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1 **Lixisenatide requires a functional gut-vagus nerve-brain axis to**
2 **trigger insulin secretion in controls and type 2 diabetic mice**

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17 **Running title: lixisenatide and gut brain axis**

18 **Key words:** GLP-1 analogues, GLP-1 receptor agonist, autonomic nervous system, incretin,
19 vagus nerve

20

21 **Abstract**

22

23 Aims/hypothesis: Endogenous glucagon-like peptide-1 (GLP-1) regulates glucose-induced
24 insulin secretion through both direct β cell-dependent and indirect gut-brain axis-dependent
25 pathways. However, little is known about the mode of action of the GLP-1 receptor agonist
26 lixisenatide.

27 Methods: We studied the effects of lixisenatide (intraperitoneal (i.p). injection) on insulin
28 secretion, gastric emptying, vagus nerve activity and brain c-Fos activation in naive,
29 chronically-vagotomized, GLP-1 receptor knock-out (KO), high-fat diet-fed (HFD) diabetic
30 mice, or *db/db* mice.

31 Results: Lixisenatide dose-dependently increased oral glucose-induced insulin secretion that
32 is correlated with a decrease of glycemia. In addition, lixisenatide inhibited gastric emptying.
33 These effects of lixisenatide were abolished in vagotomized mice, characterized by a delay
34 of gastric emptying, and in GLP-1 receptor KO mice. I.p. administration of lixisenatide also
35 increased the vagus nerve firing rate and the number of c-Fos labeled neurons in the nucleus
36 tractus solitarius (NTS) of the brainstem. In diabetic mouse models, lixisenatide increased
37 the firing rate of the vagus nerve when administrated simultaneously to an intraduodenal
38 glucose. It increased also insulin secretion and c-Fos activation in the NTS.

39 Conclusions: Altogether, our findings show that lixisenatide requires a functional vagus nerve
40 and neuronal gut-brain-islets axis as well as the GLP-1 receptor to regulate glucose-induced
41 insulin secretion in healthy and diabetic mice.

42

43 **New & Noteworthy**

44 Lixisenatide is an agonist of the GLP-1 receptor, modified from exendin 4, used to treat type
45 2 diabetic patients. However, whereas the mode of action of endogenous GLP-1 is
46 extensively studied, the mode of action of GLP-1 analog, lixisenatide, is poorly understood.
47 Here, we demonstrated that lixisenatide activates the vagus nerve and recruits the gut-brain
48 axis through the GLP-1 receptor to decrease gastric emptying and stimulate insulin secretion
49 to improve glycemia.

50

51

52 Introduction

53 The control of glucose homeostasis involves metabolic, endocrine, and neuro-endocrine
54 mechanisms. The glucagon-like peptide-1 (GLP-1) is a neuroendocrine hormone secreted by
55 the intestinal L-cells in response to a meal which enhances glucose-induced insulin secretion
56 (23). GLP-1 triggers the intestinal neuronal circuits, such as the enteric nervous system and
57 the vagus nerve, which relay the nutritional and neuro-endocrine information to the brain,
58 notably the brainstem (5). Combined with other central and peripheral signals, such as
59 changes in plasma leptin, insulin, glucagon, and nutrient concentrations (34), the brain
60 generates neural signals to regulate peripheral effector tissues such as the Langerhans islets
61 to control the insulin and glucagon secretions or the stomach to control gastric emptying (1,
62 10, 34). In addition to its neuro-endocrine activity, GLP-1 directly binds to its receptor at the
63 surface of pancreatic β cells to enhance glucose-induced insulin secretion (12) and in the
64 brainstem (mainly area postrema) and hypothalamus (arcuate nucleus, ventromedial and
65 paraventricular nuclei) (42), to control food intake and energy expenditure. Importantly, the
66 physiological relevance of the gut-secreted GLP-1 mechanism of action is still matter of
67 debate since the amino-terminal peptidase dipeptidyl peptidase 4 (DPP-4), which removes 2
68 amino acids from intact GLP-1 very quickly after its secretion, transforms GLP-1_{7-37/36amide} into
69 GLP-1_{9-37/36amide}, molecular forms unable to mimic the effects of GLP-1 (13). To circumvent
70 DPP-4 cleavage and ensure an efficient therapeutic approach, DPP-4 resistant GLP-1
71 receptor agonists have been developed. Exendin 4 is a 39 amino-acid peptide found in the
72 saliva of a lizard, the Gila monster (15) with gluco-incretin properties since it enhances
73 glucose-dependent insulin secretion by the pancreatic β -cell, suppresses supraphysiologic
74 elevation of glucagon secretion, and slows gastric emptying. Exendin 4 encompasses only
75 50% amino acid homology to GLP-1. Along the same line of pharmacological strategy
76 lixisenatide has been described as "des-38-proline-exendin-4-(1–39)-peptidylpenta-L-lysyl-L-
77 lysinamide, which is derived from the first 39 amino acids of exendin 4 omitting proline at
78 position 38 and adding six lysine residues (11). All GLP-1 analogs and GLP-1 receptor
79 agonists have some common mode of action. Whereas much is known about the mode of
80 action of endogenous GLP-1, a lot remains to be understood regarding the mode of action of
81 lixisenatide. Here we studied the acute physiological effects of lixisenatide on glucose-
82 induced insulin secretion and gastric emptying through the gut-brain axis in naive,
83 chronically-vagatomized normal chow-fed, GLP-1 receptor knock-out (KO), and in diabetic
84 mice (either genetically-modified *db/db* or HFD-fed). We also recorded the vagus nerve firing
85 rate to demonstrate the recruitment of gut-brain axis by lixisenatide.

86

87 **Material and methods**

88

89 *Animals*

90 All experiments were performed with adult males C57Bl/6J mice from Charles Rivers (Lyon,
91 France). Animals were housed in the animal facility with inverted 12/12-hours day-light
92 cycles. Experiments studying the effect of lixisenatide on control mice were performed on 12-
93 week old mice. To study the importance of the GLP-1 receptor, some experiments were
94 performed with GLP-1 receptor knock-out (KO) 12-week old mice, developed in DJ.
95 Drucker's laboratory (43) and bred in our animal facility for two decades. These mice are on
96 C57Bl/6 background and despite of their viability and normal development, they are
97 characterized by an alteration of glucose metabolism (hyperglycemia and low insulin
98 secretion after an oral glucose challenge) and an absence of GLP-1-induced satiety (43). To
99 study the impact of diabetes without obesity, 8-weeks old mice were fed for two months with
100 a ketogenic high fat diet 72% (72% corn oil and lard, 28% protein and <1% carbohydrate;
101 SAFE, Augy, France), as described in numerous instances (6, 18, 19, 36). To evaluate the
102 impact of lixisenatide on obesity and diabetes, we studied 8-week old mice mice fed a 60%
103 high-fat diet (Research Diets, Inc. New Brunswick, USA) for two months (6, 18, 19, 36).
104 Under such conditions, and in contrast to the mice fed a 72% fat-enriched diet, these mice
105 gained weight and developed hyperglycemia (18, 19). 8-week old *db/db* mice, where obesity
106 and diabetes originate from a genetic mutation in the leptin receptor gene, were also studied
107 (Charles River, Lyon, France). Lixisenatide was provided by Sanofi (Paris, France). Saline
108 solution was used as control solution (i.e. 0µg/kg). The animal models were treated acutely
109 or chronically (two weeks; 1 injection/day) with lixisenatide (10µg/kg) injected
110 intraperitoneally. In humans, the half-life of lixisenatide is 2.6 ± 1 hours after a subcutaneous
111 injection of lixisenatide (20µg) (14). No data is available for the half-time in mice. The chronic
112 treatment began 1.5 months after HFD and at 8-weeks old for *db/db* mice. At the end of
113 experiments, mice were killed by cervical dislocation. All animal experimental procedures
114 were approved by the local ethical committee of Rangueil University Hospital (Toulouse) (For
115 all experiments: 12/1048/02/21 except for vagotomy surgery: 12/1048/02/26)

116

117 *Glucose tolerance tests*

118 To evaluate the impact of lixisenatide on glycemia, oral glucose tolerance test (OGTT) and
119 intraperitoneal glucose tolerance test (IPGTT) glucose tolerance tests were performed in 6
120 hour-fasted mice. During OGTT, the glucose solution (2g/kg, as a 30% solution) was
121 administrated by intragastric gavage with a gavage needle. During IPGTT, the glucose
122 solution (1g/kg, as a 20% solution) was administrated by intraperitoneal injection with a 27G

123 needle. Lixisenatide (10µg/kg; 100µL) or saline (0µg/kg; 100µL) were injected
124 intraperitoneally 30 min before the glucose administration. Glucose solution was
125 administrated at 0 minute, and the blood glucose was quantified from the tip of the tail vein
126 with a glucose-meter (Accu-Chek Active glucose meter, Roche Diagnostics GmbH,
127 Mannheim, Germany) 30 minutes before or 0, 15, 30, 60, 90 and 120 min after the glucose
128 challenge. Tail blood was sampled 15 minutes after glucose challenge to assess insulin
129 level.

130 *Quantification of plasma insulin*

131 Plasma insulin concentration was determined in 10µL using a mouse insulin ELISA kit
132 (Merckodia, Uppsala, Sweden). The coefficient of variation within the kit is 3.1% and between
133 assay is 5.9%. The lower detection limit is 0.2ng/mL.

134 *Gastric emptying experiments*

135 Gastric emptying was measured following the oral administration of acetaminophen
136 (100µg/kg; powder diluted in 30% glucose solution) in 6 hour-fasted mice. Lixisenatide
137 (10µg/kg; 100µL) or saline (0µg/kg; 100µL) were injected intraperitoneally 30 min before the
138 acetaminophen administration and the acetaminophen solution was administered by gavage
139 at 0 minute. The blood glucose was quantified from the tip of the tail vein with a glucose-
140 meter (Accu-Chek Active glucose meter, Roche Diagnostics GmbH, Mannheim, Germany)
141 30 minutes before or 0, 15, 30, 60 and 90 after the acetaminophen gavage. 20µL of tail blood
142 was sampled at -30, 15, 30 and 60 minutes. The plasma concentration of acetaminophen
143 was assessed within 5µL as described by manufacturer (Acetaminophen assay kit, Sekisui
144 Diagnostics, Charlottetown, Canada).

145

146 *Recording of the vagus nerve firing rate activity*

147 First, mice were fasted during 6 hours. For all electrophysiology procedures, mice were
148 anesthetized with isoflurane 1.5-2% diluted in air, which is a high dose only used for surgery.
149 Abdominal surgery was performed to fix a catheter (diameter = 0.3 mm) in the duodenum
150 1cm after the pylorus to permit intra-duodenal glucose injection (2g/kg, as a 30% solution).
151 The left cervical vagus nerve was then isolated from the carotid artery and throat muscles
152 with a piece of non-adhesive tape, and two covered platinum electrodes (diameter = 0.85
153 mm, A-M Systems, Inc., WA, USA) - uncovered at the two ends for 2 at 3 mm - were set
154 directly on the nerve avoiding contact with other tissues and fixed with silicone elastomers
155 Kwik-Cast sealant (World Precision Instrument, FL, USA). Then mice were housed in a
156 Faraday cage and electrodes were connected to a high-impedance probe, and action

157 potentials were displayed and saved on a computer after initial amplification through a low-
158 noise amplifier (Bio Amp, AD Instruments). Data were digitized PowerLab 4/30 data
159 acquisition system (ADInstruments, Colorado springs, CO, USA). Then, a 3-4 times lower
160 concentration of anesthesia (0.5% diluted in the air). This procedure allows a light and
161 sufficient anesthesia of the mouse while the firing rate activity of the vagus can still be
162 recorded. A piezoelectric sensor was set on the mouse thorax to record breath movements
163 allowing controlling the quality of the anesthesia. After a stabilization period of 10-20
164 minutes, the basal vagal activity was recorded on a computer with the LabChart 7.3 software
165 (ADInstruments) and band-pass filtered between 25 and 30Hz to record slow action
166 potentials from vagal afferences (30). However, we cannot discriminate totally the
167 measurement of efferent activity through that method. Then, lixisenatide (10 μ g/kg; 100 μ L) or
168 saline (0 μ g/kg; 100 μ L) was injected intraperitoneally and the basal activity was recorded for
169 20 minutes before the intraduodenal glucose injection and the following 15 minute-recording
170 period. Mice were sacrificed at the end of the recording period by cervical dislocation.

171 *Vagus nerve activity data analysis*

172 The firing rate activity was assessed using the “event count” option of the LabChart software.
173 The number of event was counted per minute and a mean of these values was realized by
174 defined time period: basal activity (during 5min), 10min post lixisenatide injection (during
175 10min, just before glucose injection), 0-7min after glucose injection (during 7min) and 8-
176 15min after glucose injection (during 7min). The percentage from basal activity was
177 calculated as a ratio between the event count per minute from the defined time period and
178 the mean of event count per minute from the basal activity multiplied by 100.

179 *Subdiaphragmatic vagotomy*

180 To address the role of lixisenatide on the gut-brain axis we vagotomized (without
181 pyloroplasty) 8-week old mice under anesthesia (intraperitoneal injection of Ketamine,
182 100mg/kg+ Xylazin, 10mg/kg and maintained under isoflurane, 0.5 to 1% of air) and let them
183 recover for three weeks (19). After an upper midline laparotomy (2cm) the vagal trunk was
184 exposed and 1 cm of the ventral vagus nerve and of all neural and connective tissues
185 surrounding the esophagus were cut off. Sham-operated mice underwent the same
186 procedure without cutting the vagal trunk (19).

187 *C-fos detection in the brain*

188

189 Mice were fasted for 6h before they received a saline solution (0 μ g/kg) or 10 μ g/kg i.p. of
190 lixisenatide 30 min before gavage of glucose (2g/kg as a 30% solution). Ninety minutes post

191 injection all mice were sacrificed by an overdose of sodium pentobarbital (100 mg/kg, i.p.).
192 Immediately following euthanasia, all mice were infused transcardially with first, heparin
193 (25000 U.I./5mL, Sanofi) diluted at 1/500e in phosphate buffer saline (PBS) and, secondly,
194 with 60mL of a 4% paraformaldehyde (PFA) solution (PFA powder (SigmaAldrich) diluted in
195 PBS 0.1M) to fix the brains in situ. The brains were isolated and post-fixed with 4% PFA
196 solution overnight 4°C. The brains were soaked in citric buffer (pH 4.5) for 6h at room
197 temperature and heated in 80mL of citric buffer (microwave oven, 650 W for 1.5 minutes).
198 They were rinsed with PBS at RT and 4°C, and transferred to a 10% sucrose solution
199 (sucrose powder in PBS) and finally into a 30% sucrose solution overnight 4°C. The next
200 day, the tissues were frozen and stored in -80°C. The areas analyzed included the dorsal
201 vagal complex located according to the mouse brain atlas with in stereotaxic coordinates
202 (16). For NTS, one caudal (bregma-7.83), one medial (bregma-7.47), one rostral (bregma-
203 7.31) sections were chosen from each mouse (i.e. 3 non-consecutive sections/animal, n=4-5
204 per group). For medial ARC (bregma-1.69-1.91), 2-4 non-consecutive sections were chosen
205 per animal (n=4-5 per group). Sections of 25µm (Cryostat -20°C) were performed.
206 For c-Fos immunohistochemistry, after the brain sectioning, free-floating sections were
207 blocked in a blocking solution (PBS, 0,3% Triton X-100 and 2% donkey serum) and then,
208 incubated with rabbit anti-c-Fos antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz,
209 CA) for 48 h at 4 °C. After incubation, the tissue was rinsed in PBS and incubated in
210 biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) in
211 PBS with 0.3% Triton X-100 (PBST) for 1 h. The slides were then washed in PBS and
212 incubated in avidin-biotin solution (vectastain; Vector Laboratories, Burlingame, CA) for 1 h.
213 c-Fos immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB) enhanced with
214 nickel chloride. Sections were then mounted on gelatin-coated slides, dehydrated in an
215 increasing series of alcohols, defatted in xylene, and coverslipped with permount (Menzel,
216 Braunschweig, Germany).
217 Stained sections were analyzed using an Olympus AX-70 microscope (Olympus, Center
218 Valley, PA, USA). C-Fos positive cells were counted by an experimenter blind to the
219 treatment, using Volocity 4 software (Ver.4, Improvision, Coventry, UK). The analysis and
220 data are expressed as an average of "number of positive cells per section" from 3 non-
221 consecutive sections of NTS and 2-4 non-consecutive sections of ARC, for each animal.
222 The *in vivo* part of c-Fos measurement was realized in the same day for vehicle-injected and
223 lixisenatide-injected group as well as the immunostaining of the brain slices limiting the
224 picture and quantification differences linked to the difference of the time experiments.
225
226 *GLP-1 treatments*

227 Lixisenatide in a final volume of 100 μ L was administered intraperitoneally 30 minutes before
228 glucose gavage or intraduodenal injection of glucose (performed only for vagal firing studies),
229 i.e. at the beginning of the afternoon (nearly 1:00, 2:00 pm). 100 μ L of saline (0 μ g/kg) solution
230 was used as control injection. Different dose of lixisenatide (1, 3, 6, 10 μ g/kg) were tested in
231 glucose tolerance tests of adult control mice. The dose of 10 μ g/kg and saline solution were
232 used in GLP-1 receptor KO mice, chronically-vagotomized mice, HFD-fed mice and *db/db*
233 mice and for c-Fos experiments, vagus nerve recording, gastric emptying assessment. For
234 chronic treatment, lixisenatide was administered intraperitoneally (100 μ L; 10 μ g/kg), in the
235 morning (at 10:00 am), once a day during two weeks.

236 Each in vivo experiment, i.e. glucose tolerance tests and gastric emptying assays, were
237 performed on a single set of mice with a one week interval. New sets of mice were used for
238 each end-point bioassays, i.e. c-fos staining and vagal nerve activity recording.

239

240 *Statistical analysis*

241 Results are expressed as mean \pm standard error of the mean (SEM). Statistical significance
242 was evaluated by Student's t test, One-Way Anova or Two-Way Anova (followed by post hoc
243 Tukey's multiple comparisons tests) and correlations were performed by Pearson analysis
244 using GraphPad Prism version 6.00 for Mac (GraphPad Software, San Diego, CA, USA).

245 **Results**

246 *Lixisenatide decreases the glycemia through insulin secretion stimulation and gastric*
247 *emptying inhibition in adult healthy mice.*

248 To validate our approach, we first evaluated the impact of different doses of lixisenatide on
249 glucose tolerance, insulin secretion and gastric emptying in adult healthy mice. Lixisenatide
250 was administered by intraperitoneal injection 30 min before glucose challenge. We initially
251 administered glucose by oral gavage to enable the recruitment of the gut-vagus nerve- β cell
252 axis, and compare the results to data obtained following an intraperitoneal glucose
253 administration, which does not recruit the gut-vagus nerve- β cell axis (24). In response to the
254 oral administration of glucose, all doses of lixisenatide improved glycemic profiles in adult
255 healthy mice (**Figures 1A,B**) but only high doses of lixisenatide (3-10 μ g/kg), augmented
256 systemic plasma insulin levels (**Figure 1C**). The highest dose of lixisenatide (10 μ g/kg)
257 increased the plasma insulin concentration by two-fold (**Figure 1C**). When the glucose was
258 administrated intraperitoneally, high doses of lixisenatide (3-10 μ g/kg) reduced glycemic
259 excursion (**Figures 1D,E**). The dose of 3 μ g/kg of lixisenatide did not increase insulin
260 secretion. However, the dose of 10 μ g/kg modestly increased insulin secretion by 1.4 fold but

261 this effect did not reached statistical significance (**Figure 1F**). These results suggested that
262 as well as observed for GLP-1 activity, lixisenatide was more efficient to reduce the glycemia
263 if the glucose was injected orally, i.e. when glucose triggers the neural gut-brain axis. To
264 evidence an interaction between lixisenatide-stimulated insulin secretion and glycemia, we
265 correlated the glycemic indexes and the insulin concentrations in the lixisenatide and vehicle-
266 injected mice during an oral glucose tolerance test (**Figure 1G**). We observed a correlation
267 between the lixisenatide effects on insulin secretion and on glycemia ($P = 0.0009$; $R^2 = 0.40$
268 ; $R = -0.64$), further supporting our hypothesis. In addition, we also observed a correlation
269 between the insulin concentrations and glycemic indexes in vehicle-treated mice ($P = 0.043$;
270 $R^2 = 0.67$; $R = -0.82$) suggesting that lixisenatide was enhancing the well known crosstalk
271 between glycemia and insulin. This set of data also suggests that lixisenatide enhance
272 glucose-induced insulin secretion as does GLP-1.

273 The control of gastric emptying, which is in part regulated by GLP-1 (25, 31, 32), is an
274 important mechanism to dampen glycemic excursions following a meal. Therefore, we first
275 evaluated the impact of lixisenatide on the appearance of acetaminophen and glucose into
276 the blood following an oral administration of these molecules. Since the highest dose of
277 lixisenatide (10 $\mu\text{g}/\text{kg}$), was efficient to reduce the glycemia and increase insulin secretion
278 after oral glucose load, we used this dose to assess gastric emptying and in the next
279 experiments with other animal models. The data show that lixisenatide strongly reduces the
280 blood concentrations of glycemia and acetaminophen suggesting an effect on the lowering of
281 gastric emptying (**Figure 1H-J**). To evidence an interaction between lixisenatide-induced
282 gastric emptying inhibition and lixisenatide-induced glycemia reduction, we correlated the
283 glycemic index and the acetaminophen index in the lixisenatide-injected mice during the
284 gastric emptying assessment (**Figure 1K**). We observed a trend of correlation between both
285 parameters suggesting an association effect between them ($P = 0.11$; $R^2 = 0.32$; $R = 0.56$).
286 Interestingly, in the vehicle-treated groupe, the acetaminophen index is not correlated with
287 the glycemic index ($P = 0.21$; $R^2 = 0.28$; $R = -0.53$) suggesting that in a steady state, these
288 both parameters are not associated.

289 Altogether, these results showed that lixisenatide decreases glycemia by stimulating insulin
290 secretion and inhibiting gastric emptying in healthy adult mice.

291
292 *Lixisenatide-induced insulin secretion and gastric emptying inhibition are mediated by the*
293 *vagus nerve and the GLP-1 receptor.*

294 Although recent publications suggest other mechanisms of action (20, 46), GLP-1 can recruit
295 the gut-brain axis to control metabolism (19, 31, 38, 49). To study whether lixisenatide would

296 similarly involve this mode of action, we performed subdiaphragmatic vagotomy (SDVx) and
297 quantified the efficiency of lixisenatide to trigger insulin secretion and inhibit gastric emptying.
298 We observed that the peak of acetaminophen was early shifted by 15min in vagatomized
299 mice suggesting that the latter mouse model described an alteration of gastric emptying
300 (**Figure 2E&G**). With this in mind, our result show that in vagotomized mice, plasma insulin
301 concentration was not increased by lixisenatide after oral glucose challenge when compared
302 to vagatomized mice injected with saline solution (**Figure 2A**) or sham-operated mice
303 injected with lixisenatide (**Figure 2B**). After an intraperitoneal glucose challenge,
304 vagotomized mice released the same amount of insulin after injections of saline or
305 lixisenatide (**Figure 2C**) and the same amount of insulin of sham-operated mice injected with
306 lixisenatide (**Figure 2D**). These results suggest that the vagus nerve was required for the
307 control of insulin secretion after glucose gavage in response to lixisenatide. On the other
308 hand, vagotomy also dramatically blunted the impact of lixisenatide on gastric emptying
309 when compared to sham-operated mice particularly at the time-point 30 min (**Figure 2E-H**).

310 Since GLP-1 induces its actions through its specific receptor we have evaluated the
311 efficiency of lixisenatide in GLP-1 receptor KO mice. In these mice, lixisenatide did not
312 induce insulin secretion after oral or intraperitoneal glucose challenges when compared to
313 WT mice (**Figure 2I,J**). Lixisenatide also did not inhibit the gastric emptying in these KO mice
314 (**Figure 2K,L**).

315 These results suggest that in adult healthy mice, lixisenatide requires the vagus nerve and
316 the gut-brain axis as well as the GLP-1 receptor to mediate its physiological actions.

317 *Lixisenatide recruits and activates the gut-brain axis through the vagus nerve.*

318 Then, we recorded the firing rate activity of the vagus nerve in mice anesthetized with a low
319 dose of isoflurane. Lixisenatide or saline was administered 30 minutes before an intra-
320 duodenal glucose administration which avoids the drawback of gastric emptying on the
321 kinetic of glucose absorption. The firing rate activities of the vagus nerve were measured 10
322 minutes after lixisenatide injection and at different times after the glucose challenge (**Figures**
323 **3A1-3**). The results showed that lixisenatide by itself activates the vagus nerve in control
324 adult mice and the secondary intra-duodenal injection of glucose did not further potentiate
325 the firing rate of the vagus nerve. We confirmed that our set up was functional since
326 lixisenatide-induced vagus nerve activation was indeed associated with an increase of insulin
327 secretion by nearly two-fold (954 ± 324.4 pg/mL *versus* 1781 ± 309.6 pg/mL for 0 μ g/kg and
328 10 μ g/kg, respectively) (**Figure 3B**) as observed during the oral glucose tolerance test
329 (**Figure 1C**).

330 A functional evidence for the involvement of the gut-brain axis is the induction of c-Fos
331 expression in cells of the nucleus of tractus solitarius (NTS). Peripheral GLP-1 agonist
332 treatment can also activate the arcuate nucleus of the hypothalamus (ARC) (4). Therefore,
333 we quantified the number of c-Fos expressing cells in the NTS as well as ARC after
334 lixisenatide injection. The results showed that in adult healthy mice lixisenatide increases the
335 number of c-Fos positive cells in the NTS, suggesting that lixisenatide activates the vagus
336 nerve that directly innervates the NTS neurons. However, the number of c-Fos expressing
337 cells within the ARC remains unchanged (**Figure 3C1-2;3D1-2**).

338 Altogether, these results show that in healthy non-diabetic mice lixisenatide increases insulin
339 secretion and controls gastric emptying through a vagal activation.

340 *Acute lixisenatide treatment improves glycemia through insulin secretion and gastric*
341 *emptying in diabetic mouse models.*

342 Next, we validated the anti-diabetic effects of lixisenatide in two models of diet-induced type
343 2 diabetic mice fed a high-fat carbohydrate free diet (72% fat) or a high-fat carbohydrate-
344 enriched diet (60% fat). The mice fed 72 % fat diet are lean, diabetic, and may be ketotic,
345 although no excessive circulating ketone bodies concentrations were noticed (19). The
346 second animal model fed 60 % fat is obese, hyperinsulinemic, and diabetic (19). We also
347 evaluated the impact of lixisenatide on *db/db* mouse model, a genetic mouse model of
348 diabetes and obesity.

349 In both fat-enriched diet-fed mouse models, lixisenatide reduced glycemia (**Figure 4A,B**) and
350 increased insulin secretion (**Figure 4C**). In *db/db* mice, lixisenatide caused a significant
351 reduction in glucose profiles (27730 ± 589.1 pg/mL *versus* 21875 ± 1651 pg/mL for 0 μ g/kg
352 and 10 μ g/kg respectively) (**Figure 4D,E**), but had no significant effect on insulin secretion
353 (4528 ± 527.7 pg/mL *versus* 6537 ± 958.5 pg/mL for 0 μ g/kg and 10 μ g/kg, respectively)
354 (**Figure 4F**). It is noteworthy that these mice were quite hyperinsulinemic which could have
355 hampered a further enhancement of insulin secretion. On the other hand, lixisenatide tended
356 to decrease acetaminophen plasma concentration at 15 and 30min (**Figure 4H**) and the
357 acetaminophen index (**Figure 4K**) in the 72% fat-enriched diet-fed mice. In 60% fat-enriched
358 diet-fed mice, lixisenatide had a slight effect on gastric emptying by only decreasing the
359 acetaminophen plasma concentration 30min after its gavage (**Figure 4H**). In *db/db* mice,
360 lixisenatide had no impact on the lowering of gastric emptying (**Figure 4G-K**).

361 Altogether, these data show that the glycemic profiles of type 2 diabetic mice can be
362 controlled by lixisenatide.

363 *Lixisenatide effects recruits gut-brain axis in diabetic mouse models.*

364 To evaluate whether lixisenatide can recruit the gut-brain axis and control glycemia and
365 insulin secretion in type 2 diabetic mice, we measured the vagus nerve activity and quantified
366 c-Fos into the brainstem following glucose and lixisenatide acute administration. We
367 observed that lixisenatide alone is unable to activate the vagus nerve (**Figure 5A1-3**) in
368 contrast to what we observed previously in healthy mice (**Figure 3A1-3**). However, when
369 glucose was administered following lixisenatide administration the vagus nerve activity was
370 enhanced in 72% high-fat diet-fed mice (**Figure 5A1-3**). This activation was associated with
371 a two-fold increase of insulin concentration by two-fold ($451 \pm 79.97\text{pg/mL}$ versus $965 \pm$
372 267.4 pg/mL for $0 \mu\text{g/kg}$ and $10 \mu\text{g/kg}$, respectively) (**Figure 5B**). On the other hand, we
373 observed an increase of the number of c-Fos positive cells in the NTS (**Figure 5C1,2**) in all
374 diabetic mouse models. These results suggest an efficient recruitment of the gut-brain axis.
375 On the other hand, lixisenatide did not activate neurons in the arcuate nucleus (ARC) in 60%,
376 and 72% high-fat diet fed-mice further suggesting a lack of direct effect of the drug on the
377 brain (**Figure 5C1,2**). Interestingly, both basal and lixisenatide-induced c-Fos activation were
378 elevated in the ARC of *db/db* mice.

379 Therefore, we tested whether a chronic treatment with lixisenatide maintains these
380 physiological effects observed in an acute study. We treated type 2 diabetic mice with
381 lixisenatide daily for three weeks. It is important to note that lixisenatide was not administered
382 on the day of glucose tolerance tests. In these conditions, no sustained impact of lixisenatide
383 was observed on neither body weight or the glycemetic features (**Figure 6**). Additionally, we
384 observed no impact on vagus nerve activities in response to glucose (**Figure 7**).

385

386

387 Discussion

388 Our data indicated that lixisenatide controls glycemia through the stimulation of
389 glucose-induced insulin secretion and the inhibition of gastric emptying in both control and
390 diabetic mice. We show that the effects of lixisenatide requires the activation of the gut-vagus
391 nerve-brain axis and a functional GLP-1 receptor to control these physiological functions.

392 The control of glucose-induced insulin secretion by endogenous gut-released GLP-1
393 requires indirect neural and direct β cell routes (10). The neural route via the vagus nerve is
394 supported by numerous studies over the last decades (3, 7, 31, 37, 38). First, we observed
395 that when glucose was administered orally the stimulation of insulin secretion was more
396 efficient than when administered intraperitoneally describing the incretin effect and the
397 recruitment of GLP-1 and GIP. Then, we here demonstrated when glucose was administered
398 orally rather than intraperitoneally, lixisenatide produced more potent effect to trigger insulin
399 secretion. To add, Torkildsen et al. showed that I.P. injection of lixisenatide in *db/db* mice is
400 efficient to induce insulin secretion if they used 100 nmol/kg, i.e. 50 times higher than our
401 concentration (47). These results suggest that lixisenatide modulates glucose-induced
402 signals via the intestinal vagus nerve and the gut-brain axis to control insulin secretion.
403 Furthermore, DPP4 inhibitors enhanced the concentration of endogenous GLP-1 notably into
404 the portal vein and at the vicinity of the enteric nervous system to activate the firing rate of
405 the vagus nerve towards the brain (49). Consequently, a brain signal generated in the
406 brainstem stimulates insulin secretion via descending vagal efferent pathway towards the
407 pancreas. All these results points out that endogenous GLP-1 triggers the intestinal vagus
408 nerve to induce insulin secretion in healthy situation (21). Consequently, DPP4 inhibitors
409 have been developed and have an efficient therapeutic impact on the glycemic control by
410 increasing the local concentration of the active endogenous peptide around the enteric
411 nervous system and the intestinal vagal afferences (22). We previously demonstrated that
412 the oral administration of a low dose a DPP4 inhibitor still controlled glycemia through an
413 inhibition of the intestinal DPP4 activity at the vicinity of the entero-endocrine L cells (49).
414 Conversely, the blood administration of a similar dose did not neither increase glucose-
415 induced insulin secretion nor control glycemia (49). Such data confirm that the circulating
416 DPP4 and GLP-1 are not mainly involved in glucose-induced insulin secretion when
417 compared to the role of intestinal DPP4 and GLP-1 (49). Those results suggest that the
418 local/enteric action of GLP-1 on the intestinal vagus nerve and enteric nervous system is
419 more important than the endocrine action of the peptide. In support of the neural mode of
420 action of GLP-1, an impaired autonomic nervous system reduces DPP4i efficacy in humans
421 (35) and favors the early onset of metabolism alterations (28). The identification of this
422 physiological mode of action endogenous GLP-1 raises the possibility that a similar
423 mechanism might explain the pharmacological effects of systemically administered long

424 acting GLP-1 receptor agonists such as exendin 4, lixisenatide and liraglutide for the
425 treatment of type 2 diabetes. It is commonly accepted that GLP-1 receptor agonists activate
426 GLP-1 receptor at the surface of 1) the insulin-secreting β cell to trigger insulin secretion and
427 regulate glycemia (29) and 2) the brain areas outside the blood-brain barrier such as area
428 postrema (2, 4, 33, 42) or cross the blood-brain-barrier and act directly on hypothalamic
429 neurons to regulate the food intake and decrease body weight (4, 44). Moreover, a
430 recruitment of the gut-brain axis is also possible since the GLP-1 receptor is expressed in the
431 neurons of the vagus nerve localized in the nodose ganglion and innervating intestine
432 through their axons (42). Reducing GLP-1 receptor signaling in the neurons of the vagus
433 nerve *via* viral-mediated GLP-1 receptor knockdown in the nodose ganglia influences
434 glucose-induced insulin secretion and gastric emptying (31). Therefore, we challenged this
435 dogma and demonstrated unequivocally that vagotomy totally blunted lixisenatide induced-
436 insulin secretion and gastric emptying inhibition. Previous publications described that in
437 humans and rodents, vagotomy alone altered gastric emptying (39, 40) suggesting that the
438 vagus nerve integrity is critical for the stomach activity. On the other hand, it was shown that
439 vagotomy prevents GLP-1 actions particularly for the activation of the vagal gut-brain axis
440 (4), the insulin secretion and the gastric emptying (19, 40) while it increased the GLP-1 and
441 GIP levels in the blood after glucose stimulation (19, 39, 40, 51). These data suggest the
442 important role of the vagus nerve on some GLP-1 actions. Interestingly, studying vagatomized
443 mice allowed us to highlight a new endocrine role of GLP-1 on insulin sensitivity (27). The
444 neuroincretin effect is further supported by our data since lixisenatide efficiently triggers
445 insulin secretion during oral glucose administration but less efficiently following an
446 intraperitoneal glucose administration. Altogether, in healthy mice, lixisenatide requires a
447 functional gut-vagus nerve to brain axis for the control of glucose-induced insulin secretion
448 and gastric emptying lowering. Furthermore, our data show that lixisenatide increases the
449 NTS activity. These results suggest that lixisenatide, through its impact on vagal nerve,
450 would increase the NTS activity therefore, regulating gastric emptying separately from the
451 glucose lowering effect of the molecule. However, lixisenatide did not modify the number of
452 c-fos positive cells in the ARC suggesting that no signal was transmitted from the NTS to the
453 ARC despite the vagal activation. Our result also show that the circulating lixisenatide could
454 not act directly on the hypothalamus by crossing the blood-brain-barrier. Comparable results
455 were observed for exendin 4, a lixisenatide analogue (2). Other studies showed that the
456 activation of the NTS by exendin 4 was impaired in vagotomized mice (4) and GLP-1
457 receptor KO mice (2) suggesting that vagus nerve and GLP-1 receptor are important for
458 brain area activation induced by exendin 4-derived molecules. In healthy mice, the firing
459 rate of the vagus nerve in response to lixisenatide alone was most likely maximal since the
460 subsequent glucose administration did not further augment the firing rate. It was shown that

461 the GLP-1 receptor has a basal activity which is sufficient to maintain high concentration of
462 cAMP ensuring a significant basal insulin secretion rate (45). Furthermore, we previously
463 described that in the portal vein the molecular mechanisms of glucose sensing i.e. the
464 glucose transporter GLUT2, the somatostatin receptor and the GLP-1 receptor can be
465 maximally triggered by low rates of glucose infusion directly into the portal vein (7-9, 41).
466 Therefore, in healthy situation, the neural GLP-1 dependent route for the triggering of insulin
467 secretion requires most likely a low physiological enteric concentration of GLP-1. Conversely,
468 in case of type 2 diabetes, we and others recently showed that high doses of GLP-1 are
469 required to control glycemia in diabetic mouse models and in diabetic patients (19, 50).
470 These previous results are further supported by our present data since in our animal models
471 of type 2 diabetes, lixisenatide alone is unable to stimulate glucose-induced firing rate activity
472 of the vagus nerve, and needs a concomitant intestinal glucose signal. We evidenced that
473 the co-administration of both lixisenatide and glucose activated the vagus nerve in type 2
474 diabetic mice. This is in agreement with the increased brainstem c-Fos labeling suggesting a
475 strong activation of the gut brain axis by lixisenatide. Furthermore, the lack of c-Fos labeling
476 in the ARC suggests that lixisenatide does not regulate glycemia, food intake or body weight
477 via a direct activation of ARC neurons.

478 Our data also show that, although lixisenatide efficiently activates the gut-brain axis
479 and regulates insulin secretion in high-fat diet-fed diabetic mice, it only modestly inhibits
480 gastric emptying. This could be due to some degree of autonomic neuropathy affecting the
481 enteric nervous system leading to gastroparesis as observed during type 2 diabetes (48). On
482 the other hand, lixisenatide was unable to trigger insulin secretion and gastric emptying
483 inhibition in *db/db* mice since these mice were already dramatically hyperinsulinemic and had
484 a very strong gastroparesis and gastric dysmotility (26). Additionally, a higher sensitivity to
485 glucose and lixisenatide in the ARC were observed in *db/db* mice. These results may be
486 explained by the fact that *db/db* mice have an alteration of the blood-brain-barrier permitting
487 some brain area to have a greater access to the glucose and circulating lixisenatide (17).
488 Although lixisenatide might cross the blood-brain-barrier in these genetically-modified mice,
489 we did not observe a decrease of weight with a chronic treatment. These results can be
490 explained by the fact that GLP-1 signaling need to interact with leptin signaling to induce a
491 decrease in food intake and body weight (52).

492 Altogether, we here showed that lixisenatide requires the gut-brain-vagus nerve axis
493 to control gastric emptying and glucose-stimulated insulin secretion in both healthy and
494 hyperglycemic mice.

495

496 **Author contributions**

497 E.G. and R.B. conceived and designed the experiments; J.C, P.K., S.J.L, E.G. an C.C.G
498 performed the experiments, acquired and analyzed the data; E.G., C.M., C.C.G., S.J.L. and
499 R.B. interpreted the data, wrote and corrected the manuscript. All authors read and agreed
500 on the final version of the manuscript.

501

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509

510 **Conflict of Interest**

511 R.B. received honorarium from Sanofi.

512

513 **Figure Legends**

514 **Figure 1: Lixisenatide decreases the glycemia through insulin secretion and gastric emptying**
515 **inhibition in adult control mice.** n=5-10/group. **A-C:** Oral glucose tolerance test (2g/kg) with
516 different concentrations of lixisenatide (1, 3, 6, 10ug/kg, diluted in saline solution; 0ug/kg =
517 saline solution). **A:** Glycemia; **B:** Glycemic index (calculated as follow: sum of glycemia
518 between 15 and 120min divided by the number of minutes between 15 and 120min, i.e. 105
519 and multiplied by 1000); **C:** Plasma insulin concentration 15min after glucose gavage. **D-F:**
520 Intraperitoneal glucose tolerance test (1g/kg) with different concentrations of lixisenatide (1,
521 3, 6, 10ug/kg, diluted in saline solution; 0ug/kg = saline solution). **D:** Glycemia; **E:** Glycemic
522 index; **F:** Plasma insulin concentration 15min after glucose injection. **G-I:** Gastric emptying
523 assesment (solution of acetaminophen (100ug/kg) and glucose (2g/kg)) with lixisenatide
524 (10ug/kg) (0ug/kg = saline solution). **G:** Correlation between glycemic index and insulin level
525 vehicle (open circle) and lixisenatide (dark cycle)-injected mice (Pearson analysis). **H:**
526 Glycemia; **I:** Plasma acetaminophen concentration at -30, 0, 15 and 30min after gavage of
527 acetaminophen/glucose solution. **J:** Acetaminophen index (calculated as follow: sum of
528 acetaminophen concentrations between -30 and 60min divided by the number of minutes
529 between -30 and 60min, i.e. 90, and multiplied by 1000). **K:** Correlation between glycemic
530 index and acetaminophen index in lixisenatide-injected mice (Pearson analysis). Data are
531 mean \pm SEM and statistical significance was evaluated for figure J, by t-Student; for glycemia
532 and acetaminophen curves; by Two-Way Anova (followed by post hoc Tukey’s multiple

533 comparisons tests) and for all the rest, by One-Way Anova. Different letters indicate a
534 significant difference between the treatment groups ($p < 0.05$).

535 **Figure 2:** Lixisenatide-induced insulin secretion and gastric emptying inhibition are mediated
536 by the vagus nerve and the GLP-1 receptor. **A-H:** Subdiaphragmatic-vagotomized (SDVx)
537 and sham-operated mice ($n=5/\text{group}$). **A&B:** Plasma insulin concentration 15min after
538 glucose gavage. **C&D:** Plasma insulin concentration 15min after intra-peritoneal glucose
539 injection. **E-H:** Gastric emptying assessment (solution of acetaminophen (100ug/kg) and
540 glucose (2g/kg)) with lixisenatide (10ug/kg) (0ug/kg = saline solution). **E&G:** Plasma
541 acetaminophen concentration at -30, 0, 15 and 30min after gavage of
542 acetaminophen/glucose solution. **F&H:** Acetaminophen index (calculated as follow: sum of
543 acetaminophen concentrations between -30 and 60min divided by the number of minutes
544 between -30 and 60min, i.e. 90, and multiplied by 1000). **I-L:** GLP-1 receptor knock-out
545 (GLP-1r KO) mice and wild-type (WT) mice ($n=6-10/\text{group}$). **I:** Plasma insulin concentration
546 15min after glucose gavage. **J:** Plasma insulin concentration 15min after intra-peritoneal
547 glucose injection. **K-L:** Gastric emptying assessment (solution of acetaminophen (100ug/kg)
548 and glucose (2g/kg)) with lixisenatide (10ug/kg) (0ug/kg = saline solution). **K:** Plasma
549 acetaminophen concentration at -30, 0, 15 and 30min after gavage of
550 acetaminophen/glucose solution. **L:** Acetaminophen index (calculated as follow: sum of
551 acetaminophen concentrations between -30 and 60min divided by the number of minutes
552 between -30 and 60min, i.e. 90, and multiplied by 1000). Data are mean \pm SEM and
553 statistical significance was evaluated for figure A, B and D, by t-Student; for C, E, F, G and
554 H, by Two-Way Anova (followed by post hoc Tukey's multiple comparisons tests). Different
555 letters indicate a significant difference between the treatment groups ($p < 0.05$).

556 **Figure 3:** Lixisenatide recruits the gut-brain axis through the vagus nerve. **A:** Vagus nerve
557 recordings in adult control mice ($n=10/\text{group}$). **A1:** Representative vagus nerve recordings
558 following intraperitoneal administration of saline or lixisenatide (10 $\mu\text{g}/\text{kg}$) and in glucose
559 stimulated state (arrow) (i.e. intraduodenal (ID) injection of glucose). **A2:** an example of an
560 action potential. **A3:** number of events/second following intraperitoneal administration of
561 saline or lixisenatide (10 $\mu\text{g}/\text{kg}$) and in glucose stimulated state. **A4:** Quantification of the
562 firing rate activity over basal activity following intraperitoneal administration of saline or
563 lixisenatide (10 $\mu\text{g}/\text{kg}$) and in glucose stimulated state. **B:** Plasma insulin concentration 15min
564 after intraduodenal injection of glucose under anesthesia. c-Fos staining in adult control mice
565 ($n=5$ mice/group) of the NTS (**C1-2**) and ARC (**D1-2**) following saline (0 $\mu\text{g}/\text{kg}$) or 10 $\mu\text{g}/\text{kg}$ of
566 lixisenatide and the corresponding quantification of the number of c-Fos positive cells in
567 these brain areas. Scale bar is 20micron and the images are taken by 40x bright field. Data
568 are mean \pm SEM and statistical significance was evaluated for figure B, C2 and D2 by t-

569 Student; for A2 and A3, by Two-Way Anova (followed by post hoc Tukey's multiple
570 comparisons tests). Different letters indicate a significant difference between the treatment
571 groups ($p < 0.05$).

572 **Figure 4:** Acute lixisenatide treatment induces a better glucose tolerance through insulin
573 secretion but not gastric emptying in diabetic mouse models. $n=5$ mice/group. **A-C:** Oral
574 glucose tolerance test (2g/kg) with lixisenatide (10 μ g/kg) or without (0 μ g/kg = saline solution)
575 in high-fat fed-mice (60%: 60% of fat; 72%: 72% of fat, high-fat fed-mice). **A:** Glycemia; **B:**
576 Glycemic index (calculated as follow: sum of glycemia between 15 and 120min divided by
577 the number of minutes between 15 and 120min, i.e. 105 and multiplied by 1000); **C:** Plasma
578 insulin concentration 15min after glucose gavage. **D-F:** Oral glucose tolerance test (2g/kg)
579 with lixisenatide (10 μ g/kg) or without (0 μ g/kg = saline solution) in *db/db* mice. **D:** Glycemia;
580 **E:** Glycemic index (calculated as follow: sum of glycemia between 15 and 120min divided by
581 the number of minutes between 15 and 120min, i.e. 105 and multiplied by 1000); **F:** Plasma
582 insulin concentration 15min after glucose gavage. **G-K:** Gastric emptying assessment
583 (solution of acetaminophen (100 μ g/kg) and glucose (2g/kg)) with lixisenatide (10 μ g/kg)
584 (0 μ g/kg = saline solution). Glycemia in high-fat fed-mice (**G**) and in *db/db* mice (**I**); Plasma
585 acetaminophen concentration at -30, 0, 15 and 30min after gavage of
586 acetaminophen/glucose solution in high-fat fed-mice (**H**) and in *db/db* mice (**J**). **K:**
587 Acetaminophen index (calculated as follow: sum of acetaminophen concentrations between -
588 30 and 60min divided by the number of minutes between -30 and 60min, i.e. 90, and
589 multiplied by 1000). Data are mean \pm SEM and statistical significance was evaluated for
590 figure E and F by t-Student; for B, C and K, by Two-Way Anova (followed by post hoc
591 Tukey's multiple comparisons tests). Different letters indicate a significant difference between
592 the treatment groups ($p < 0.05$).

593 **Figure 5:** Lixisenatide effects recruits gut-brain axis in diabetic mouse models. **A:** Vagus
594 nerve recordings in 72% high-fat fed-mice ($n=5$ /group). **A1:** Representative vagus nerve
595 recordings following intraperitoneal administration of saline or lixisenatide (10 μ g/kg) and in
596 glucose stimulated state (arrow) (i.e. intraduodenal (ID) injection of glucose). **A2:** number of
597 events/second following intraperitoneal administration of saline or lixisenatide (10 μ g/kg) and
598 in glucose stimulated state. **A3:** Quantification of the firing rate activity over basal activity
599 following intraperitoneal administration of saline or lixisenatide (10 μ g/kg) and in glucose
600 stimulated state. **B:** Plasma insulin concentration 15min after intraduodenal injection of
601 glucose under anesthesia. c-Fos staining in adult control mice ($n=5$ mice/group) of the NTS
602 (**C1-2**) and ARC (**D1-2**) following saline (0 μ g/kg) or 10 μ g/kg of lixisenatide and the
603 corresponding quantification of the number of c-Fos positive cells in these brain areas. Scale
604 bar is 20micron and the images are taken by 40x bright field. Data are mean \pm SEM and

605 statistical significance was evaluated for figure B by t-Student; for A2, A3, C2 and D2 by
606 Two-Way Anova (followed by post hoc Tukey's multiple comparisons tests). Different letters
607 indicate a significant difference between the treatment groups ($p < 0.05$).

608 **Figure 6:** Lixisenatide chronic treatment has no effects on weight gain and glycemia. **A:**
609 Body weight during chronic lixisenatide treatment (21 days; 10ug/kg/day); n=5mice/group. **B-**
610 **D:** Oral glucose tolerance test (2g/kg) with lixisenatide (10ug/kg) or without (0ug/kg = saline
611 solution) in high-fat fed-mice. **B:** Glycemia; **C:** Glycemic index (calculated as follow: sum of
612 glycemia between 15 and 120min divided by the number of minutes between 15 and 120min,
613 i.e. 105 and multiplied by 1000); **D:** Plasma insulin concentration 15min after glucose
614 gavage. **E-G:** Oral glucose tolerance test (2g/kg) with lixisenatide (10 μ g/kg) or without
615 (0ug/kg = saline solution) in *db/db* mice. **E:** Glycemia; **F:** Glycemic index (calculated as
616 follow: sum of glycemia between 15 and 120min divided by the number of minutes between
617 15 and 120min, i.e. 105 and multiplied by 1000); **G:** Plasma insulin concentration 15min after
618 glucose gavage. **H-J:** Intraperitoneal glucose tolerance test (1g/kg) with lixisenatide
619 (10ug/kg) or without (0ug/kg = saline solution) in high-fat fed-mice. **H:** Glycemia; **I:** Glycemic
620 index; **J:** Plasma insulin concentration 15min after glucose injection. **K-M:** Intraperitoneal
621 glucose tolerance test (1g/kg) with lixisenatide (10 μ g/kg) or without (0ug/kg = saline solution)
622 in *db/db* mice. **G:** Glycemia; **H:** Glycemic index (calculated as follow: sum of glycemia
623 between 15 and 120min divided by the number of minutes between 15 and 120min, i.e. 105
624 and multiplied by 1000); **I:** Plasma insulin concentration 15min after glucose injection. Data
625 are mean \pm SEM. Different letters indicate a significant difference between the treatment
626 groups ($p < 0.05$).

627 **Figure 7:** Lixisenatide chronic treatment has no effect vagus nerve activity. Quantification of
628 the firing rate activity over basal activity following intraperitoneal administration of saline or
629 lixisenatide (10 μ g/kg) and in glucose stimulated state in high-fat fed-mice and *db/db* mice
630 between 0-7min (**A**) and 8-15min (**B**) after intraduodenal injection of glucose (n=3-
631 4mice/group).

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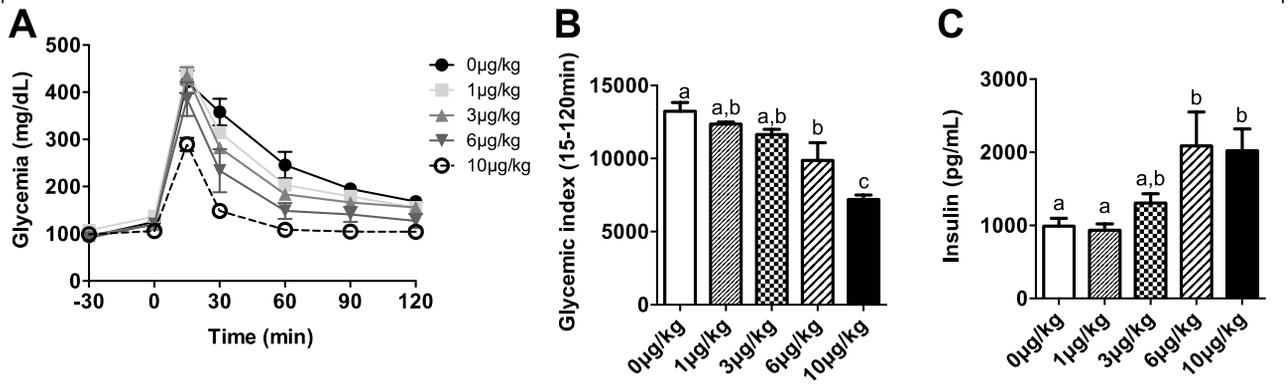
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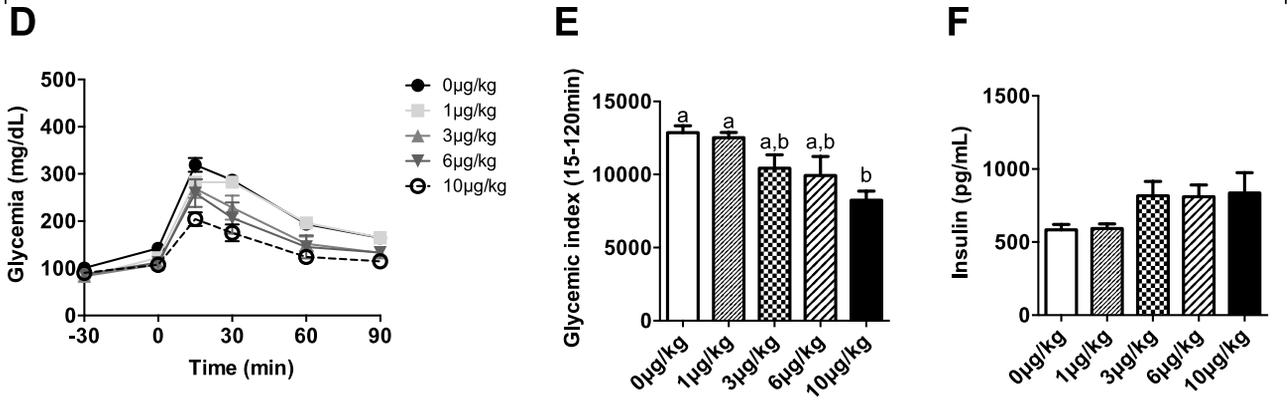
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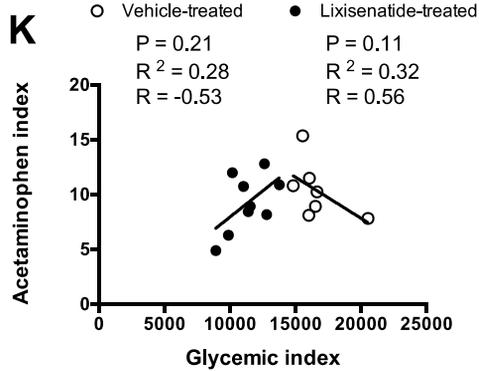
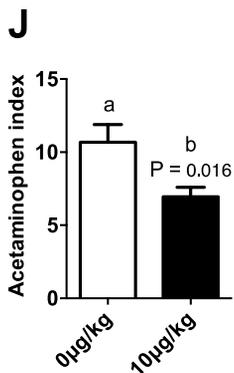
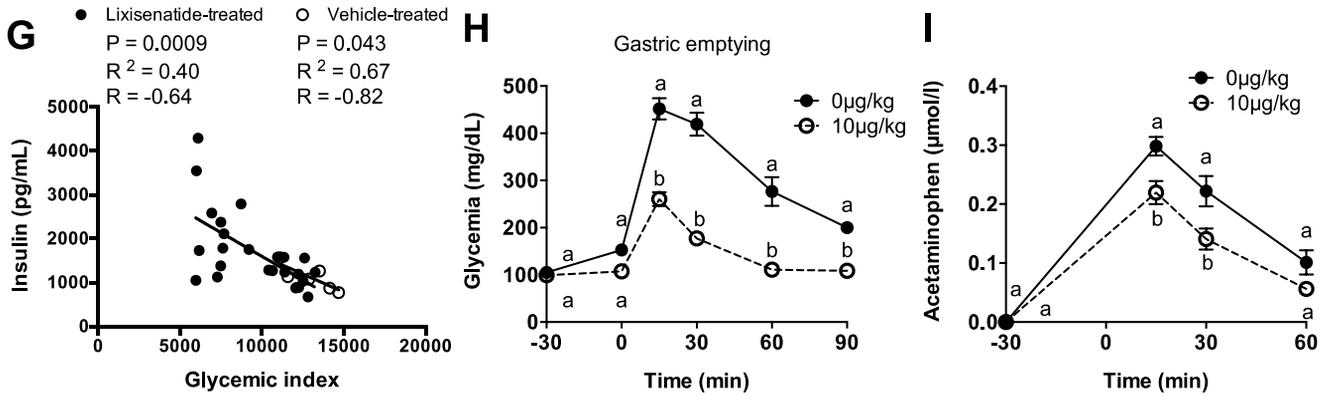
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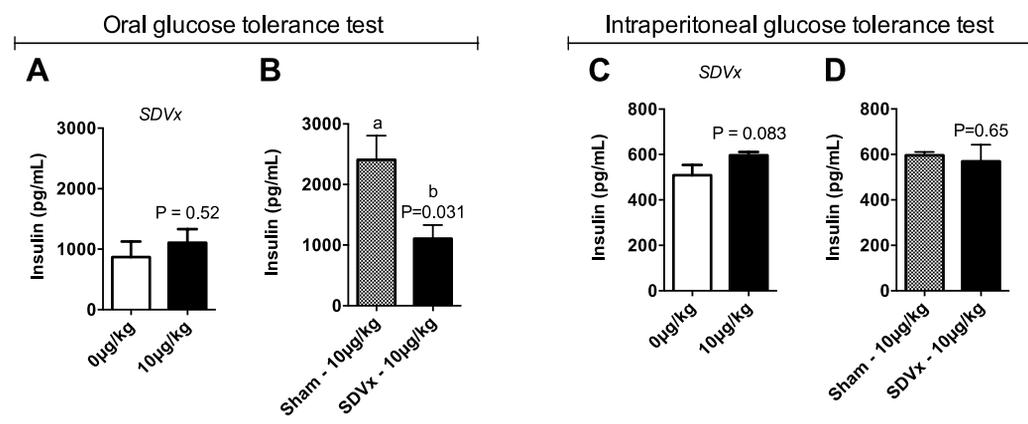


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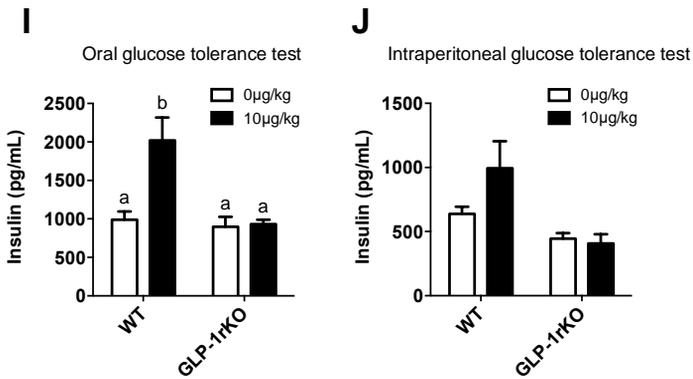
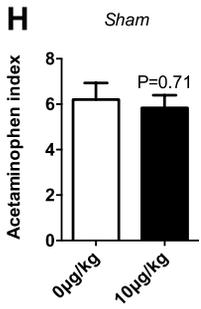
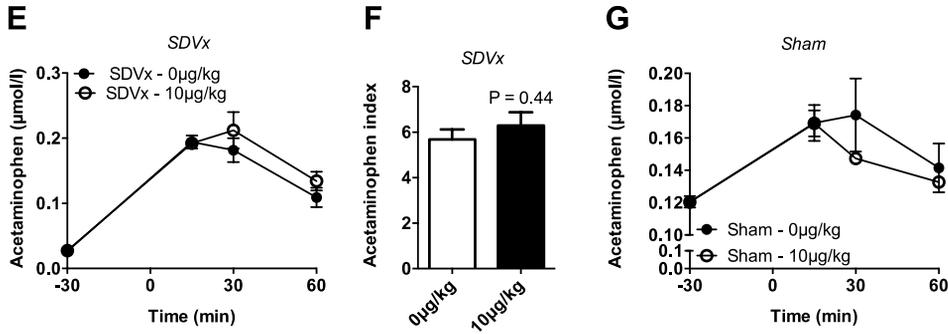


Gastric emptying assessment

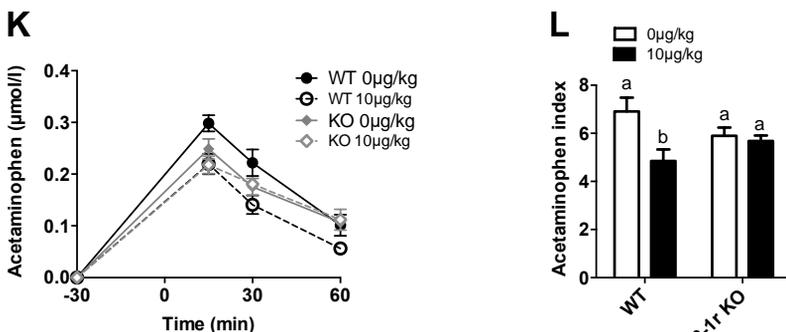


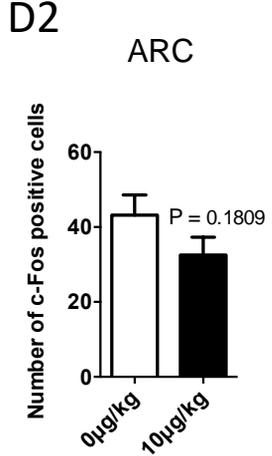
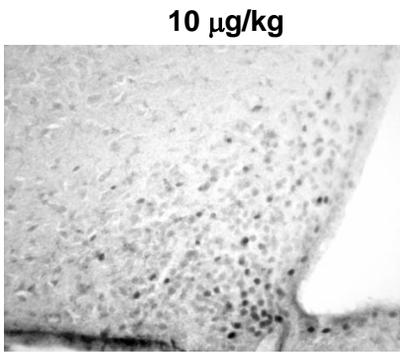
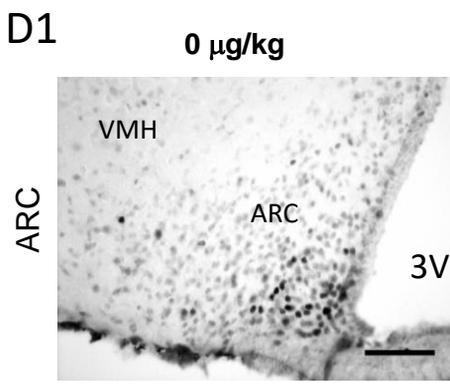
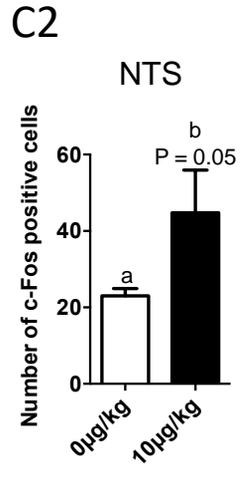
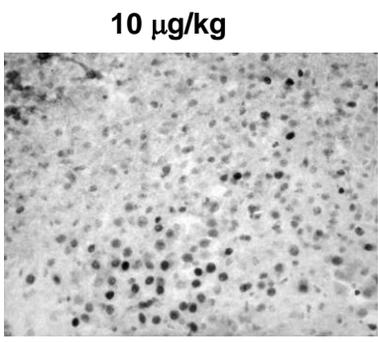
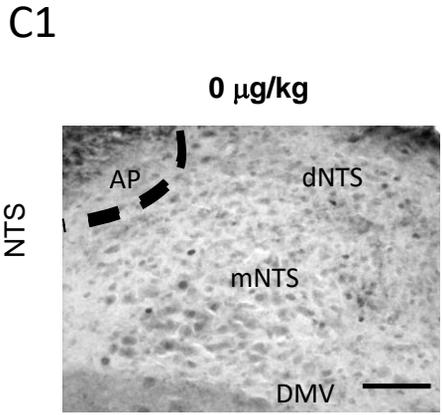
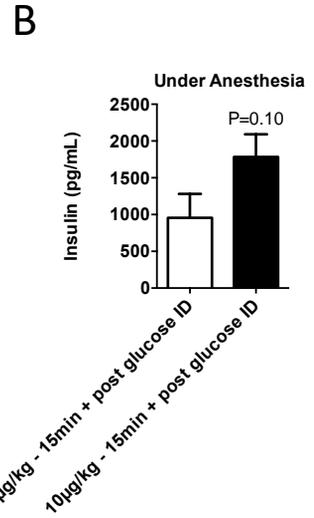
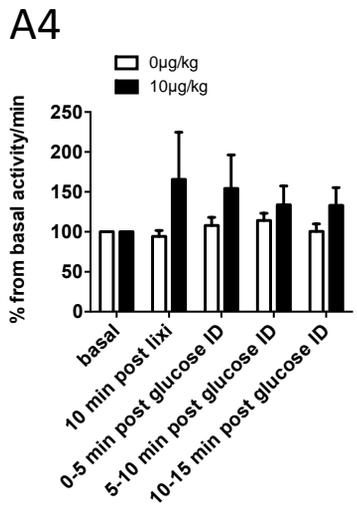
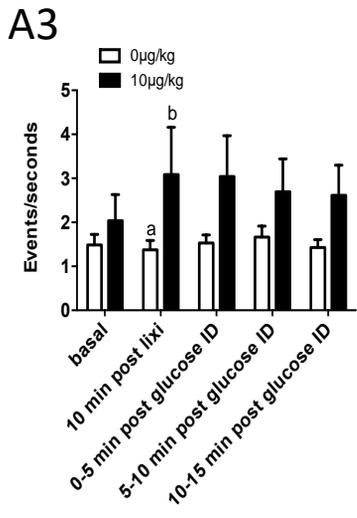
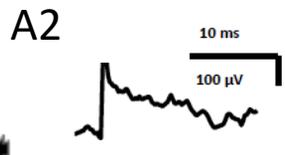
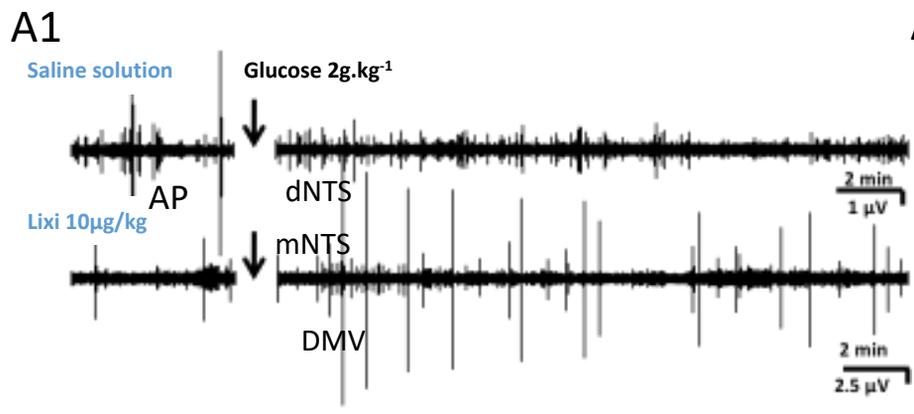


Gastric emptying assessment

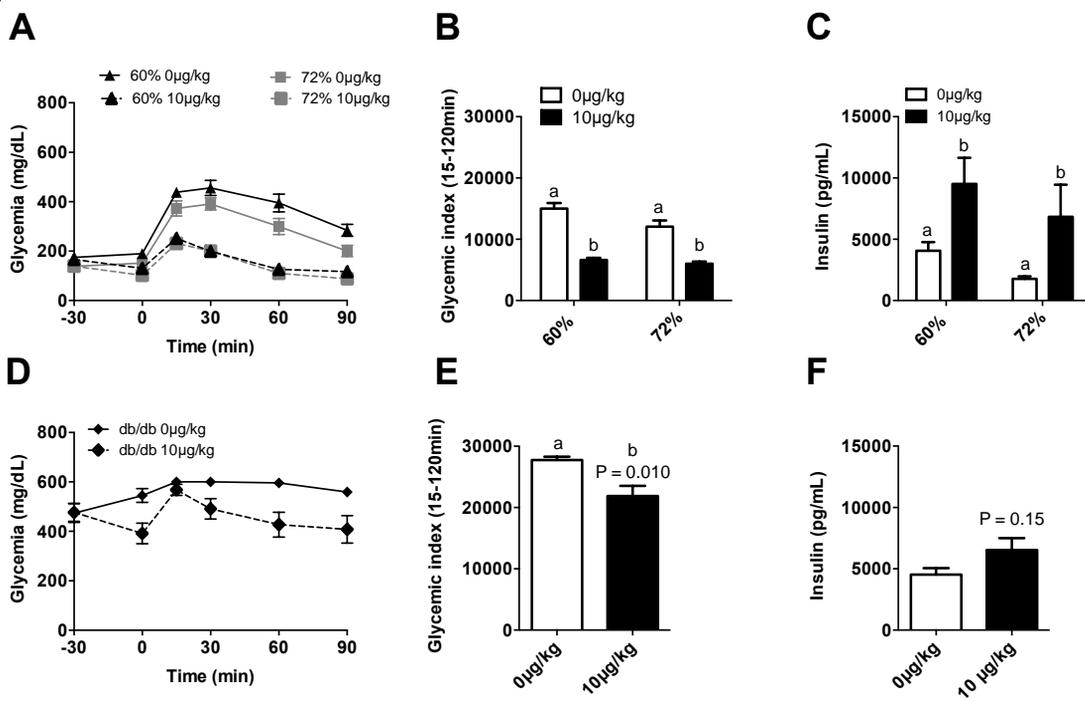


Gastric emptying assessment

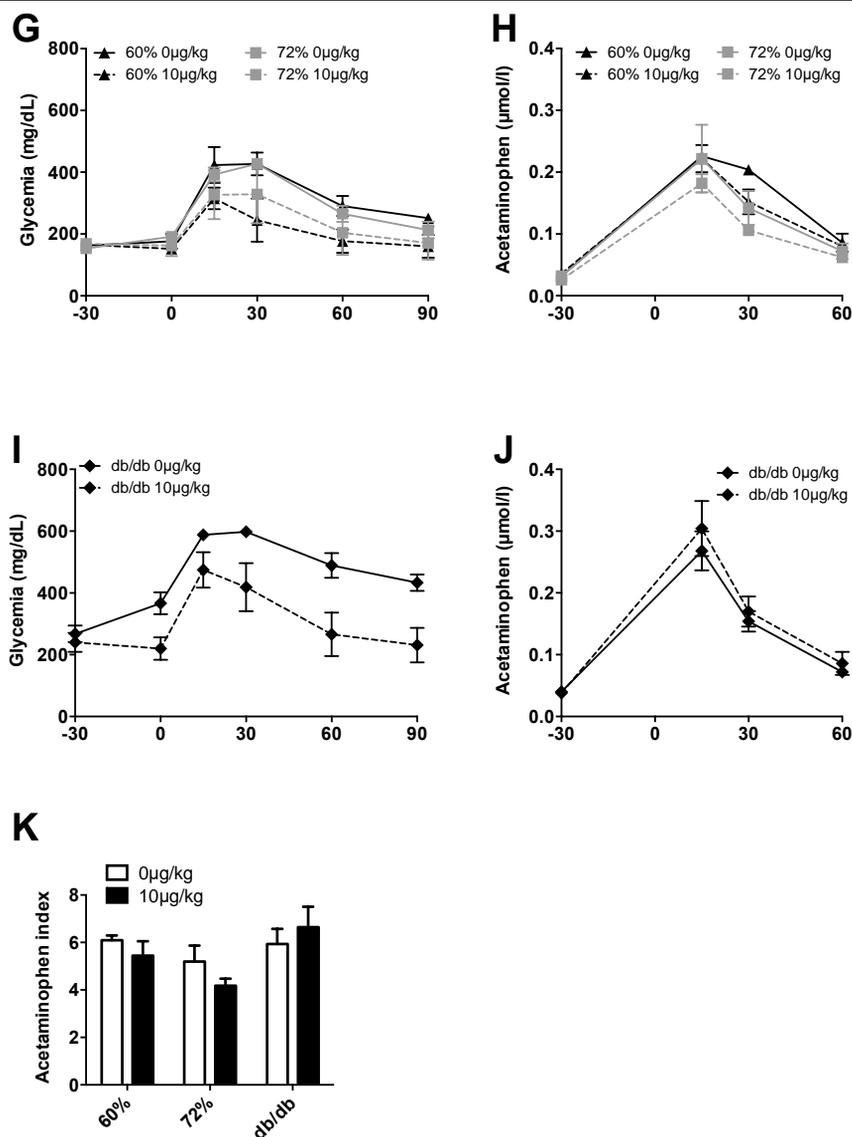


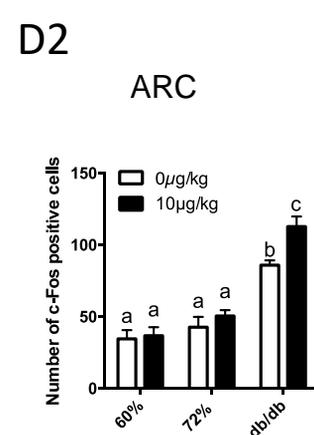
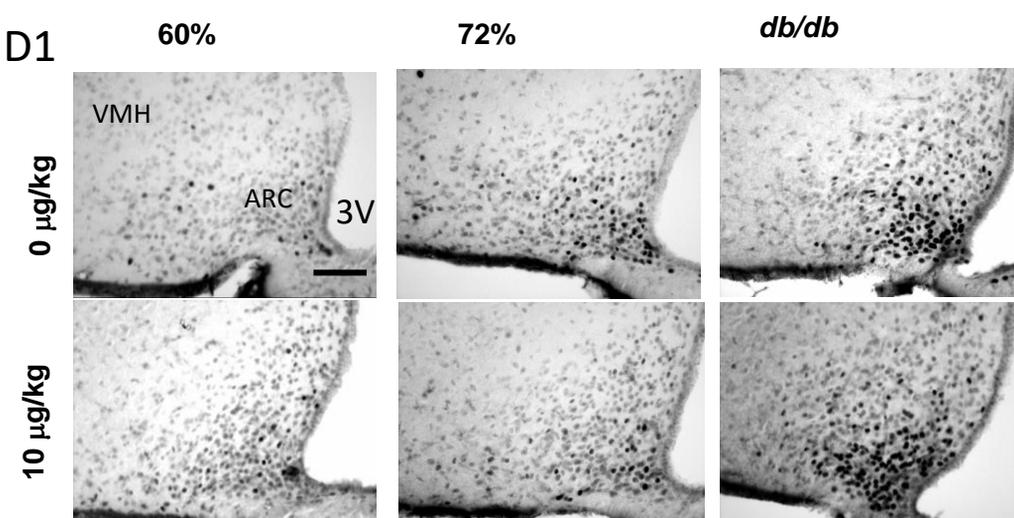
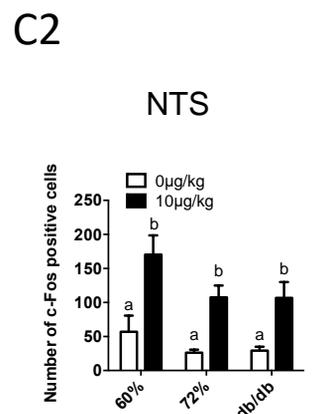
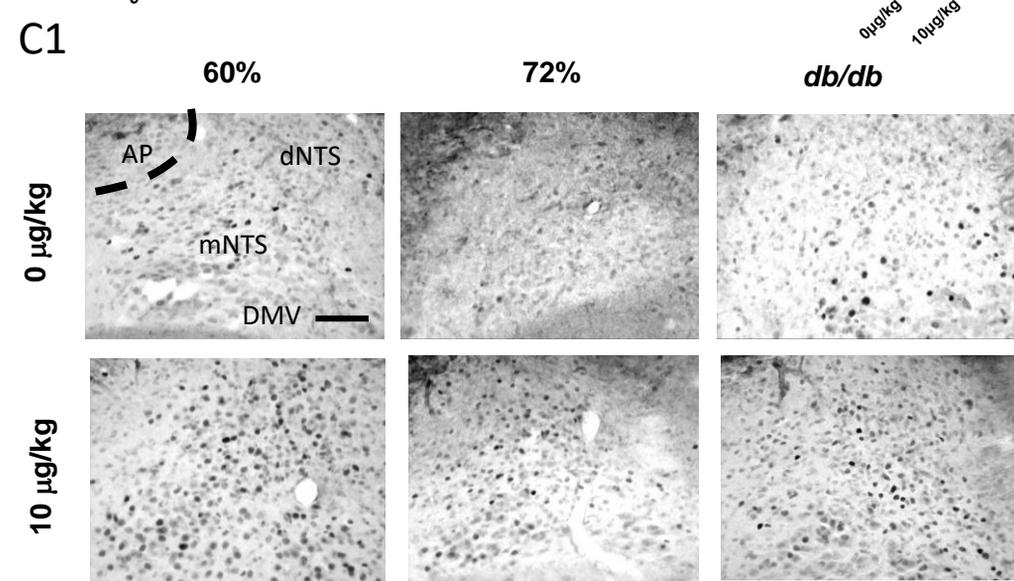
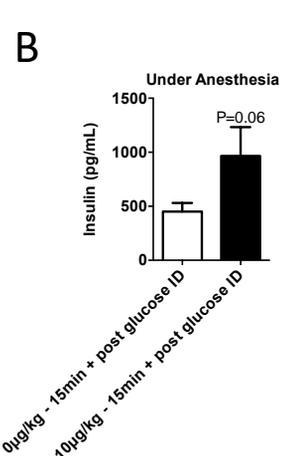
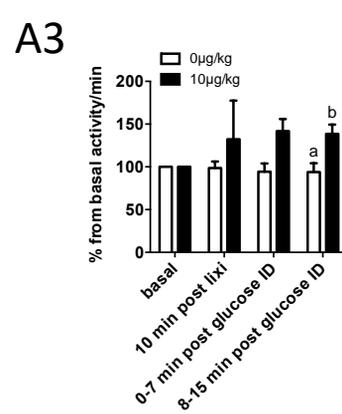
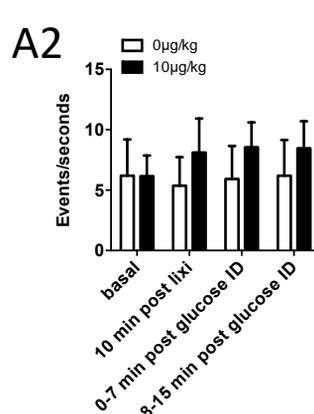
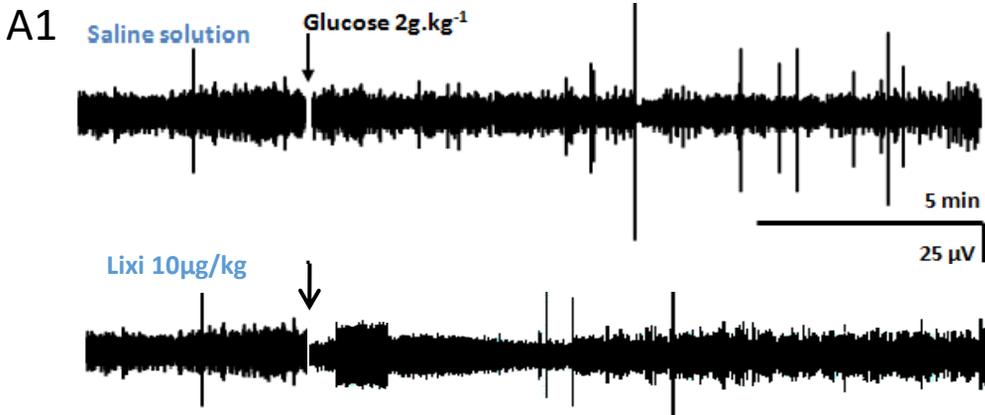


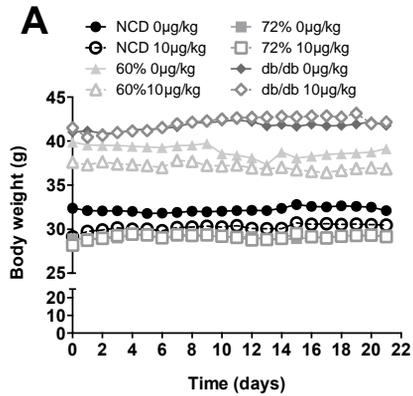
Oral glucose tolerance test



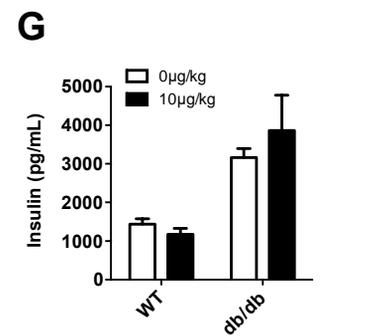
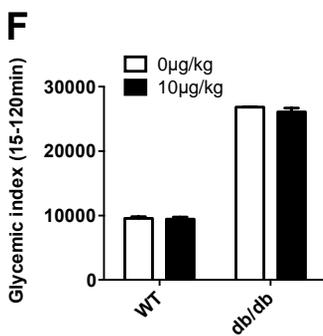
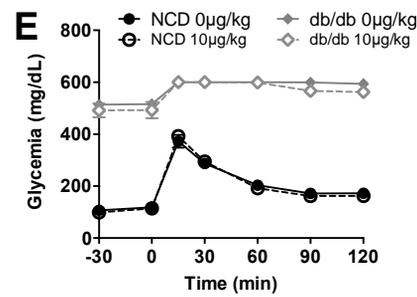
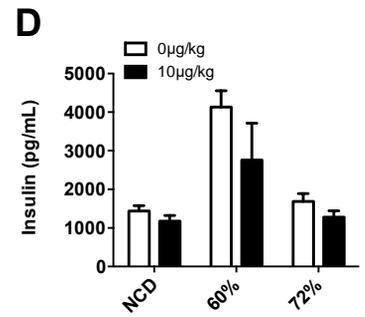
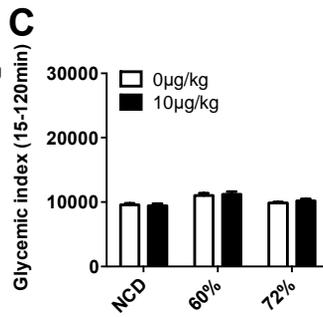
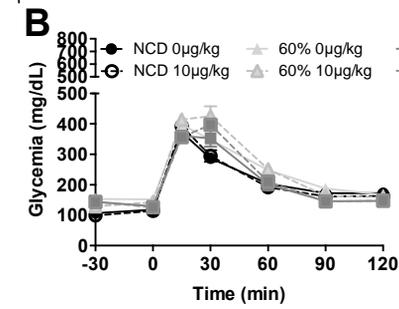
Gastric emptying assessment



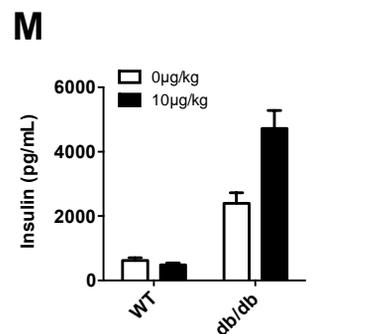
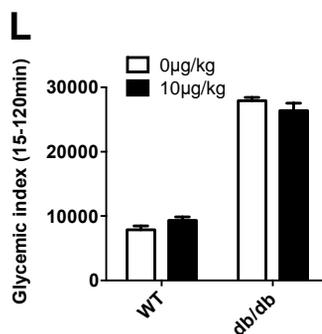
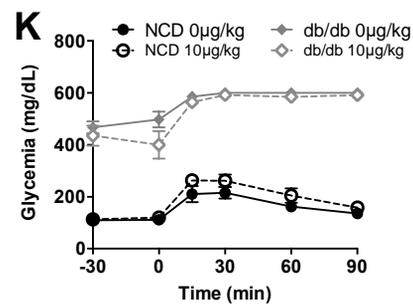
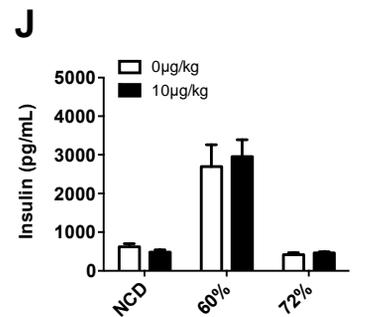
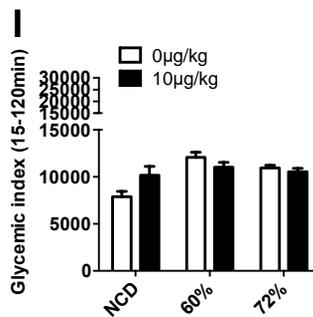
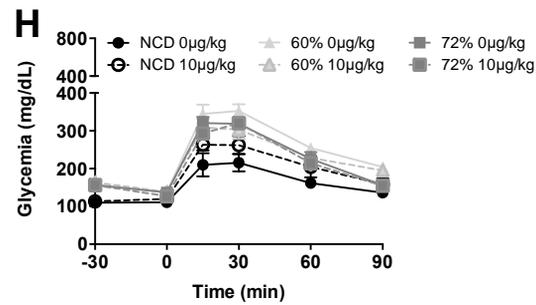


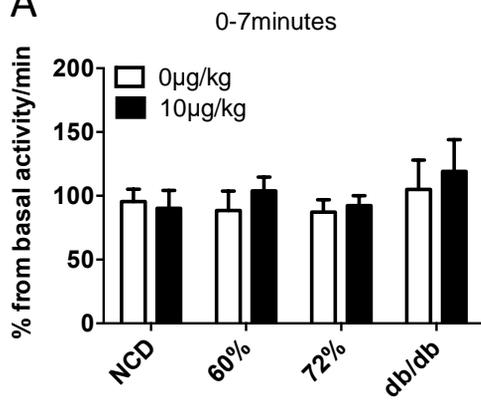


Oral glucose tolerance test



Intraperitoneal glucose tolerance test



A**B**