were compared, a one-way ANOVA corrected for

232   multiple testing using the Sidak-Holm algorithm was applied. To analyze data from two

233   independent groups un-paired t-test were used. Calculations were made using GraphPad Prism

234   (version 7.02 for Windows; GraphPad Software, La Jolla, CA) and STAT14 (SE) (StataCorp,

235   College station, TX 77845, USA). All data are presented as mean±SEM unless otherwise stated.

236

Results

Biochemical and morphometric characterization of *Gcgr*$^{-/-}$ and WT mice

The body weight of *Gcgr*$^{-/-}$ mice did not differ from WT littermates (31±6 g vs. 30±4 g, P=0.6) (Fig. 1*A*). Blood glucose concentrations were lower in *Gcgr*$^{-/-}$ mice compared to WT littermates (7±1 mmol/L vs. 9±0.8 mmol/L, P<0.0001) (Fig. 1*B*). Plasma glucagon concentrations were increased ~20-fold in *Gcgr*$^{-/-}$ mice compared to WT littermates (378±158 pmol/L vs. 21±15 pmol/L, P<0.0001) (Fig. 1*C*). Plasma concentrations of total L-amino acids were ~three-fold increased in *Gcgr*$^{-/-}$ mice compared to WT littermates (7.4±1.4 mmol/L vs. 2.5±0.6 mmol/L, P< 0.0001) (Fig. 1*D*). Ammonia/ammonium concentrations were increased ~1.5 fold in *Gcgr*$^{-/-}$ mice compared to WT littermates (27±4 μmol/L vs. 18±4 μmol/L, P=0.0002) (Fig. 1*E*). Plasma concentrations of corticosterone and bile acids did not differ between *Gcgr*$^{-/-}$ mice and WT littermates (corticosterone: 176±64 ng/mL vs. 173±100 ng/mL, P=0.9) (Fig. 1*F*) (bile acids: 6±4 μmol/L vs. 4±0.6 μmol/L, P=0.3), but the concentration of bile acids showed greater variation in *Gcgr*$^{-/-}$ (Fig. 1*G*).

Plasma metabolomics analysis

A principle component analysis identified pooled concentrations of amino acids as the primary component separating *Gcgr*$^{-/-}$ mice from WT littermates (Fig. 2*A*) and was, furthermore, in an additional set of analysis, the predictor with most effect (~eight-fold) as well as the most significant predictor (P=7.7×10^{-9}) across the 188 identified metabolites (Fig. 2*B*). Alanine (Ala), glutamine (Gln), glycine (Gly), threonine (Thr), and serine (Ser) were, listed in decreasing order, the amino acids found in the highest concentration in *Gcgr*$^{-/-}$ mice (Fig. 2*C*).

Gcgr$^{-/-}$ mice show alpha-cell hyperplasia and hypertrophy

The mean islet size (area) was significantly larger in the *Gcgr*$^{-/-}$ mice compared to WT littermates (43,206±2,054 μm^2 vs. 1,1939±697 μm^2, P<0,0001). In the WT mice, the mean beta-cell area was 5,555±423 μm^2 amounting to a mean 46% of the mean total islet area, and the mean beta-cell area

261 in the $Gcgr^{-/-}$ mice was 7,047±465 μm^2, amounting to a mean 16% of the total islet area. The

262 percent beta-cell areas was thus smaller in the $Gcgr^{-/-}$ mice (P<0.05). In the WT mice, the mean

263 alpha-cell area was 1,150±70 μm^2 amounting to a mean 10% of the mean total islet area. The mean

264 alpha-cell area in the $Gcgr^{-/-}$ mice was 23,013±1,207 μm^2, corresponding to a mean 53% of the total

265 islet area. The alpha-cell area in the WT mice was significantly smaller than in the $Gcgr^{-/-}$ mice

266 (P<0,0001). Representative double stained (insulin and glucagon) pancreatic islets from $Gcgr^{-/-}$

267 mice and WT littermates are shown in Fig. 3*A* and *D*. The individual beta-cell size was slightly

268 larger in the$Gcgr^{-/-}$ mice than in the WT littermates (142±0.2 μm^2 vs. 102±0.1 μm^2, P< 0.001)

269 whereas the mean individual alpha-cell size was much larger in the $Gcgr^{-/-}$ mice than in the WT

270 littermates (240±0.3 μm^2 vs. 87±0.1 μm^2, P<0.0001).

271 Using a specific antibody against the murine GLP-1 receptor (37), the GLP-1 receptor was

272 identified in pancreatic islets of both WT and $Gcgr^{-/-}$ mice. Staining for the GLP-1 receptor,

273 combined with insulin, glucagon, and somatostatin immunohistochemistry, revealed that the GLP-1

274 receptor was localized to the beta-cells in both groups of mice, however, GLP-1 receptor staining of

275 the somatostatin and glucagon producing cells could not be excluded. The GLP-1 receptor was

276 confined to the plasma membrane of insulin positive cells in WT mice whereas in $Gcgr^{-/-}$ mice both

277 plasma membrane and cytosolic staining was detected (Fig. 4).

278 **Pancreatic concentrations of glucagon, GLP-1, insulin, and somatostatin in $Gcgr^{-/-}$ mice and**

279 **WT littermates**

280 Pancreatic concentrations of extractable glucagon were higher in $Gcgr^{-/-}$ mice compared to WT

281 littermates (6,851±2,361 pmol/g vs. 366±285 pmol/g, P=0.0003). Similarly, pancreatic

282 concentrations of extractable, amidated total GLP-1 were significantly higher in $Gcgr^{-/-}$ mice

283 compared to WT littermates (388±66 pmol/g vs. 69±56 pmol/g, P<0.0001). Concentrations of

284 active extractable GLP-1 were 63±22 pmol/g in the $Gcgr^{-/-}$ mice, whereas active GLP-1 was not

285   detectable in WT littermate pancreata). The analytic detection limit was calculated to be 5 pmol/g.

286   The pancreatic concentrations of extractable insulin did not differ significantly between $Gcgr^{-/-}$

287   mice and WT littermates (5218±1782 pmol/g vs. 7218±676.3 pmol/g, P=0.2). The pancreatic

288   concentration of extractable somatostatin was increased in $Gcgr^{-/-}$ mice compared to WT littermates

289   (1,943±231 pmol/g vs. 1,071±91 pmol/g, P=0.002) (data not shown).

290   **Amino acids stimulate glucagon secretion and proliferation of alphaTC1.9 cells**

291   Incubation with amino acids stimulated secretion of glucagon ~six fold from the alphaTC1.9 cells

292   compared with PBS stimulated controls (59±26 pmol/L vs. 9±8 pmol/L, P=0.0006) (Fig. 5*A*). A

293   prolonged amino acid stimulation of the alphaTC1.9 cells led to an increased proliferation by ~two-

294   fold compared to proliferation of cells not exposed to amino acids (31±8 % vs. 17±4 % BrdU

295   positive cells, P=0.01) (Fig. 5*B*).

296   **Genetic disruption of glucagon receptor signaling influence on amino acid clearance**

297   $Gcgr^{-/-}$ mice and WT littermates received identical amino acid loads (1 μmol/g) at time 0 min. At

298   time 12 min, the plasma Δ amino acid concentration in WT littermates reached 0.8±0.5 mmol/L,

299   whereas the $Gcgr^{-/-}$ mice had a Δ concentration of 6±1 mmol/L (data not shown).

300   **The effects of pharmacological blockade of glucagon receptor signaling on amino acid**

301   **clearance in mice**

302   In mice treated with a glucagon receptor antagonist (GRA), fasting plasma concentrations of amino

303   acids tended to be higher than in vehicle treated mice (3.7±0.3 mmol/L vs. 3.0±0.3 mmol/L, P=0.1).

304   In GRA+Vamin treated mice, plasma concentrations of amino acids, at time 12 min, were

305   significantly higher compared to vehicle+Vamin treated mice (9±1 mmol/L vs. 5±1 mmol/L,

306   P=0.006). In the latter group, concentrations returned to near baseline (4.8±0.6 mmol/L) 20 min

307   after the Vamin injection whereas concentrations in GRA+Vamin treated mice remained elevated

308   (7±2 mmol/L) (Fig. 6*A*). The incremental area under the curve (iAUC$_{0\text{-}20\ min}$) was significantly

309   higher in GRA+Vamin treated mice compared to vehicle+Vamin treated mice (P=0.006) (Fig. 6*B*).

310   **The effects of pharmacological blockade of glucagon receptor signaling on glucagon secretion**

311   **in mice**

312   In GRA treated mice, fasting plasma concentrations of glucagon were significantly higher than in

313   vehicle treated mice (11±1 pmol/L vs. 7±2 pmol/L, P=0.02). Glucagon concentrations increased in

314   Vamin treated groups (vehicle+Vamin and GRA+Vamin). The glucagon response at time 2 min

315   tended to be larger (~two-fold) in GRA treated mice compared to vehicle treated mice (39±11

316   pmol/L vs. 17±4 pmol/L, P=0.1) (Fig. 6*C*) and the glucagon secretory response was prolonged in

317   GRA treated mice compared to vehicle treated mice (Fig. 6*C*). The iAUC$_{0\text{-}20\ min}$ of GRA+Vamin

318   treated mice was four-fold larger when compared to vehicle+Vamin treated mice (82 min×pmol/L

319   vs. 19 min×pmol/L, P=0.02) (Fig. 6*D*).

320   **The effect of pharmacological blockade of glucagon receptor signaling on insulin secretion in**

321   **mice**

322   Fasting plasma concentrations of insulin did not differ from GRA and vehicle treated mice (0.9±0.2

323   ng/mL vs. 0.6±0.5 ng/mL, P=0.3). Plasma concentrations of insulin increased in response to the

324   amino acid stimulation in both groups. GRA treated mice had a smaller response in insulin secretion

325   compared to vehicle treated mice, although the difference was not significant (13±3 pmol/L vs.

326   19±5 pmol/L, P=0.3) at time 2 min (Fig. 7*A*). The iAUC$_{0\text{-}20\ min}$ was smaller (~20 min×ng/mL,

327   P=0.1) in the GRA treated group compared to the vehicle treated group after the amino acid

328   stimulation (Fig. 7*B*).

329

330

331 **The effect of pharmacological disruption of glucagon receptor signaling on blood glucose**

332 **concentrations in mice**

333 Fasting blood glucose concentrations of GRA and vehicle treated mice differed significantly

334 (6.5±0.2 mmol/L vs. 7.5±0.3 mmol/L, P=0.008). Blood glucose concentrations increased in

335 response to the amino acid stimulation in both groups. Blood glucose concentrations also increased

336 in response to PBS in both groups. At time 2 min, the blood glucose levels were significantly lower

337 in GRA+PBS treated mice compared to vehicle+PBS treated mice (8±0.3 mmol/L vs. 11±0.9

338 mmol/L, P=0.02) (Fig. 7*C)*. The total area under the curve ($_TAUC_{20\ min}$) was ~two-fold larger in the

339 vehicle+Vamin treated group compared to the GRA+Vamin treated group (P=0.06) (Fig.7*D*).

340 **Amino acid clearance after ligation of the kidneys**

341 Plasma concentrations of amino acids 12 min after Vamin injection did not differ significantly

342 between mice subjected to ligation of both kidneys and control mice (4.4±0.6 mmol/L vs. 3.7±0.3

343 mmol/L, P=0.4) (data not shown).

344

Discussion

Here we demonstrate that normal clearance of amino acids in mice requires glucagon receptor signaling, and that disruption of the latter, by a glucagon receptor antagonist (GRA, 25-2648) or genetic deletion of the glucagon receptor ($Gcgr^{-/-}$), results in significantly higher plasma concentrations of amino acids (hyperaminoacidemia) possibly due to decreased hepatic ureagenesis.

Firstly, we *re*-characterized the $Gcgr^{-/-}$ mouse with a focus on amino acid metabolism using plasma metabolomics, histology and measurements of pancreatic peptide hormones. As previously reported (11, 14) $Gcgr^{-/-}$ mice had lower blood glucose concentrations, elevated plasma concentrations of glucagon, increased alpha-cell mass, increased pancreatic content of glucagon, total - and active - GLP-1, somatostatin, and an equal content of insulin compared to WT littermates. Importantly, we found that $Gcgr^{-/-}$ mice had pronounced increases in plasma amino acid and ammonia concentrations. In line with this, a metabolomics driven principal component analysis showed that among 188 metabolites measured after glucagon receptor signaling disruption, the amino acid concentrations showed the most dramatic changes (~41%). In particular, the glucogenic amino acids showed the largest changes. We were, in contrast to what have been reported previously (53), unable to detect significantly higher plasma concentrations of bile acids in $Gcgr^{-/-}$ mice compared to WT littermates, but we did observe a greater variability in the $Gcgr^{-/-}$ mice and bile acids were indeed higher in some animals. In this report, corticosterone plasma concentrations were found to be similar in $Gcgr^{-/-}$ mice and WT mice after a short-term fast (four hours), and others (11, 14) have reported similar corticosterone concentrations under similar conditions. However, the corticosterone levels of $Gcgr^{-/-}$ mice were found to be increased two-fold compared to WT mice upon a prolonged fast (>12 hours), suggesting that increased levels of corticosteronemay function to prevent hypoglycemia during prolonged fasting, but are less important in the fed state.

368   Remodeling of the pancreatic islets has been suggested to be of importance for maintaining

369   adequate metabolism and in particular normal glucose concentrations (6).

370   We found the average area of the pancreatic islets to be significantly larger in the $Gcgr^{-/-}$ mice than

371   in the WT littermates, in accordance with previous studies (8, 14). The larger alpha-cell area was

372   due to both hyperplasia and hypertrophy, as the individual alpha-cells in the $Gcgr^{-/-}$ mice were

373   significantly larger than in WT littermates, and there were also more alpha cells. In the pancreatic

374   alpha-cells, proglucagon is primarily processed to glucagon by pro-hormone convertase (PC) 2 (39),

375   and very little, if any, of proglucagon is processed to GLP-1 (20). Active GLP-1 (7-36NH$_2$) is

376   therefore *not* a product of the alpha-cell proglucagon processing under normal conditions, according

377   to the present studies. However, we detected significant amounts of fully processed active GLP-1 in

378   pancreatic tissue from $Gcgr^{-/-}$ mice. A plasticity in the proglucagon producing cells, may therefore

379   exist, as has previuosly been reported (19, 39), which be activated upon metabolic or anatomical

380   alterations. However, GLP-1 remained a minor product of proglucagon amounting to only about 1%

381   of glucagon. If active GLP-1 was present in the WT pancreas in the same proportion, the

382   concentration of active GLP-1 would be below detection limit (0.6 pmol/g). The larger amount of

383   active GLP-1 in the pancreas may result in increased concentrations of circulating GLP-1 (14) in

384   $Gcgr^{-/-}$ mice, and this has been suggested as an underlying reason for the improved glucose

385   tolerance of these mice (11, 22). In connection with this finding we thought that it would be

386   interesting to investigate the expression of the GLP-1 receptor in the markedly abnormal islets of

387   the $Gcgr^{-/-}$ mice compared to the WT littermates. For this we used specific murine GLP-1 receptor

388   antibody (37) but the receptor was found exclusively on the beta-cells. The sensitivity of the

389   immunohistochemical approach does not allow us to exclude expression of a small (and therefore

390   undetectable) number of GLP-1 receptors on the glucagon and somatostatin producing cells, but at

391   least the hyperplastic and hypertrophic alpha-cells do not seem to express the receptor in large

392   amounts, and expression in the beta-cells was apparently not down regulated by the larger than

393   normal exposure to the ligand. Interestingly, the GLP-1 receptor staining in WT mice was confined

394   to the cell membrane of the beta-cells, whereas both membrane and cytoplasmic staining was found

395   in *Gcgr*$^{-/-}$ islets, due to internalization of the GLP-1 receptor. We speculate that this may be due to

396   increased secretion of pancreatic GLP-1 in the *Gcgr*$^{-/-}$ mice thereby resulting in an increased

397   internalization of the GLP-1 receptor. However, further studies are needed to clarify this.

398   We found an increased density of somatostatin cells in the *Gcgr*$^{-/-}$ islets compared with the WT

399   islets, as also reported in (14) and in accordance with the higher somatostatin content measured in

400   the pancreatic extracts from these mice. The delta-cells may therefore contribute to the increased

401   islet area detected in *Gcgr*$^{-/-}$ mice. Finally, it has been suggested that some alpha-cells, contributing

402   to the hyperplasia, may transdifferentiate to beta-cells (30, 44). Our data showed, as reported in (14)

403   that the pancreatic insulin content did not differ significantly between *Gcgr*$^{-/-}$ mice and WT

404   littermates. However, insulin immunostaining showed a slightly larger beta-cell area in *Gcgr*$^{-/-}$ mice

405   than in WT littermates.

406   GRA treated WT mice had reduced clearance of an intravenous load of mixed amino acids,

407   compared to vehicle treated mice suggesting that intact glucagon receptor signaling is required for

408   adequate clearance of exogenous amino acids. Both groups responded with an increase in glucagon

409   concentrations upon amino acid stimulation. However, the glucagon increase was larger in GRA

410   treated mice compared to vehicle treated mice. The greater and longer lasting glucagon response

411   observed in GRA treated mice might be due to the persistently higher level of amino acids in GRA

412   treated mice. Both groups responded with an increase in insulin concentrations upon amino acid

413   stimulation. However, a tendency to a decreased insulin response in GRA treated mice was

414   observed, perhaps due to GRA inhibition of glucagon-induced insulin secretion (15, 23).

415 In the *Gcgr*^{-/-} mice, the receptor deletion can lead to lifelong metabolic adaptions. These adaptions

416 may skew the results, and make the results from the model difficult to interpret in terms of

417 physiology and less relevant for the understanding of human metabolic disease (8). However, we

418 were able to demonstrate decreased clearance of amino acids using both *Gcgr*^{-/-} as well as a highly

419 specific glucagon receptor antagonist. It is therefore unlikely that the decreased clearance of amino

420 acids is a result of biological adaption following knockout of the glucagon receptor gene.

421 Furthermore, the glucagon receptor is not expressed in human or mouse adipose tissue or in muscles

422 (there is a single aberrant report of receptor expression in muscles (17), and we were also able to

423 demonstrate that ligation of the kidneys did not acutely influence amino acid clearance in WT mice.

424 We therefore suggest that the observed effects of glucagon receptor blockade on amino acid

425 metabolism are a consequence of disrupted glucagon signaling in the liver.

426 Cultured alpha cells (alphaTC1.9) incubated with amino acids showed increased proliferation and

427 glucagon secretion, supporting a role for amino acids as growth factors for the alpha-cells (32, 41)

428 and as stimulators of glucagon secretion as previously suggested (3, 26, 36, 38, 41, 45).

429 The amino acid clearance experiments were performed using mice anesthetized with isoflurane.

430 Isoflurane has, in contrast to other rodent anesthetics, previously been shown *not* to attenuate

431 arginine-stimulated glucagon secretion in mice (52), supporting that the glucagon responses

432 observed are physiologically relevant. The use of anesthetized mice allowed us to obtain sufficient

433 plasma to perform accurate measurements of amino acids, glucagon, and insulin. In addition, by

434 using anesthetized mice we were also able to exclude the effects of muscular contractions on

435 plasma amino acids (46).

436 Some of the findings in *Gcgr*^{-/-} mice are reflected in human studies: patients with inactivating

437 glucagon receptor knockout mutations show pancreatic swelling, hyperaminoacidemia, and

438 hyperglucagonemia (27). Subjects with non-alcoholic fatty liver disease (NAFLD) and type 2

439 diabetes have been shown to have elevated concentrations of plasma amino acids and

440 hyperglucagonemia (49). These findings suggest that, also in humans, impaired liver function leads

441 to hyperglucagonemia, perhaps as a consequence of impaired glucagon action on hepatic amino

442 acid turnover, leading to elevated concentrations of circulating amino acids that stimulate alpha-

443 cells. In line with this, patients with glucagon producing tumors (glucagonomas) show

444 hypoaminoacidemia (9, 50). This may be caused by the high rate of glucagon accelerated hepatic

445 amino acid turnover and ureagenesis.

446 In conclusion, both pharmacological and genetic disruption of glucagon receptor signaling leads to

447 severely impaired amino acid clearance in mice, supporting an essentiel role for glucagon receptor

448 signaling in acute amino acid turnover in mice. Furthermore, mice lacking glucagon receptor

449 signaling ($Gcgr^{-/-}$ mice) have hyperaminoacidemia, hyperglucagonemia, and alpha-cell hyperplasia.

450 These findings support the existence of a liver - alpha-cell axis with amino acid and glucagon

451 feedback loops in mice . This feedback circuitry may be particularly important during ingestion of

452 protein rich meals that raise the concentration of circulating amino acids. Disruption of the axis, be

453 it by liver dysfunction as seen in NAFLD patients or in defects in glucagon receptor signaling as

454 seen in patients with inctivating glucagon receptor mutations or treatment with a glucagon receptor

455 antagonist, leads to hyperaminoacidemia and is, in this way, responsible for the apparent

456 hypersecretion of glucagon (hyperglucagonemia) rather than disturbances in glucose metabolism.

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Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

Conceived and designed research: K.D.G., J.P., N.J.W.A. and J.J.H. Performed experiments: K.D.G., M.W.S., C.Ø., H.K., S.S.P, C.P., J.A., S.L.J., B.H., J.H., J.P., and N.J.W.A., Analyzed data: K.D.G., M.W.S., C.Ø., S.S.P., J.A., J.P., N.J.W.A., and J.J.H. Interpreted results of experiments: K.D.G., M.W.S., C.Ø., H.K., S.S.P, H.V., J.P., N.J.W.A., and J.J.H. Prepared figures: K.D.G., and N.J.W.A. Drafted manuscript: K.D.G. and N.J.W.A. Edited and revised manuscript: M.W.S., C.Ø., H.K., S.S.P., H.V., B.H., M.J.C., J.P., and J.J.H.. Approved final version of manuscript: K.D.G., M.W.S., C.Ø., H.K., S.S.P., H.V., C.P., J.A., S.L.J., B.H., J.H., M.J.C., J.P., N.J.W.A., and J.J.H..

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Figure captions and legends

Fig. 1: Biochemical characterization of glucagon receptor knockout mice and wild-type littermates

(*A*) The body weight of glucagon receptor knockout mice (*Gcgr*$^{-/-}$) male (red empty circles) or female mice (red full circles) did not differ significantly, P=0.4, from wild-type (WT) male (black empty squares) or female littermates (black full squares). The body weight of female *Gcgr*$^{-/-}$ did not differ from *Gcgr*$^{-/-}$ males (P=0.2). The body weight of female WT differed from WT males (25±3 g vs. 33±5 g, P=0.0002). (*B*) Blood glucose concentrations in *Gcgr*$^{-/-}$ mice were significantly lower compared to WT, P<0.0001, and did not differ between males and females. (*C*) Plasma concentrations of glucagon were significantly increased in *Gcgr*$^{-/-}$ mice compared to WT, P<0.0001, and did not differ between males and females. (*D*) Plasma amino acid concentrations were significantly increased in *Gcgr*$^{-/-}$ mice compared to WT, P<0.0001, and did not differ between males and females. (*E*) Plasma ammonia/ammonium concentrations were significantly increased in *Gcgr*$^{-/-}$ mice compared to WT, P=0.0002, and did not differ between males and females. (*F*) Plasma concentrations of corticosterone did not differ between *Gcgr*$^{-/-}$ mice and WT, P=0.8. Plasma corticosterone concentrations of *Gcgr*$^{-/-}$ females did not differ from *Gcgr*$^{-/-}$ males, P=0.2. Plasma corticosterone concentrations of WT females differed from WT males (251±72 ng/mL vs. 85±25 ng/mL, P<0.0001). (*G*) Plasma concentrations of bile acids did not differ between *Gcgr*$^{-/-}$ mice and WT, P=0.3, and did not differ between males and females. *Gcgr*$^{-/-}$ mice n=11 (15-28 weeks of age), WT littermates n= 15 (15-25 weeks of age). Data is presented as mean±SD, ****P < 0.0001.

669

670 **Fig. 2:** Glucagon receptor knockout mice show hyperaminoacidemia

671 *(A)* A principal component analysis revealed that plasma amino acid concentrations (component 1)

672 separated glucagon receptor knockout mice (*Gcgr*$^{-/-}$) and wild-type (WT) littermates completely.

673 (*B*) A principal component analysis revealed that amino acids (red dot) were the principal

674 component showing the greatest difference when *Gcgr*$^{-/-}$ mice were compared to WT littermates.

675 Green dots indicate the metabolites that were significantly increased in *Gcgr*$^{-/-}$ compared to WT

676 littermates, blue dots indicate metabolites that were significantly decreased in *Gcgr*$^{-/-}$ compared to

677 WT littermates. (*C*) In *Gcgr*$^{-/-}$, mice the concentrations of certain amino acids were elevated to a

678 larger degree than those of other amino acids. All amino acid concentrations, except the

679 concentration of tryptophan (Trp), were significantly higher in *Gcgr*$^{-/-}$ mice (red bars) compared to

680 WT littermates (black bars). Data presented as mean±SEM. *Gcgr*$^{-/-}$ mice n=11 (15-28 weeks of

681 age), WT littermates n=11 (18-25 weeks of age).

682

683

684   **Fig. 3.** Glucagon receptor knockout mice show alpha-cell hyperplasia and hypertrophy

685   (*A*) A typical immunohistochemical staining for glucagon (red, *B*) and insulin (green, *C*) in

686   glucagon receptor knockout (*Gcgr*$^{-/-}$) mice. (*D*) A typical immunohistochemical staining for

687   glucagon (red, *E*) and insulin (green, *F*) in wild-type (WT) littermates. The dotted arrows indicate a

688   glucagon positive cell and the solid arrows indicate an insulin positive cell, the *Gcgr*$^{-/-}$ mice showed

689   an increased size of glucagon positive cells compared to WT littermates. All images are shown at

690   X200 magnification. The scale bar indicates 50 μm.

691

692 **Fig. 4:** Glucagon receptor knockout mice show an internalization of the glucagon-like-peptide-1

693 receptor compared to wild-type littermates

694 (*A*) A typical insulin staining pattern in wild-type (WT) littermates and (*C*) in glucagon receptor

695 knockout (*Gcgr$^{-/-}$*) mice. (*B*) A typical glucagon-like-peptide-1 (GLP-1) receptor staining pattern in

696 WT littermates and (*D, F, H*) in *Gcgr$^{-/-}$* mice. (*E*) A typical somatostatin and (*G*) glucagon staining

697 pattern in *Gcgr$^{-/-}$* mice. The arrows (in *A, B*, *C* and *D*) indicate cells that stain positive for both

698 insulin and the GLP-1 receptor. GLP-1 receptor staining was localized to insulin positive cells in

699 both WT and *Gcgr$^{-/-}$* mice. The GLP-1 receptor staining was confined to the plasma membrane in

700 WT mice whereas both membrane and cytosolic staining was observed in *Gcgr$^{-/-}$* mice. All images

701 are shown at X200magnification. The scale bar indicates 50 μm.

702

703

704 **Fig. 5:** Amino acids stimulate proliferation and secretion in cultured alpha-cells

705 (*A*) The cultured alphaTC1.9 cells stimulated four hours with Vamin (1 %) (red circles) showed a

706 significant increase in glucagon secretion compared to PBS stimulated control cells (black squares).

707 (*B*) The alphaTC1.9 cells stimulated 48 hours with Vamin (red circles) showed an increased

708 proliferation compared to control cells stimulated with phosphate-buffered saline (PBS) (black

709 squares). Data presented as mean±SD, *P < 0.05, ***P < 0.001.

710   **Fig. 6:** Total L-Amino acids and glucagon plasma concentrations following amino acid stimulation

711   during pharmacological blockade of the glucagon receptor

712   (*A*) Mice treated with the glucagon receptor antagonist (GRA) (red line and circles) were not able

713   to clear the amino acid load (Vamin, 1 μmol/g body weight) administered at time 0 min whereas

714   mice treated with vehicle (blue line and upwards triangles) almost completely cleared the same

715   amino acid load within 12 min. The control groups receiving phosphate-buffered saline (PBS)

716   instead of Vamin after GRA treatment (pink line and squares) or vehicle administration (light blue

717   line and downwards triangles) showed no acute changes in the concentration of amino acids. (*B*)

718   The incremental areas under the curve ($iAUC_{0-20\ min}$) are shown for the four groups. (*C*) Mice

719   treated with GRA responded to the amino acid stimulation with a larger increase in glucagon

720   concentrations compared to mice treated with vehicle. The controls groups receiving PBS after

721   GRA or vehicle treatment showed no change in glucagon concentrations. (*D*) The $iAUC_{0-20\ min}$ are

722   shown for the four groups. Data presented as mean±SEM (n=3-7), $*P < 0.05$, $**P < 0.01$.
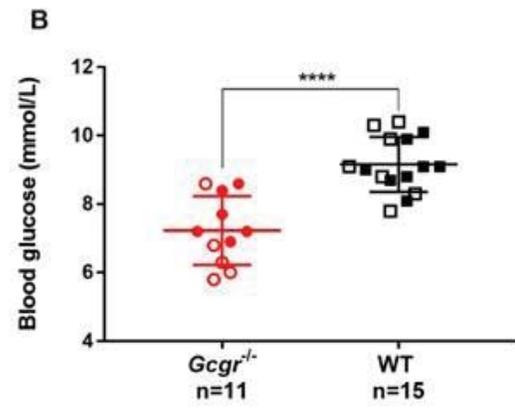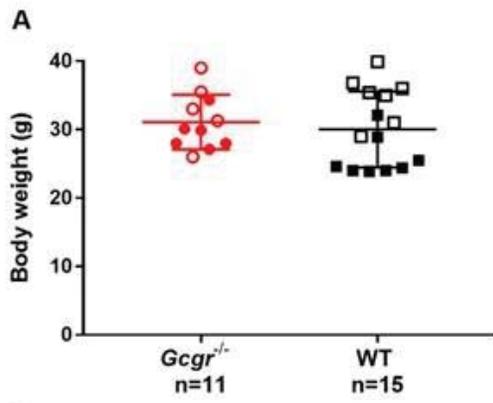
723

724

725 **Fig. 7:** Plasma insulin and blood glucose concentrations following amino acid stimulation during
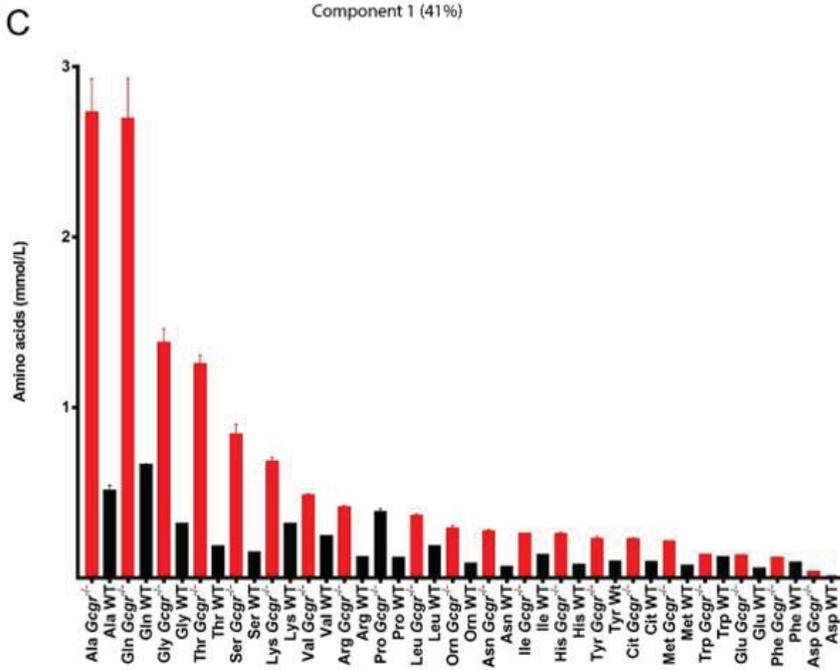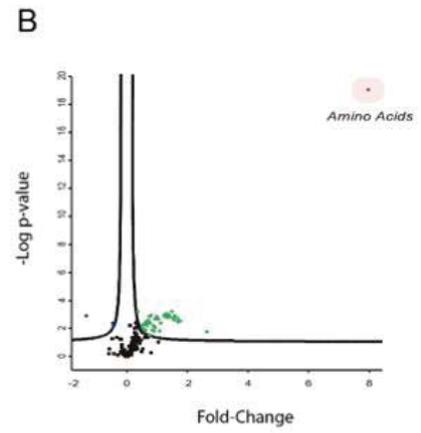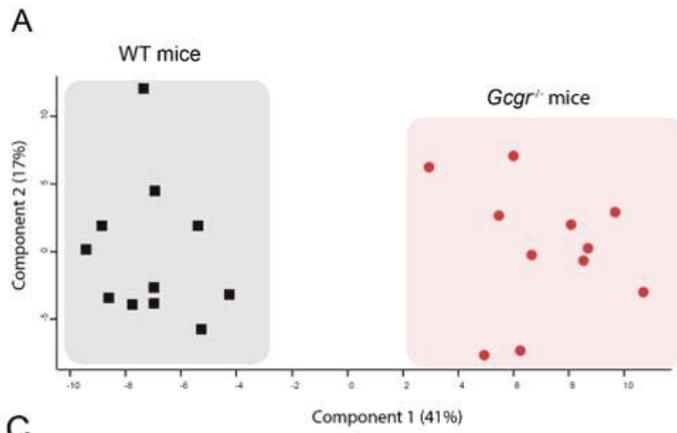
726 pharmacological disruption of the glucagon receptor

727 (*A*) Both glucagon receptor antagonist (GRA, red line and circles) and vehicle (blue line and

728 upward triangles) treated mice responded with a fast increase in plasma insulin concentration

729 following amino acid infusion (Vamin, 1 μmol/g body weight). Control groups receiving

730 phosphate-buffered saline (PBS) instead of amino acids (GRA+PBS, pink line and squares, and

731 vehicle+PBS, light blue line and downward triangles) showed no change in insulin concentrations.

732 (*B*) The incremental areas under the curve (iAUC$_{0-20 \text{ min}}$) are shown for the four groups. The amino

733 acid stimulation resulted in an increase in insulin concentrations in both groups. (*C*) Blood glucose

734 concentrations increased in response to the amino acid stimulation in both groups. Blood glucose

735 concentrations also increased in the control groups receiving PBS. (*D*) The total area under the

736 curve ($_\text{T}$AUC$_{0-20 \text{ min}}$) are shown for blood glucose levels. Data presented as mean±SEM (n=3-7).

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738

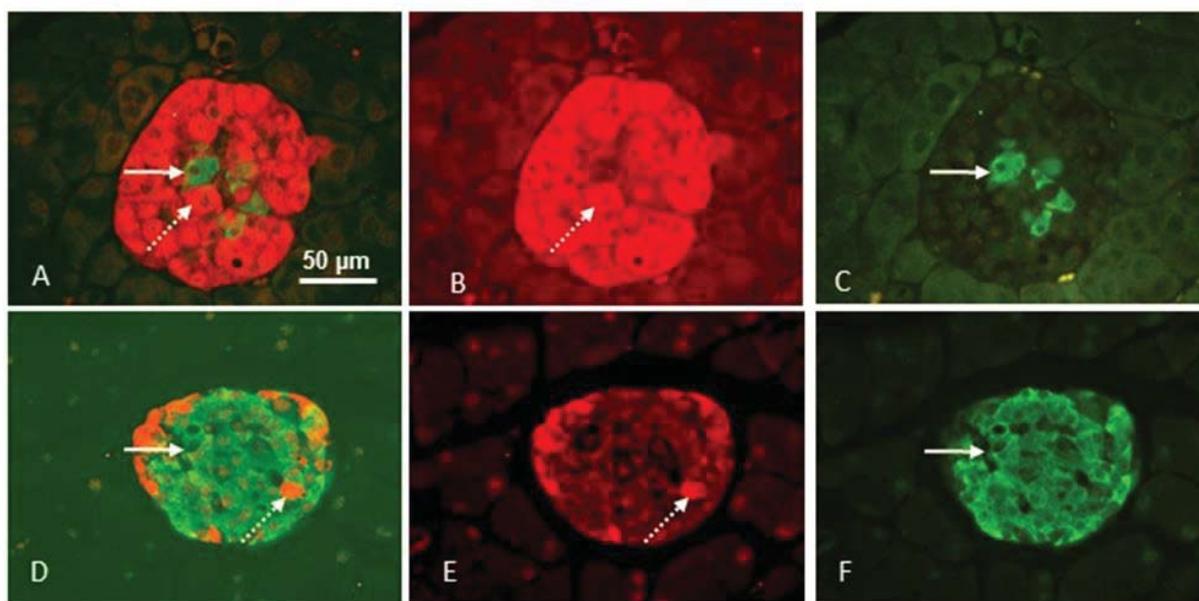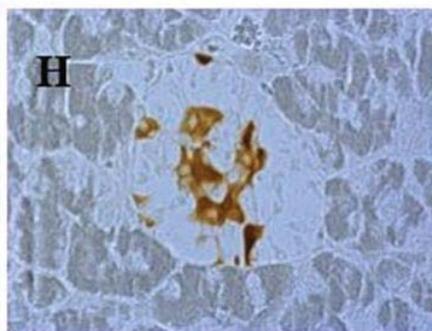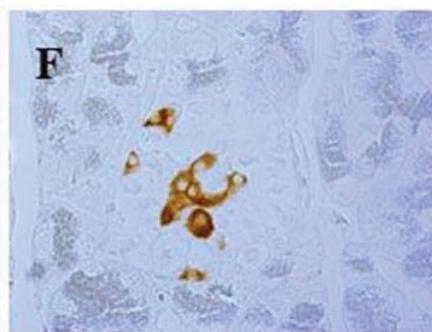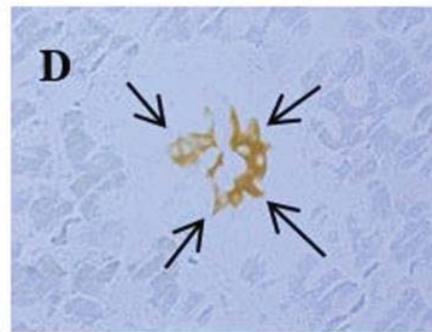739 **Table 1**: The components and concentrations of Vamin 14 Electrolytefree

Amino acid	g/L
Isoleucine	4.2
Leucine	5.9
Valine	5.5
Phenylalanine	5.9
Methionine	4.2
Lysine	6.8
Threonine	4.2
Tryptophan	1.4
Cysteine	0.42
Histidine	5.1
Tyrosine	0.17
Alanine	12.0
Arginine	8.4
Aspartic Acid	2.5
Glutamic Acid	4.2
Glycine	5.9
Proline	5.1
Serine	3.4

A

Body weight (g)

Gcgr⁻/⁻
n=11

WT
n=15

B

Blood glucose (mmol/L)

****

Gcgr⁻/⁻
n=11

WT
n=15

C

Glucagon (pmol/L)

****

Gcgr⁻/⁻
n=11

WT
n=15

D

Amino acids (mmol/L)

****

Gcgr⁻/⁻
n=11

WT
n=15

E

Ammonia (µmol/L)

****

Gcgr⁻/⁻
n=11

WT
n=15

F

Corticosterone (ng/mL)

Gcgr⁻/⁻
n=11

WT
n=15

G

Bile acids (µmol/L)

Gcgr⁻/⁻
n=5

WT
n=5

50 µm

A

Glucagon (pmol/L)

+Amino acids
n=4

-Amino acids
n=4

*

B

BrdU pos. (%)

+ Amino acids
n=12

- Amino acids
n=6

***

A

Insulin (ng/mL)

GRA + Vamin
GRA + PBS
Vehicle + Vamin
Vehicle + PBS

Time (min.)

B

$IAUC_{0-20\ min.}$ insulin (min×ng/mL)

GRA + Vamin
Vehicle + Vamin
GRA + PBS
Vehicle + PBS

C

Blood glucose (mmol/L)

GRA + Vamin
GRA + PBS
Vehicle + Vamin
Vehicle + PBS

Time (min.)

D

$_{T}AUC_{0-20\ min}$ blood glucose (min×mmol/L)

GRA + Vamin
Vehicle + Vamin
GRA + PBS
Vehicle + PBS