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J Charpentier, A Waget, P Klopp, C Magnan, C Cruciani-Guglielmacci, et al.. Lixisenatide requires a functional gut-vagus nerve-brain axis to trigger insulin secretion in controls and type 2 diabetic mice. AJP - Gastrointestinal and Liver Physiology, 2018, 315 (5), pp.G671-G684. 10.1152/ajpgi.00348.2017. hal-03105441

HAL Id: hal-03105441 https://cnrs.hal.science/hal-03105441

Submitted on 15 Nov 2022

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Lixisenatide requires a functional gut-vagus nerve-brain axis to 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

- 21 Abstract
- 22

23 <u>Aims/hypothesis</u>: 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.

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

Results: Lixisenatide dose-dependently increased oral glucose-induced insulin secretion that 31 32 is correlated with a decrease of glycemia. In addition, lixisenatide inhibited gastric emptying. These effects of lixisenatide were abolished in vagotomized mice, characterized by a delay 33 of gastric emptying, and in GLP-1 receptor KO mice. I.p. administration of lixisenatide also 34 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 glucose. It increased also insulin secretion and c-Fos activation in the NTS. 38

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

42

43 New & Noteworthy

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

50

52 Introduction

The control of glucose homeostasis involves metabolic, endocrine, and neuro-endocrine 53 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 (23). GLP-1 triggers the intestinal neuronal circuits, such as the enteric nervous system and 56 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 changes in plasma leptin, insulin, glucagon, and nutrient concentrations (34), the brain 59 generates neural signals to regulate peripheral effector tissues such as the Langerhans islets 60 61 to control the insulin and glucagon secretions or the stomach to control gastric emptying (1, 10, 34). In addition to its neuro-endocrine activity, GLP-1 directly binds to its receptor at the 62 surface of pancreatic β cells to enhance glucose-induced insulin secretion (12) and in the 63 brainstem (mainly area postrema) and hypothalamus (arcuate nucleus, ventromedial and 64 paraventricular nuclei) (42), to control food intake and energy expenditure. Importantly, the 65 physiological relevance of the gut-secreted GLP-1 mechanism of action is still matter of 66 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-17-37/36amide into GLP-1_{9-37/36amide}, molecular forms unable to mimic the effects of GLP-1 (13). To circumvent 69 DPP-4 cleavage and ensure an efficient therapeutic approach, DPP-4 resistant GLP-1 70 receptor agonists have been developed. Exendin 4 is a 39 amino-acid peptide found in the 71 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 elevation of glucagon secretion, and slows gastric emptying. Exendin 4 encompasses only 74 50% amino acid homology to GLP-1. Along the same line of pharmacological strategy 75 lixisenatide has been described as "des-38-proline-exendin-4-(1-39)-peptidylpenta-L-lysyl-L-76 77 lysinamide, which is derived from the first 39 amino acids of exendin 4 omitting proline at position 38 and adding six lysine residues (11). All GLP-1 analogs and GLP-1 receptor 78 79 agonists have some common mode of action. Whereas much is known about the mode of action of endogenous GLP-1, a lot remains to be understood regarding the mode of action of 80 81 lixisenatide. Here we studied the acute physiological effects of lixisenatide on glucoseinduced insulin secretion and gastric emptying through the gut-brain axis in naive, 82 chronically-vagatomized normal chow-fed, GLP-1 receptor knock-out (KO), and in diabetic 83 mice (either genetically-modified *db/db* or HFD-fed). We also recorded the vagus nerve firing 84 rate to demonstrate the recruitment of gut-brain axis by lixisenatide. 85

87 Material and methods

88

89 Animals

All experiments were performed with adult males C57BI/6J mice from Charles Rivers (Lyon, 90 France). Animals were housed in the animal facility with inverted 12/12-hours day-light 91 cycles. Experiments studying the effect of lixisenatide on control mice were performed on 12-92 93 week old mice. To study the importance of the GLP-1 receptor, some experiments were performed with GLP-1 receptor knock-out (KO) 12-week old mice, developed in DJ. 94 Drucker's laboratory (43) and bred in our animal facility for two decades. These mice are on 95 C57Bl/6 background and despite of their viability and normal development, they are 96 97 characterized by an alteration of glucose metabolism (hyperglycemia and low insulin secretion after an oral glucose challenge) and an absence of GLP-1-induced satiety (43). To 98 study the impact of diabetes without obesity, 8-weeks old mice were fed for two months with 99 a ketogenic high fat diet 72% (72% corn oil and lard, 28% protein and <1% carbohydrate; 100 SAFE, Augy, France), as described in numerous instances (6, 18, 19, 36). To evaluate the 101 102 impact of lixisenatide on obesity and diabetes, we studied 8-week old mice mice fed a 60% high-fat diet (Research Diets, Inc. New Brunswick, USA) for two months (6, 18, 19, 36). 103 Under such conditions, and in contrast to the mice fed a 72% fat-enriched diet, these mice 104 gained weight and developed hyperglycemia (18, 19). 8-week old db/db mice, where obesity 105 106 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 solution was used as control solution (i.e. 0µg/kg). The animal models were treated acutely 108 chronically (two weeks; 1 injection/day) with lixisenatide (10µg/kg) injected 109 or 110 intraperitoneally. In humans, the half-life of lixisenatide is 2.6 ± 1 hours after a subcutaneous injection of lixisenatide (20µg) (14). No data is avalaible for the half-time in mice. The chronic 111 treatment began 1.5 months after HFD and at 8-weeks old for db/db mice. At the end of 112 experiments, mice were killed by cervical dislocation. All animal experimental procedures 113 114 were approved by the local ethical committee of Rangueil University Hospital (Toulouse) (For all experiments: 12/1048/02/21 except for vagotomy surgery: 12/1048/02/26) 115

116

117 Glucose tolerance tests

To evaluate the impact of lixisenatide on glycemia, oral glucose tolerance test (OGTT) and intraperitoneal glucose tolerance test (IPGTT) glucose tolerance tests were performed in 6 hour-fasted mice. During OGTT, the glucose solution (2g/kg, as a 30% solution) was administrated by intragastric gavage with a gavage needle. During IPGTT, the glucose solution (1g/kg, as a 20% solution) was administrated by intraperitoneal injection with a 27G needle. Lixisenatide (10µg/kg; 100µL) or saline (0µg/kg; 100µL) were injected intraperitoneally 30 min before the glucose administration. Glucose solution was administrated at 0 minute, and the blood glucose was quantified from the tip of the tail vein with a glucose-meter (Accu-Chek Active glucose meter, Roche Diagnostics GmbH, Mannheim, Germany) 30 minutes before or 0, 15, 30, 60, 90 and 120 min after the glucose challenge. Tail blood was sampled 15 minutes after glucose challenge to assess insulin level.

130 Quantification of plasma insulin

Plasma insulin concentration was determined in 10µL using a mouse insulin ELISA kit
(Mercodia, Uppsala, Sweden). The coefficient of variation within the kit is 3.1% and between
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 (100µg/kg; powder diluted in 30% glucose solution) in 6 hour-fasted mice. Lixisenatide 136 (10µg/kg; 100µL) or saline (0µg/kg; 100µL) were injected intraperitoneally 30 min before the 137 acetaminophen administration and the acetaminophen solution was administered by gavage 138 139 at 0 minute. The blood glucose was quantified from the tip of the tail vein with a glucosemeter (Accu-Chek Active glucose meter, Roche Diagnostics GmbH, Mannheim, Germany) 140 141 30 minutes before or 0, 15, 30, 60 and 90 after the acetaminophen gavage. 20µL of tail blood was sampled at -30, 15, 30 and 60 minutes. The plasma concentration of acetaminophen 142 was assessed within 5µL as described by manufacturer (Acetaminophen assay kit, Sekisui 143 144 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 anesthetized with isoflurane 1.5-2% diluted in air, which is a high dose only used for surgery. 148 149 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). The left cervical vagus nerve was then isolated from the carotid artery and throat muscles 151 with a piece of non-adhesive tape, and two covered platinum electrodes (diameter = 0.85152 mm, A-M Systems, Inc., WA, USA) - uncovered at the two ends for 2 at 3 mm - were set 153 directly on the nerve avoiding contact with other tissues and fixed with silicone elastomers 154 155 Kwik-Cast sealant (World Precision Instrument, FL, USA). Then mice were housed in a Faraday cage and electrodes were connected to a high-impedance probe, and action 156

potentials were displayed and saved on a computer after initial amplification through a low-157 noise amplifier (Bio Amp, AD Instruments). Data were digitized PowerLab 4/30 data 158 acquisition system (ADInstruments, Colorado springs, CO, USA). Then, a 3-4 times lower 159 160 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 minutes, the basal vagal activity was recorded on a computer with the LabChart 7.3 software 164 (ADInstruments) and band-pass filtered between 25 and 30Hz to record slow action 165 potentials from vagal afferences (30). However, we cannot discriminate totally the 166 167 measurement of efferent activity through that method. Then, lixisenatide (10µg/kg; 100µL) or saline (0µg/kg; 100µL) was injected intraperitoneally and the basal activity was recorded for 168 20 minutes before the intraduodenal glucose injection and the following 15 minute-recording 169 170 period. Mice were sacrificed at the end of the recording period by cervical dislocation.

171 Vagus nerve activity data analysis

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

179 Subdiaphragmatic vagotomy

To address the role of lixisenatide on the gut-brain axis we vagotomized (without pyloroplasty) 8-week old mice under anesthesia (intraperitoneal injection of Ketamine, 100mg/kg+ Xylazin, 10mg/kg and maintained under isoflurane, 0.5 to 1% of air) and let them recover for three weeks (19). After an upper midline laparotomy (2cm) the vagal trunk was exposed and 1 cm of the ventral vagus nerve and of all neural and connective tissues surrounding the esophagus were cut off. Sham-operated mice underwent the same 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 (0µg/kg) or 10µg/kg i.p. of 190 lixisenatide 30 min before gavage of glucose (2g/kg as a 30% solution). Ninety minutes post

injection all mice were sacrificed by an overdose of sodium pentobarbital (100 mg/kg, i.p.). 191 Immediately following euthanasia, all mice were infused transcardially with first, heparin 192 (25000 U.I./5mL, Sanofi) diluted at 1/500e in phosphate buffer saline (PBS) and, secondly, 193 194 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 solution overnight 4°C. The brains were soaked in citric buffer (pH 4.5) for 6h at room 196 197 temperature and heated in 80mL of citric buffer (microwave oven, 650 W for 1.5 minutes). They were rinsed with PBS at RT and 4°C, and transferred to a 10% sucrose solution 198 199 (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 (16). For NTS, one caudal (bregma-7.83), one medial (bregma-7.47), one rostral (bregma-202 7.31) sections were chosen from each mouse (i.e. 3 non-consecutive sections/animal, n=4-5 203 per group). For medial ARC (bregma-1.69-1.91), 2-4 non-consecutive sections were chosen 204 per animal (n=4-5 per group). Sections of 25µm (Cryostat -20°C) were performed. 205

206 For c-Fos immunohistochemistry, after the brain sectioning, free-floating sections were blocked in a blocking solution (PBS, 0,3% Triton X-100 and 2% donkey serum) and then, 207 incubated with rabbit anti-c-Fos antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, 208 CA) for 48 h at 4 °C. After incubation, the tissue was rinsed in PBS and incubated in 209 biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) in 210 PBS with 0.3% Triton X-100 (PBST) for 1 h. The slides were then washed in PBS and 211 incubated in avidin-biotin solution (vectastain; Vector Laboratories, Burlingame, CA) for 1 h. 212 213 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, Braunschweig, Germany). 216

Stained sections were analyzed using an Olympus AX-70 microscope (Olympus, Center Valley, PA, USA). C-Fos positive cells were counted by an experimenter blind to the treatment, using Volocity 4 software (Ver.4, Improvision, Coventry, UK). The analysis and data are expressed as an average of "number of positive cells per section" from 3 nonconsecutive sections of NTS and 2-4 non-consecutive sections of ARC, for each animal.

The *in vivo* part of c-Fos measurement was realized in the same day for vehicle-injected and lixisenatide-injected group as well as the immunostaining of the brain slices limiting the picture and quantification differences linked to the difference of the time experiments.

225

226 GLP-1 treatments

Lixisenatide in a final volume of 100µL was administered intraperitoneally 30 minutes before 227 glucose gavage or intraduodenal injection of glucose (performed only for vagal firing studies), 228 i.e. at the beginning of the afternoon (nearly 1:00, 2:00 pm). 100µL of saline (0µg/kg) solution 229 230 was used as control injection. Different dose of lixisenatide (1, 3, 6, 10µg/kg) were tested in 231 glucose tolerance tests of adult control mice. The dose of 10µg/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 chronic treatment, lixisenatide was administered intraperitoneally (100µL; 10µg/kg), in the 234 235 morning (at 10:00 am), once a day during two weeks.

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

239

240 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated by Student's t test, One-Way Anova or Two-Way Anova (followed by post hoc Tukey's multiple comparisons tests) and correlations were performed by Pearson analysis

using GraphPad Prism version 6.00 for Mac (GraphPad Software, San Diego, CA, USA).

245 Results

Lixisenatide decreases the glycemia through insulin secretion stimulation and gastricemptying inhibition in adult healthy mice.

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

this effect did not reached statistical significance (Figure 1F). These results suggested that 261 as well as observed for GLP-1 activity, lixisenatide was more efficient to reduce the glycemia 262 if the glucose was injected orally, i.e. when glucose triggers the neural gut-brain axis. To 263 264 evidence an interaction between lixisenatide-stimulated insulin secretion and glycemia, we 265 correlated the glycemic indexes and the insulin concentrations in the lixisenatide and vehicleinjected mice during an oral glucose tolerance test (Figure 1G). We observed a correlation 266 267 between the lixisenatide effects on insulin secretion and on glycemia (P = 0.0009; R2 = 0.40 ; R = -0.64), further supporting our hypothesis. In addition, we also observed a correlation 268 269 between the insulin concentrations and glycemic indexes in vehicle-treated mice (P = 0.043; 270 R2 = 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 lixisenatide (10 µg/kg), was efficient to reduce the glycemia and increase insulin secretion 277 278 after oral glucose load, we used this dose to assess gastric emptying and in the next experiments with other animal models. The data show that lixisenatide strongly reduces the 279 blood concentrations of glycemia and acetaminophen suggesting an effect on the lowering of 280 281 gastric emptying (Figure 1H-J). To evidence an interaction between lixisenatide-induced gastric emptying inhibition and lixisenatide-induced glycemia reduction, we correlated the 282 283 glycemic index and the acetaminophen index in the lixisenatide-injected mice during the gastric emptying assessment (Figure 1K). We observed a trend of correlation between both 284 parameters suggesting an association effect between them (P = 0.11; $R^2 = 0.32$; R = 0.56). 285 286 Interestingly, in the vehicle-treated groupe, the acetaminophen index is not correlated with the glycemic index (P = 0.21; $R^2 = 0.28$; R = -0.53) suggesting that in a steady state, these 287 both parameters are not associated. 288

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

291

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

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

similarly involve this mode of action, we performed subdiaphragmatic vagotomy (SDVx) and 296 quantified the efficiency of lixisenatide to trigger insulin secretion and inhibit gastric emptying. 297 We observed that the peak of acetaminophen was early shifted by 15min in vagatomized 298 299 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 concentration was not increased by lixisenatide after oral glucose challenge when compared 301 302 to vagatomized mice injected with saline solution (Figure 2A) or sham-operated mice injected with lixisenatide (Figure 2B). After an intraperitoneal glucose challenge, 303 vagotomized mice released the same amount of insulin after injections of saline or 304 305 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 control of insulin secretion after glucose gavage in response to lixisenatide. On the other 307 hand, vagotomy also dramatically blunted the impact of lixisenatide on gastric emptying 308 309 when compared to sham-operated mice particularly at the time-point 30 min (Figure 2E-H).

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

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

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

Then, we recorded the firing rate activity of the vagus nerve in mice anesthetized with a low 318 319 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 adult mice and the secondary intra-duodenal injection of glucose did not further potentiate 324 the firing rate of the vagus nerve. We confirmed that our set up was functional since 325 326 lixisenatide-induced vagus nerve activation was indeed associated with an increase of insulin secretion by nearly two-fold (954 \pm 324.4 pg/mL versus 1781 \pm 309.6pg/mL for 0 µg/kg and 327 10 µg/kg, respectively) (Figure 3B) as observed during the oral glucose tolerance test 328 329 (Figure 1C).

A functional evidence for the involvement of the gut-brain axis is the induction of c-Fos 330 expression in cells of the nucleus of tractus solitarius (NTS). Peripheral GLP-1 agonist 331 treatment can also activate the arcuate nucleus of the hypothalamus (ARC) (4). Therefore, 332 333 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).

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

Acute lixisenatide treatment improves glycemia through insulin secretion and gastricemptying in diabetic mouse models.

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

In both fat-enriched diet-fed mouse models, lixisenatide reduced glycemia (Figure 4A,B) and 349 increased insulin secretion (Figure 4C). In db/db mice, lixisenatide caused a significant 350 351 reduction in glucose profiles (27730 \pm 589.1pg/mL versus 21875 \pm 1651pg/mL for 0 μ g/kg and 10 µg/kg respectively) (Figure 4D,E), but had no significant effect on insulin secretion 352 (4528 \pm 527.7 pg/mL versus 6537 \pm 958.5 pg/mL for 0 μ g/kg and 10 μ g/kg, respectively) 353 (Figure 4F). It is noteworthy that these mice were quite hyperinsulinemic which could have 354 hampered a further enhancement of insulin secretion. On the other hand, lixisenatide tended 355 356 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).

Altogether, these data show that the glycemic profiles of type 2 diabetic mice can be controlled by lixisenatide. 363 Lixisenatide effects recruits gut-brain axis in diabetic mouse models.

To evaluate whether lixisenatide can recruit the gut-brain axis and control glycemia and 364 insulin secretion in type 2 diabetic mice, we measured the vagus nerve activity and quantified 365 366 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 contrast to what we observed previously in healthy mice (Figure 3A1-3). However, when 368 369 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 a two-fold increase of insulin concentration by two-fold (451 \pm 79.97pg/mL versus 965 \pm 371 267.4 pg/mL for 0 µg/kg and 10 µg/kg, respectively) (Figure 5B). On the other hand, we 372 observed an increase of the number of c-Fos positive cells in the NTS (Figure 5C1.2) in all 373 374 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.

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

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387 Discussion

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

acting GLP-1 receptor agonists such as exendin 4, lixisenatide and liraglutide for the 424 treatment of type 2 diabetes. It is commonly accepted that GLP-1 receptor agonists activate 425 GLP-1 receptor at the surface of 1) the insulin-secreting β cell to trigger insulin secretion and 426 regulate glycemia (29) and 2) the brain areas outside the blood-brain barrier such as area 427 428 postrema (2, 4, 33, 42) or cross the blood-brain-barrier and act directly on hypothalamic neurons to regulate the food intake and decrease body weight (4, 44). Moreover, a 429 recruitment of the gut-brain axis is also possible since the GLP-1 receptor is expressed in the 430 neurons of the vagus nerve localized in the nodose ganglion and innervating intestine 431 432 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 glucose-induced insulin secretion and gastric emptying (31). Therefore, we challenged this 434 dogma and demonstrated unequivocally that vagotomy totally blunted lixisenatide induced-435 insulin secretion and gastric emptying inhibition. Previous publications described that in 436 humans and rodents, vagotomy alone altered gastric emptying (39, 40) suggesting that the 437 vagus nerve integrity is critical for the stomach activity. On the other hand, it was shown that 438 439 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. Interstingly, stydying vagatomized mice allowed us to highlight a new endocrine role of GLP-1 on insulin sensitivity (27). The 443 neuroincretin effect is further supported by our data since lixisenatide efficiently triggers 444 insulin secretion during oral glucose administration but less efficiently following an 445 intraperitoneal glucose administration. Altogether, in healthy mice, lixisenatide requires a 446 functional gut-vagus nerve to brain axis for the control of glucose-induced insulin secretion 447 and gastric emptying lowering. Furthermore, our data show that lixisenatide increases the 448 449 NTS activity. These results suggest that lixisenatide, through its impact on vagal nerve, would increase the NTS activity therefore, regulating gastric emptying separately from the 450 glucose lowering effect of the molecule. However, lixisenatide did not modify the number of 451 c-fos positive cells in the ARC suggesting that no signal was transmitted from the NTS to the 452 453 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 activation of the NTS by exendin 4 was impaired in vagotomized mice (4) and GLP-1 456 457 receptor KO mice (2) suggesting that vagus nerve and GLP-1 receptor are important for brain area activation induced by exendin 4-derivated molecules. In healthy mice, the firing 458 459 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

the GLP-1 receptor has a basal activity which is sufficient to maintain high concentration of 461 cAMP ensuring a significant basal insulin secretion rate (45). Furthermore, we previously 462 described that in the portal vein the molecular mechanisms of glucose sensing i.e. the 463 464 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, in case of type 2 diabetes, we and others recently showed that high doses of GLP-1 are 468 required to control glycemia in diabetic mouse models and in diabetic patients (19, 50). 469 470 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 diabetic mice. This is in agreement with the increased brainstem c-Fos labeling suggesting a 474 strong activation of the gut brain axis by lixisenatide. Furthermore, the lack of c-Fos labeling 475 in the ARC suggests that lixisenatide does not regulate glycemia, food intake or body weight 476 477 via a direct activation of ARC neurons.

Our data also show that, although lixisenatide efficiently activates the gut-brain axis 478 and regulates insulin secretion in high-fat diet-fed diabetic mice, it only modestly inhibits 479 480 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 the other hand, lixisenatide was unable to trigger insulin secretion and gastric emptying 482 483 inhibition in *db/db* mice since these mice were already dramatically hyperinsulinemic and had a very strong gastroparesis and gastric dysmotility (26). Additionally, a higher sensitivity to 484 glucose and lixisenatide in the ARC were observed in *db/db* mice. These results may be 485 explained by the fact that *db/db* mice have an alteration of the blood-brain-barrier permitting 486 some brain area to have a greater access to the glucose and circulating lixisenatide (17). 487 488 Although lixisenatide might cross the blood-brain-barrier in these genetically-modified mice, we did not observe a decrease of weight with a chronic treatment. These results can be 489 490 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

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

501

502 Acknowledgement

Inserm received funds from Sanofi through an Investigator-Sponsored pre-clincal agreement "Role of lixisenatide on the regulation of the gut to brain neural axis for the control of glucose induced-insulin secretion and gastric emptying. A mechanistic preclinical study". We would like to thank Daniel J. Drucker for his kind gift of GLP-1 receptor KO mice, the technical expertise from Y. Barreira and her team from the Animal Care Facility of Rangueil Hospital (UMS US006/Inserm).

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 inhibition in adult control mice. n=5-10/group. A-C: Oral glucose tolerance test (2g/kg) with 515 different concentrations of lixisenatide (1, 3, 6, 10ug/kg, diluted in saline solution; 0ug/kg = 516 saline solution). A: Glycemia; B: Glycemic index (calculated as follow: sum of glycemia 517 between 15 and 120min divided by the number of minutes between 15 and 120min, i.e. 105 518 and multiplied by 1000); C: Plasma insulin concentration 15min after glucose gavage. D-F: 519 520 Intraperitoneal glucose tolerance test (1g/kg) with different concentrations of lixisenatide (1, 3, 6, 10ug/kg, diluted in saline solution; 0ug/kg = saline solution). D: Glycemia; E: Glycemic 521 index; F: Plasma insulin concentration 15min after glucose injection. G-I: Gastric emptying 522 assessement (solution of acetaminophen (100ug/kg) and glucose (2g/kg)) with lixisenatide 523 (10ug/kg) (0ug/kg = saline solution). **G**: Correlation between glycemic index and insulin level 524 525 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 acetaminophen/glucose solution. J: Acetaminophen index (calculated as follow: sum of 527 acetaminophen concentrations between -30 and 60min divided by the number of minutes 528 529 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 mean ± SEM and statistical significance was evaluated for figure J, by t-Student; for glycemia 531 532 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).

Figure 2: Lixisenatide-induced insulin secretion and gastric emptying inhibition are mediated 535 by the vagus nerve and the GLP-1 receptor. A-H: Subdiaphragmatic-vagotomized (SDVx) 536 and sham-operated mice (n=5/group). A&B: Plasma insulin concentration 15min after 537 glucose gavage. C&D: Plasma insulin concentration 15min after intra-peritoneal glucose 538 539 injection. E-H: Gastric emptying assessment (solution of acetaminophen (100ug/kg) and glucose (2g/kg)) with lixisenatide (10ug/kg) (0ug/kg = saline solution). E&G: Plasma 540 15 and 541 acetaminophen concentration at -30, 0, 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/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) and glucose (2g/kg) with lixisenatide (10ug/kg) (0ug/kg = saline solution). K: Plasma 548 549 acetaminophen concentration at -30, 0, 15 and 30min after of gavage 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 between -30 and 60min, i.e. 90, and multiplied by 1000). Data are mean ± SEM and 552 statistical significance was evaluated for figure A, B and D, by t-Student; for C, E, F, G and 553 H, by Two-Way Anova (followed by post hoc Tukey's multiple comparisons tests). Different 554 letters indicate a significant difference between the treatment groups (p < 0.05). 555

Figure 3: Lixisenatide recruits the gut-brain axis through the vagus nerve. A: Vagus nerve 556 recordings in adult control mice (n=10/group). A1: Representative vagus nerve recordings 557 following intraperitoneal administration of saline or lixisenatide (10µg/kg) and in glucose 558 stimulated state (arrow) (i.e. intraduodenal (ID) injection of glucose). A2: an example of an 559 action potential. A3: number of events/second following intraperitoneal administration of 560 saline or lixisenatide (10µg/kg) and in glucose stimulated state. A4: Quantification of the 561 562 firing rate activity over basal activity following intraperitoneal administration of saline or lixisenatide (10µg/kg) and in glucose stimulated state. B: Plasma insulin concentration 15min 563 after intraduodenal injection of glucose under anesthesia. c-Fos staining in adult control mice 564 565 (n=5 mice/group) of the NTS (C1-2) and ARC (D1-2) following saline (0µg/kg) or 10µg/kg of lixisenatide and the corresponding quantification of the number of c-Fos positive cells in 566 these brain areas. Scale bar is 20micron and the images are taken by 40x bright field. Data 567 568 are mean ± SEM and statistical significance was evaluated for figure B, C2 and D2 by t569 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).

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

593 Figure 5: Lixisenatide effects recruits gut-brain axis in diabetic mouse models. A: Vagus nerve recordings in 72% high-fat fed-mice (n=5/group). A1: Representative vagus nerve 594 recordings following intraperitoneal administration of saline or lixisenatide (10µg/kg) and in 595 glucose stimulated state (arrow) (i.e. intraduodenal (ID) injection of glucose). A2: number of 596 events/second following intraperitoneal administration of saline or lixisenatide (10µg/kg) and 597 598 in glucose stimulated state. A3: Quantification of the firing rate activity over basal activity following intraperitoneal administration of saline or lixisenatide (10µg/kg) and in glucose 599 stimulated state. B: Plasma insulin concentration 15min after intraduodenal injection of 600 601 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 (0µg/kg) or 10µg/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 ± SEM and statistical significance was evaluated for figure B by t-Student; for A2, A3, C2 and D2 by
Two-Way Anova (followed by post hoc Tukey's multiple comparisons tests). Different letters
indicate a significant difference between the treatment groups (p <0.05).

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

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

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