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Research Article

Absence of the Peptide transporter 1 induces a prediabetic and depressive-like phenotype in mice

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Short Title: Pept1^{-/-} mice, a prediabetes and depressive-like phenotype model

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Abstract

Introduction: Protein-enriched diets improve glycemic control in diabetes or emotional behavior in depressive patients. In mice, these benefits depend on intestinal gluconeogenesis activation by di/tripeptides. Intestinal di/tripeptides absorption is carried out by the Peptide transporter 1, PEPT1. The lack of PEPT1 might thus alter glucose and emotional balance.

Methods: To determine the effects of PEPT1 deficiency under standard dietary conditions or during a dietary challenge known to promote both metabolic and cognitive dysfunction, insulin sensitivity, anxiety and depressive-like traits, hippocampal serotonin (5-HT) and insulin signaling pathway were measured in wild-type (WT) and *Pept1*^{-/-} mice fed either a chow or a high- fat high-sucrose (HF-HS) diet.

Results: *Pept1*^{-/-} mice exhibited slight defects in insulin sensitivity and emotional behavior, which were aggravated by a HF-HS diet. *Pept1*^{-/-} mice fed a chow diet had lower hippocampal 5-HT levels and exhibited cerebral insulin resistance under HF-HS diet. These defects were independent of intestinal gluconeogenesis but might be linked to increased plasma amino acids levels.

Conclusion: *Pept1*^{-/-} mice develop prediabetic and depressive-like traits and could thus be used to develop strategies to prevent or cure both diseases.

Introduction

Those last two decades have seen type 2 diabetes to emerge as a dramatic epidemic concerning more than 10% of the worldwide population. This disease is defined by high blood glucose along with a loss of peripheral insulin sensitivity and a default of insulin production by pancreatic β islets. The complications of diabetes are what make this pathology so threatening to public health: blindness, kidney disease, infection, neurological disease and cardiovascular diseases including an increased risk of stroke and inferior limb amputation [1]. In parallel, major depression is the most widespread psychiatric disorder [2]. Its prevalence during a lifetime is 16% in men and 25% in women and has an important social, economic and sanitary impact [3]. According to the DSM-V (Diagnostic and Statistical Manual of Mental Disorders), depression is diagnosed by the appearance of at least 5 symptoms in the same period, including at least anhedonia and depressed mood. Other potential symptoms include changes in weight and appetite, chronic fatigue, slowed thinking and reduced physical movement, feelings of worthlessness and excessive guilt, or recurrent suicidal ideation.

At first sight, diabetes and depression do not have much in common as diabetes is a metabolic disease and depression a psychological one, but they both share epidemic characteristics and are a matter of public health. Beyond these epidemiological similarities, several studies have highlighted a bidirectional link between diabetes and depression [4]. Indeed, depression is a risk factor for the onset of obesity and diabetes since depressed subjects have a 60% increased risk of developing type 2 diabetes. Conversely, diabetic patients have a twofold increase odds of depression [5,6]. This bidirectional link is particularly dramatic, since diabetic patients suffering from depression have a bad treatment observance and it is the same for hygienic-dietary recommendations [7]. Therefore, they have increased risks of complications [8].

Several biological mechanisms have been associated with these two diseases and proposed as a causal link explaining the bidirectional relationship. Firstly, depression and diabetes are closely linked to the functioning of the hypothalamus, a cerebral region involved in the regulation of many physiological functions, including energy homeostasis and stress management. One of the mechanisms described involves the elevation of hypothalamus-pituitary-adrenal (HPA) axis activity, which causes a chronic increase in cortisol plasma levels, possibly contributing to the development of diabetic features [9]. Activation of the HPA axis is accompanied by stimulation of catecholamine secretion and activation of pro-inflammatory cytokines such as IL-6 [9]. So, like obesity and diabetes, depression also causes low-grade inflammation, and would be a common mechanism for these two pathologies [10]. More recently, defects in insulin sensitivity have been proposed as a key mechanistic link between both depression and diabetes. Indeed, several preclinical studies performed either in diet-induced insulin resistance (i.e. hypercaloric diet) [11–13] or in mouse models lacking insulin receptor [14] highlight the

crucial role of brain insulin signaling for the regulation of emotional behaviors. Interestingly, diabetes treatments, such as insulin [15] or anti-diabetic drugs such as metformin [16] improve also anxiety and depressive-like behaviors.

Changes in dietary habits, combined with increased physical activity, have been proposed as the first line of treatment for diabetic patients in order to improve their glycemic parameters for many years [17], while the study of the impact of diet on mental health is an emerging field of psychiatry [18]. More specifically, several studies show that specific diets, particularly protein-enriched diets, improve type 2 diabetes, anxiety and depressive disorders. Firstly, dietary protein intake promotes satiety and therefore reduces body weight gain in animals and humans [19–21]. Protein improves glucose homeostasis by improving glucose tolerance (e.g. evaluated from glycated hemoglobin) in the short term [22–24], and insulin sensitivity in the long term, in patients suffering from type 2 diabetes [25]. Secondly, low-calorie diets with a high-protein percentage (30% more protein) can significantly improve psychometric parameters, as depression, stress and anxiety, in obese people [26]. Although it is difficult to distinguish the effects of reduced caloric intake from protein supplementation in the latter study, increased consumption of protein-enriched foods has been associated with benefits for depressed mood in a few studies [27,28].

Proteins from food are digested in small peptides and amino acids that are then transferred across the brush-border membrane of the absorptive enterocytes. Our laboratory demonstrated that protein-enriched diets exert their beneficial effects on energy homeostasis and emotional behavior through the induction of intestinal gluconeogenesis [29,30]. The latter is a biological function inducing a gut-brain signal targeting the hypothalamus to promote anti-diabetic, anti-obesity and anti-anxiety effects [31,32]. Interestingly, the capacity of protein-enriched diets to induce the production of glucose by the intestine is mediated by the detection of di/tri peptides in the portal vein [33]. While several transporters ensure the uptake of free amino acids across the brush membrane, di- and tripeptides are selectively transported into enterocytes by the high capacity A1 peptide transporter, PEPT1, a member of the SLC15 family [34,35]. More than 400 dipeptides and 8,000 tripeptides can be transported by this low affinity transporter [36]. Due to its broad substrate specificity, PEPT1 recognizes a variety of peptide-like drugs with a structure similar to small peptides and has therefore been used as a target to improve intestinal absorption of poorly absorbed drugs [37]. This transporter is highly expressed in the small intestine (duodenum, jejunum, and ileum) and in certain situations in the distal part of the colon [38]. Indeed, an increase in PEPT1 expression in the colon is notably involved in the pathogenesis of inflammatory bowel disease [39]. Intestinal PEPT1 expression and function may be regulated by various nutritional, hormonal and inflammatory parameters [37,40,41] contributing to maintain and adapt intestinal functions to the different environment [42]. For example, in the proximal

part of the intestine, PEPT1 participates in the regulation of glucose tolerance mediated by the proteins, by controlling the release of gut peptides, including Glucagon Peptide-Like 1 (GLP-1) [43].

Interestingly, PEPT1 expression levels are reduced in pathophysiological conditions such as diabetes and obesity but are increased by both dietary protein and fasting, two conditions also responsible for activation of intestinal gluconeogenesis [33,44–48]. Therefore, we hypothesized that PEPT1 may be a key player in linking the beneficial effects of dietary protein and intestinal gluconeogenesis on both metabolism and mood. To evaluate the role of PEPT1 in the regulation of glucose homeostasis and emotional behavior *per se*, we here studied whether mice deleted for *Pept1* develop metabolic and emotional disorders characteristic of diabetes and anxio-depressive states. Since both diseases are favored by a high-fat, high-sucrose diet [13], we also performed the same study under a nutritional challenge.

Methods

Animals

We used male adult *Pept1*^{-/-} [49] and wild-type (WT) (C57Bl/6J; Charles River Laboratories, L'Arbresle, France) mice in the present study. *Pept1*^{-/-} mice were obtained from Deltagen (San Mateo, CA). Briefly, a mutation of the *Slc15a1n* gene was performed to obtain the deletion of amino acids 16 to 24 on the PEPT1 protein, thereby rendering the protein inactive [49].

All mice were housed in groups in the animal facility of Lyon 1 University (Animaleries Lyon Est Conventiionnelle and Specific Pathogen Free) in controlled temperature (22°C) conditions, with a 12-h light-12-h dark cycle. Mice had free access to water and standard rodent chow diet. For specific experiments, 10-week-old mice were fed a protein-enriched diet during 15 days [50] or a high-fat high-sucrose diet (HF-HS, 36% fat and 36% sucrose, UE SAAJ INRAE, Jouy-en-Josas, France) during 11 weeks [51]. The first panel of each figure summarizes the experiments performed on each group of animals. All the procedures were performed in accordance with the ARRIVES principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Diet composition

Standard rodent chow diet (SAFE A04, Augis, France) is composed of 3.1% fat, 60.4% carbohydrates (including 43.5% starch content) and 16.1% proteins. Protein-enriched diet (Safe U8958P Version 0121) is composed of 7.5% fat, 19.5% carbohydrates (including 8.7% starch content) and 51.5% proteins.

HF-HS diet is composed of 36.1% fat, 35% carbohydrates (50% maltodextrine + 50% sucrose) and 19.8% proteins and was produced by the Unité de Préparation des Aliments Expérimentaux (INRA, UE0300, Jouy-en-Josas, France).

Tissue Sampling, Biochemical Assays, and Protein Analyses

Blood or tissue sampling was performed on mice that were not tested in behavioral procedures to avoid the possible effect of stressful procedures on protein expression levels. After 6-h fasting, blood was collected from mice into EDTA (0.5 M, pH 8) by submandibular bleeding, and mice were killed by cervical dislocation. The hippocampus was immediately removed and frozen in liquid N₂ and stored at -80°C. Tissue samples were lysed in Standard Lysis Buffer supplemented with protease inhibitor cocktail (1%), EDTA (5mM), Na₃VO₄ (1mM), NaH (20mM) and DTT (1mM). Western blot was performed from 30 µg of protein homogenate using brain-derived neurotrophic factor (BDNF; Santa Cruz Biotechnology), pGSK3 (Ser9), GSK3, pAKT (Ser473), AKT or β-actin (Cell Signaling Technologies) antibodies. Quantification of the β-actin or non-phosphorylated form of proteins was used for normalization.

Glucose and insulin Tolerance Test (GTT/ITT):

Blood glucose levels were measured, and glucose and insulin tolerance tests were performed in 6-h fasted mice, as previously described [52,53]. For mice fed a standard chow diet (Group 1), tests were performed at 11 and 12 weeks of age : after intraperitoneal injection of glucose (1 g/kg b.w.) or insulin (0.5 U/kg b.w.), blood glucose was monitored for 120 min using a glucometer from blood samples collected from the tail vein. For mice fed a HF-HS diet (Group 7), tests were performed after 4 and 5 weeks of HF-HS diet : after intraperitoneal injection of glucose (0.75 g/kg b.w.) or insulin (0.75 U/kg b.w.), blood glucose was monitored for 120 min using a glucometer from blood samples collected from the tail vein [53].

Insulin release (IR):

Animals of the Group 1 (10 weeks of age) were fasted for 16 h and then received an intraperitoneal injection of glucose (3 g/kg b.w.). Blood was withdrawn from the tail vein at 0, 3, 6 and 12 min after injection for insulin assessment.

Hyperinsulinemic euglycaemic clamp

Clamp was performed in conscious, unrestrained, catheterized 13-week old mice after 6-h of fasting (Group 2), as previously described [54]. A catheter was inserted into the right jugular vein under anesthesia (2% isoflurane), and mice were allowed to recover for 7 days. After 6-h of fasting, blood was withdrawn from the tail vein to measure glycemia (with an Accu-Check glucometer, Roche) and a bolus of [3-3H] glucose (2.5 mCi, specific activity 0.74 TBq/mmol, Perkin Elmer, Boston, USA) and

insulin (1.25 mU) was administered via the catheter to the mice. We then infused [3-3H] glucose (0.17 mCi) and insulin (3 mU/kg/min) at a rate of 1 mL/min in the jugular vein. In the same way, euglycemia was maintained through a variable infusion of 15% (weight/volume) glucose. Glycemia was monitored every 20 min until the end of the clamp. After 120 min infusion, 20 μ L of blood sampled from the tail vein was collected to determine [3-3H] glucose specific activity, as described previously [54]. We checked that a steady state in plasma glucose and [3-3H] glucose specific activity was established at this time, as previously reported [54]. GIR (glucose infusion rate), EGP (endogenous glucose production) and Rd (rate of whole-body glucose disappearance) were determined using radioactivity and glycemia values, as described previously [54].

Insulin secretion

Islets were obtained from 13-week-old mice (Group 3), isolated through collagenase perfusion and Histopaque[®] gradient (Sigma) and cultured, as described previously [55]. Eight islets were pre-incubated in HEPES-buffered Krebs Ringer bicarbonate medium without glucose for 30 min and then replaced for 1 h in the same medium supplemented with 5.5 or 16.7 mM glucose at 37 C with 5% CO₂. At the end of the experiment, the supernatant was recovered to measure the insulin released, and islets were extracted with acid-ethanol solution to measure insulin content. Insulin was quantified using Mouse Insulin Elisa kits (Merckodia - detection limit: 0.043 μ g/L).

Glucose-6-phosphatase activity analysis

The intestine were sampled as previously described from 12-week old mice fed either a standard chow diet or a protein-enriched diet for 15 days (Group 9) [53]. Frozen tissues were homogenized using Fast Prep[®] in 10 mM HEPES and 0.25 M sucrose, pH 7.4 (9 vol/g tissue). G6Pase activity was assayed in homogenates for 10 min at 30°C at pH 7.3 in the presence of a saturating glucose-6-phosphate concentration (20 mM). Released P_i was determined by complexometry [56]. More specifically, the released inorganic phosphate was complexed with ammonium molybdate (0.1g/L). This complex was reduced by ascorbic acid (5g/L), then excess free molybdate was trapped by the addition of arsenite (25g/L). The specific activity of G6Pase was obtained by subtracting the non-specific phosphatase activity obtained by hydrolysis of β -glycerophosphate (100mM).

Behavioral Tests

Animals were tested only once in each paradigm after 6-h fasting (except the sucrose preference test [SPT]). Each mouse fed a standard chow diet was tested in the open field test (OFT) and 2-days later in the forced swim test (FST). Each mouse fed a HF-HS diet was tested in the OFT, 2-days later in the SPT and 5 days later in the FST. For OFT, each mouse was placed into the center of a Plexiglas box (57 \times 39 \times 28 cm), which was virtually delimited by a center area (220 cm²) and the periphery, and was allowed

to freely explore it for a session of 15 min. Total ambulatory distance, time spent immobile, and center entries were recorded using a video tracking device. The quantification was performed on the first 12 min (Viewpoint, Lyon, France). For FST, the movement (swimming + climbing) of each mouse immersed in vertical cylinder (17 cm diameter and 20 cm height) in the three-quarters filled with warm water ($+28 \pm 3^\circ\text{C}$) was recorded during a 6-min session with a computerized device (BIOSEB's FST system, Bioseb, France). Prior to the SPT, mice were habituated to the presence of 2 drinking bottles (water vs. 1% sucrose solution) in their home cage for 3 days. Bottles were switched each day to avoid the position bias. Following the acclimation, bottles were weighed the next day. Sucrose preference was calculated as a percentage of the consumed sucrose solution over the total volume of fluid.

In vivo Intracerebral Microdialysis

Under anesthesia (chloral hydrate, 400 mg/kg, i.p.), 13-week old mice were stereotaxically implanted with concentric microdialysis probes (active membrane length: 2.0 mm, molecular weight cut-off: 4.5 kD) in the ventral hippocampus (coordinates in mm from bregma: AP: -3.4, L: ± 3.4 , V: 4.0). The next day, mice were connected to a swivel system and the probes were connected to a microinjection pump, allowing a continuous perfusion of artificial cerebrospinal fluid (composition: NaCl 147 mM, KCl 3.5 mM, CaCl_2 1.26 mM, MgCl_2 1.2 mM, NaH_2PO_4 1.0 mM, NaHCO_3 25.0 mM; pH 7.4 ± 0.2) at a flow rate of 1.5 $\mu\text{l}/\text{min}$, while animals were awake and freely moving in their cage. After a 1-h stabilization period of the aCSF perfusion (t_0 to t_{60}), four 15-min fractions were collected to determine 5-HT baseline values for the individual mice. Subsequent dialysate samples were then collected after systemic administration of citalopram (10mg/kg) for a 60-min period, that is, t_{60} to t_{120} . Microdialysates were kept at -80°C until analysis of 5-HT content by high performance liquid chromatography (HPLC) coupled to an amperometric detector (VT03; Antec Leyden, Netherlands). The amounts of 5-HT in microdialysates (19 μl) were calculated by measurement of peak heights relative to external standards. The limit of sensitivity for 5-HT was ~ 0.5 fmol/sample (signal-to-noise ratio = 2) [13,57].

Statistical Analyses

All data are presented as mean \pm SEM. Data were tested for normality and homogeneity of variance prior analyses. Two-group comparisons were analyzed using Student's *t* test. Mixed-effect analysis followed by Bonferroni's or Sidak's post hoc test was used for multiple comparisons. Significance level was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical details and the exact value of *n* can be found in the figure legends.

Results

The absence of PEPT1 in a standard dietary context has no major effects on metabolism.

We first characterized the metabolism of PEPT1-deficient mice. We showed that the absence of PEPT1 in *Pept1*^{-/-} mice had no effect on body weight, blood glucose levels at 6-h of fasting, or on glucose (GTT) tolerance (Fig. 1c-d). Nevertheless, we noted that the absence of PEPT1 under standard diet caused small deterioration in insulin sensitivity (Fig. 1e). We further assessed whole body insulin sensitivity using a euglycaemic hyperinsulinaemic clamp. The rate of glucose infusion (GIR) required to maintain euglycemia was 27% lower in *Pept1*^{-/-} mice compared to WT mice ($p=0.08$) (Fig. 1f). In contrast, endogenous glucose production (EGP) during the clamp tended to be higher in *Pept1*^{-/-} mice ($p=0.07$) (Fig. 1f). Therefore, these results suggest that the absence of PEPT1 had a slight deleterious effect on insulin sensitivity and EGP in a standard dietary context.

Defects in glucose homeostasis, and particularly EGP, has been linked to altered insulin secretion in type 2 diabetic patients [58]. To better understand the effects of the absence of PEPT1 on glycemic control and insulin sensitivity, we studied the secretion profile of this hypoglycemic hormone.

First, *in vivo*, we measured insulin secretion levels in response to an intraperitoneal injection of glucose. In WT mice, in the absence of an insulin secretion defect, there was a massive increase in insulin secretion 3 minutes after glucose injection (Fig. 1g), then a drop at 6 minutes and a second peak in insulin secretion at 12 minutes (Fig. 1g). Interestingly, 16h-fasted *Pept1*^{-/-} mice had a 2-fold higher basal insulin levels than WT mice (Fig. 1h). Moreover, the typical insulin secretion profile of WT was not observed in *Pept1*^{-/-} mice. Indeed, in the absence of PEPT1, the early peak of insulin secretion (at 3 minutes) was absent, reflecting impaired insulin secretion in response to glucose (Fig. 1g). However, insulin levels increased at 12 minutes, showing the persistence of the second phase of insulin secretion in *Pept1*^{-/-} mice (Fig. 1g). Second, we studied, *ex vivo*, the ability of pancreatic islets isolated from *Pept1*^{-/-} and WT mice to secrete insulin in response to glucose (Fig. 1h). Independently of the genotype of the mice from which they were derived, pancreatic islets secreted more insulin when the glucose concentration in the culture medium was increased (Fig. 1h). Thus, the absence of *Pept1* did not affect the insulin-secreting capacity of isolated pancreatic islets (Fig. 1h). In summary, *Pept1*^{-/-} mice exhibited higher fasting insulinemia and had a defect in the first phase of insulin secretion *in vivo*, which might be associated with alteration of insulin sensitivity.

Pept1 deficiency alters behavior independently of nutritional challenge.

We then assessed whether the lack of PEPT1 could be sufficient to alter emotional behavior.

To evaluate anxiety-like behaviour, we carried out an open-field test (OFT) (Fig. 2b). In this test, the mouse must overcome its natural anxiety when placed in an empty, brightly lit arena. In this test, we studied the spontaneous behavior of mice to explore the brightly lit center of the arena or the less brightly lit periphery. In this test, avoidance of the central area is interpreted as an increase in anxiety. Thus, the less anxious they are, the more they will venture into the center, and the more anxious they are, the more they will stay along the walls (the least lit part). Both *Pept1*^{-/-} and WT mice travelled the same distance during the test (Fig. 2b) and spent the same time immobile (Fig. 2c). Moreover, the number of entries (Fig. 2d) and the time spent in the hypercenter (Fig. 2e) were the same for *Pept1*^{-/-} and WT mice. These results suggested that the deficiency of PEPT1 had no effect on anxiety.

To evaluate depressive-like behavior, we assessed the resignation of *Pept1*^{-/-} mice using the forced swim test (FST). In this test, mice are placed in a container filled with water, with no way out. They then swam to try to get out, before resigning themselves and stop swimming. The time to first immobilization did not vary according to genotype. However, *Pept1*^{-/-} mice spend more time immobile than WT mice (Fig. 2g), indicating a trend to resignation. The development of anxiety and depressive-like states are associated with neurobiological defects, and especially decreased serotonin (5-HT) and neurotrophin levels in the hippocampus. Using *in vivo* intracerebral microdialysis in freely moving mice, we measured the extracellular 5-HT levels in the hippocampus of WT and *Pept1*^{-/-} mice. Consistent with their increased resignation, the levels of 5-HT were 42% decreased in the hippocampus of *Pept1*^{-/-} mice compared to WT (Fig. 2h). We then examined whether the inhibition of 5-HT reuptake by citalopram would rescue hippocampal 5-HT levels in *Pept1*^{-/-} mice. Surprisingly, citalopram induced a stronger induction of extracellular hippocampal 5-HT levels in *Pept1*^{-/-} compared to WT mice (Fig. 2i), suggesting that the decrease in hippocampal 5-HT could result from an increase in 5-HT reuptake rather than a decrease in 5-HT secretion. Alternative mechanisms could have also contributed to these neurochemical effects such as a higher activity of the inhibitory 5-HT_{1A} autoreceptor [59] but this aspect warrants further investigations. This decrease in hippocampal 5-HT levels was associated with decreased Brain derived Neurotrophin Factor expression (Fig. 2j). In summary, PEPT1 deficiency had no effect on anxiety-like behavior, but altered depressive-like behavior, probably through molecular changes in the hippocampus.

Hypercaloric diet reveals the deleterious effect of the deficiency of PEPT1 on glycemic and behavioral balances.

Hypercaloric diet has been shown to alter glucose homeostasis and emotional behavior [13]. We thus assessed whether the small defects in insulin sensitivity and the depressive-like traits of *Pept1*^{-/-} mice could be worsened by a HF-HS diet. After 5 weeks on HF-HS diet, *Pept1*^{-/-} mice showed a slight worsening of insulin sensitivity (Fig. 3b) and a similar glucose tolerance (Fig. 3c), compared to WT mice.

Consistent with the worsening effect of the HF-HS diet on glucose control in absence of PEPT1, *Pept1*^{-/-} mice had a fasting glycaemia higher than that of WT mice (Fig. 3d), with no difference in body weight (Fig. 3e).

On the other hand, regarding anxious and depressive behaviors, the phenotype of *Pept1*^{-/-} mice on the HF-HS diet was also worsened compared to the absence of nutritional challenge. In the OFT, *Pept1*^{-/-} mice were more active than WT mice (Fig. 4b and c) but spent less time in the center of the arena than WT mice (Fig. 4d and e). Moreover, during the FST, *Pept1*^{-/-} mice stopped swimming earlier (Fig. 4f) and spent more time immobile than WT mice (Fig. 4g).

Finally, we performed a sucrose preference test, to assess anhedonia, which is also a major feature of anxiety and depression states. As expected, WT mice consumed more sucrose than water while *Pept1*^{-/-} mice consumed as much sucrose as water, reflecting a loss of the preference for sucrose (Fig. 4h).

Taken together, these data showed that *Pept1*^{-/-} mice displayed anxiety, resignation, and anhedonia in a context of a HF-HS diet, suggesting that their anxiety-depressive-like phenotype worsened under a nutritional challenge.

Mechanisms responsible for the metabolic and behavioral effects of PEPT1 deficiency.

We previously demonstrated that protein-enriched diets improve both energy homeostasis and emotional behavior through the induction of intestinal gluconeogenesis [29,30]. Proteins are metabolized to di/tripeptides, the latter activating intestinal gluconeogenesis [33]. However, di/tripeptides are mainly transported by PEPT1 [38]. Accordingly, we suggested that the lack of PEPT1 might blunt intestinal gluconeogenesis and promote prediabetic and anxio-depressive-like states. Contrarily to our hypothesis, the activity of intestinal glucose-6-phosphatase (G6Pase), the mandatory enzyme of gluconeogenesis, was comparable in WT and *Pept1*^{-/-} mice (Fig. 5a). Moreover, the capacity of protein-enriched diets to induce G6Pase activity was not altered by the deficiency of PEPT1 (Fig. 5a). These results thus showed that the phenotype of *Pept1*^{-/-} mice was not due to a decrease in G6Pase activity.

Another mechanism that could underlie the effects of PEPT1 deficiency on metabolism and behavior could involve GLP1. Indeed, recently, PEPT1 has been involved in the acute beneficial effect of ingested protein on glucose tolerance, via a mechanism that could involve GLP1 secretion [60]. However, we did not measure any change in GLP1 levels in the absence of PEPT1 (Fig. 5b).

Mice lacking PEPT1 surprisingly exhibited high amino acids plasma levels [61]. Elevated plasma amino acids and branched-chain amino acids in particular have been involved in the development of insulin

resistance [62]. However, no variation in branched-chain amino acids was observed in *Pept1*^{-/-} mice (Fig. 5c).

Lastly, the effects observed could be due to reduced insulin sensitivity. Indeed, decrease in insulin sensitivity, particularly in a mouse model of high fat diet, has been linked to defects in hippocampal insulin signaling [63]. We thus analyze the phosphorylation levels of the major kinases involved, namely AKT and GSK3. Interestingly, the phosphorylation level of GSK3 was decreased in the hippocampus of *Pept1*^{-/-} mice fed HF-HS diet (Fig. 5d), suggesting an impairment in brain insulin sensitivity.

Discussion

The comorbidity of type 2 diabetes and depression is a major challenge for healthcare worldwide. A better characterization of the bidirectional relationship between these two diseases is a key step towards developing common preventive or curative therapeutic strategies. Among currently used strategies, protein-enriched diets are considered as an efficient nutritional approach to improve either physiological parameters of diabetic or emotional scores of depressive patients. Using a mouse model lacking the peptide transporter PEPT1, we here confirmed the key role of proteins, and their products (i.e. di/tripeptides) in both diseases. Our results demonstrated that the deficiency of PEPT1 was sufficient to promote defects in insulin sensitivity and emotional behavior under a standard chow diet, which were further exacerbated by a hypercaloric diet.

Data from the literature showed that the acute inhibition of PEPT1 in mice blunted the short time beneficial effects of proteins on glucose tolerance [60]. Consistently, our results suggest that the lack of PEPT1 would be sufficient to alter carbohydrate homeostasis. Indeed, deficiency of PEPT1 in mice reduced insulin sensitivity and resulted in the loss of the early peak of insulin secretion in response to glucose. This early peak plays a crucial role in regulating endogenous glucose production, particularly in the liver, which is known to be involved in the emergence of hyperglycemia in diabetics [58]. Thus, the absence of this early peak could lead to delayed inhibition of hepatic glucose production, and might contribute to the hyperglycemia observed in *Pept1*^{-/-} mice fed a hypercaloric diet.

The novelty of our work lies in the fact that we highlighted for the first time the role of PEPT1 in the regulation of emotional behavior. Indeed, mice lacking *Pept1* developed despair traits together with hippocampal alterations specific of depression. Clinical studies and animal models highlight the important role of the hippocampus in the etiology of major depression, and particularly the role of the serotonergic system. Concentrations of brain-derived neurotrophic factor are decreased in the postmortem brains of depressed patients [64] and the capacity of serotonin release is reduced in patients experiencing major depression [65]. Moreover, challenging mice with a high fat diet induced

the development of anxio-depressive like states associated with reduced serotonin levels in the hippocampus [13]. Consistently, our results showed that *Pept1*^{-/-} mice exhibited a lower hippocampal BDNF expression and serotonin levels, that might be related to their altered behavioral phenotype. Of note, the decrease in serotonin levels could not be attributed to a decrease in tryptophan content, which does not change between WT and *Pept1*^{-/-} mice [61].

On the other hand, several studies suggest that the bidirectional link between diabetes and depression may be related to defects in insulin signaling. Indeed, deletion of the insulin receptor specifically in neurons or in glial cells is sufficient to promote anxio-depressive like states, related to altered monoamine signaling [14,66]. The defects in insulin secretion and insulin sensitivity observed in *Pept1*^{-/-} mice could thus be a mechanistic link promoting their emotional behavior defects. At the molecular level, depressive-like phenotype are linked to defects in the insulin-induced phosphorylation of AKT and GSK3 in the hippocampus [63]. Accordingly, in our study, *Pept1*^{-/-} mice fed a HF-HS had lower GSK3 phosphorylation levels in their hippocampus.

Induction of a low grade inflammation by a specific gut microbiome has also been pinpointed as a driver of the glucose homeostasis dysfunctions associated with type 2 diabetes and of mental health [67]. PEPT1 plays an important role in the induction of colitis in irritable bowel diseases [39]. More precisely, under inflammatory conditions, PEPT1 might aggravate intestinal inflammation by transporting proinflammatory bacterial peptides into cells [68]. Consistently, mice lacking *Pept1* are protected against the inflammation induced by colitis [69]. Increased intestinal inflammation does not therefore cause the diabetic and depressive features of *Pept1*^{-/-} mice.

Altogether, our results showed that *Pept1*^{-/-} mice developed molecular and metabolic defects characteristic of prediabetic and depressive-like states that might be related to a primary defect in peripheral and central insulin sensitivity.

Since the absence of PEPT1 appeared to modulate both metabolism and behavior, we wished to better understand the mechanisms involved. We previously showed that the beneficial effects of protein enriched diet on emotional behavior depends on the activation of intestinal gluconeogenesis [30]. Moreover, this biological function has also anti-diabetic effects, by improving insulin sensitivity [55,70,71]. Protein-enriched diets stimulate intestinal gluconeogenesis gene expression through the release of di/tripeptides [33]. The lack of PEPT1 leads to a decrease in dipeptide transport through the intestinal mucosa [49], and might thus alter, or even suppress intestinal gluconeogenesis. Thus, the absence of PEPT1 could be related to impairment in intestinal gluconeogenesis. Of note, intestinal gluconeogenesis-deficient mice also develop prediabetes [55]. However, mice lacking *Pept1* were still able to induce intestinal gluconeogenesis when fed protein-enriched diet, suggesting that the prediabetic phenotype of *Pept1*^{-/-} mice was not due to an absence or a decrease in this function.

Since peptones and dipeptides induce the release of the incretin GLP-1 *in vitro* and *in vivo* [72,73], a lack of PEPT1 might result in a decrease of GLP1 secretion. However, GLP1-induced secretion by peptones is not prevented by an acute PEPT1 inhibition [60,73] and we did not measure a decrease in GLP1 plasma levels in *Pept1*^{-/-} mice, suggesting that the defect in insulin secretion was not due to a decreased incretin effect of GLP1.

The development of insulin resistance has been mechanistically associated with high plasma levels of BCAA and specific amino acids in clinical or animal studies [74,75]. Specifically, the plasma levels of the amino acids Phenylalanine (Phe) and Tyrosin (Tyr) are strongly associated with diabetes [76]. They are also associated with the beneficial effects of gastric by-pass on glucose homeostasis [62,77]. Moreover, elevated amino acid plasma levels appear to have synergistic effect with hyperlipidemia on the development of insulin resistance [62]. Despite lacking PEPT1, *Pept1*^{-/-} mice exhibit a higher plasma levels of amino acids, which is likely due to changes in their hepatic nitrogen metabolism [61]. These mice do not exhibit increased BCAA plasma levels, compared to WT mice [61], but show significantly increased levels of Phe and Tyr, which might thus contribute to their decreased insulin sensitivity [61]. Interestingly, insulin controls dorsal raphe neuronal activity and hippocampal serotonin release through a 5-HT_{1A} autoreceptor-dependent mechanism [78]. Consistently, mice with reduced insulin sensitivity (induced by a high-fat diet) have lower basal hippocampal 5-HT levels associated with 5-HT_{1A} autoreceptor hypersensitivity [13]. A similar mechanism may occur in *Pept1*^{-/-} mice. Indeed, neurochemical analyses performed in *Pept1*^{-/-} mice suggest that their basal hippocampal 5-HT levels may be mainly due to overactivation of the inhibitory 5-HT_{1A} autoreceptor. Reduced hippocampal 5-HT levels could then contribute to the reduced brain-derived neurotrophic factor levels measured in the *Pept1*^{-/-} hippocampus [79]. From all these data, we could therefore speculate that the defects in glucose homeostasis and emotional behavior, exacerbated by HF-HS feeding in *Pept1*^{-/-} mice, could be due to changes in specific amino acids (e.g. Phe and Tyr), which would result in decreasing peripheral and central insulin sensitivity and subsequent decreased serotonin levels.

The bi-directional links between diabetes and depression were suggested almost 15 years ago, and are now taken into account in therapeutic approaches [80]. Antidiabetic drugs (GLP1 analogs and metformin) improve emotional behavior in rodents [81] and humans [82]. The potential use of these insulin-sensitizing agents to treat both diabetes and mood disorders opens up a new avenue of research. In this context, *Pept1*^{-/-} mice, as a model of both prediabetes and most importantly depressive-like states, could represent an innovative tool for testing or developing effective treatments for glycemic and emotional balance, and thus participate in the construction of preventive or curative strategies common to both pathologies.

Statements

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Statement of Ethics

This study protocol was reviewed and approved by the regional Animal Care Committee (CE2A-55, Lyon-1 University, France), approval number DR2015-01.

Conflict of Interest Statement

The authors have no conflicts of interest to declare

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Author Contributions

M.S. F.S. and A.G.-S. designed the experiments, and M.S, A.T. and F.S. conducted them and performed data analyses. G.Ma. helped in the experiments and contributed to the interpretation of data. M.S. performed the surgical procedures. J.Z. and B.G. performed the microdialysis experiments. A.S. assisted in animal breeding, housing, and animal experimentation. J.V-P., G.Mi. and A.G.-S. discussed and interpreted the data, supervised the study, and wrote the manuscript.

Data Availability Statement

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request

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Figure Legends

Figure 1 : PEPT1 deficiency moderately alters insulin secretion and sensitivity in a standard dietary context

(a) Timeline of experiments performed on the different groups of mice (IR : insulin release, GTT : glucose tolerance test, ITT: insulin tolerance test) **(b)** Body weight (mean \pm SEM, n= 7-8) **(c)** Blood glucose level after 6-hours fasting (mean \pm SEM, n=7-8) **(d)** Glucose and **(e)** insulin tolerance test (mean \pm SEM, n=12-13) **(f)** Insulin sensitivity assessed by hyperinsulinemic-euglycemic clamp (means \pm SEM, n=4-7) **(g)** Plasma insulin secretion profile after intraperitoneal glucose injection (3 g/kg) (means \pm SEM, n=6-7) **(h)** Insulin secretion measured at 5.5 mM and 16.7 mM glucose from islets isolated from *Pept1*^{-/-} and WT mice (means \pm SEM, n=6-7). Simple **(b,c)** or multiple **(f)** Student t-test, or Mixed-effect analysis (considering repeated measures) **(d, e, g, h)** was performed as a statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001 versus WT mice, # p < 0.05 versus T0.

Figure 2 : PEPT1 deficiency alters behavior by increasing anxiety and resignation in a standard food context

(a) Timeline of experiments performed on the different groups of mice (OFT : open field test, FST : forced swim test). **(b-e)** Travelled distance (b), Immobility (c), number of entries (d) and time spent (e) in the hypercenter during an OFT in WT and *Pept1*^{-/-} mice (mean \pm SEM, n=8-11) **(f-g)** Latency to first immobilization (f) and time spent immobile (g) during an FST in WT and *Pept1*^{-/-} mice (mean \pm SEM, n=7-8) **(h)** hippocampal basal serotonin (5-HT) in WT and *Pept1*^{-/-} mice (mean \pm SEM, n=8) **(i)** Data are means \pm SEM of extracellular 5-HT levels expressed as percent of baseline. The arrow indicates the time of the subcutaneous injection of citalopram (10 mg/kg) **(j)** Relative protein expression levels of BDNF (mean \pm SEM, n=4-5). **(b-h, j)** Student t-test was performed as a statistical analysis. **(i)** Mixed-effect (considering repeated measures) was performed as a statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001 versus WT mice.

Figure 3 : Absence of PEPT1 worsens metabolic impairment in mice fed a high-fat, high-sucrose diet

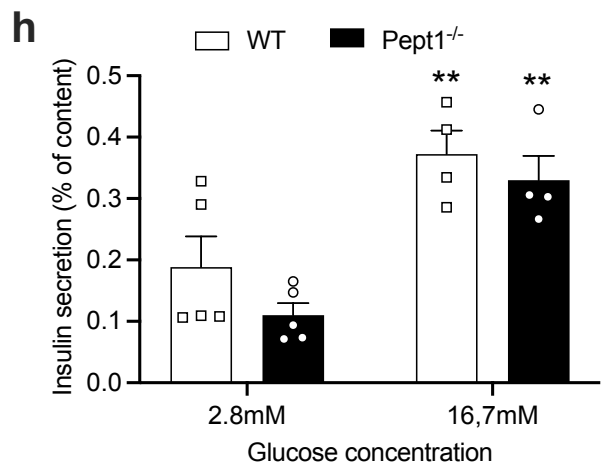
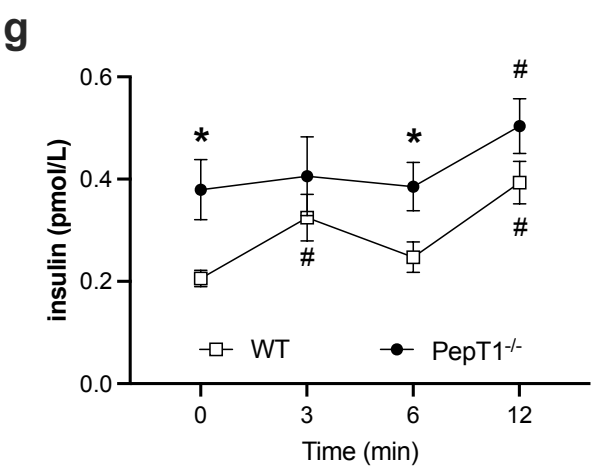
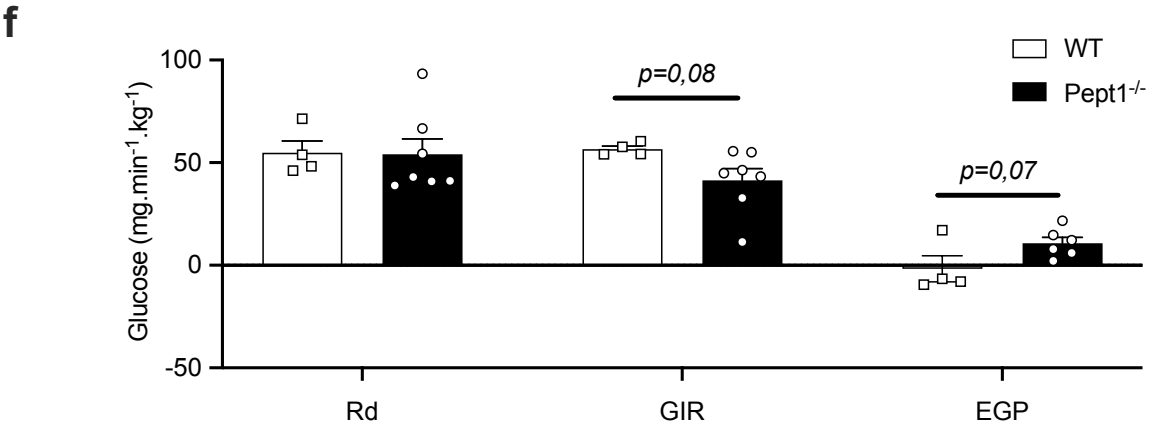
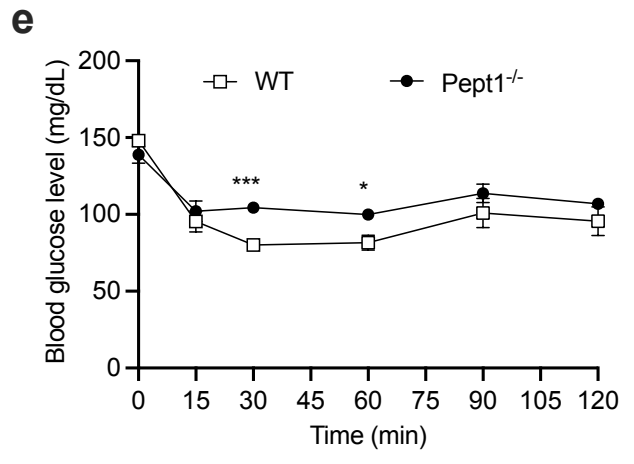
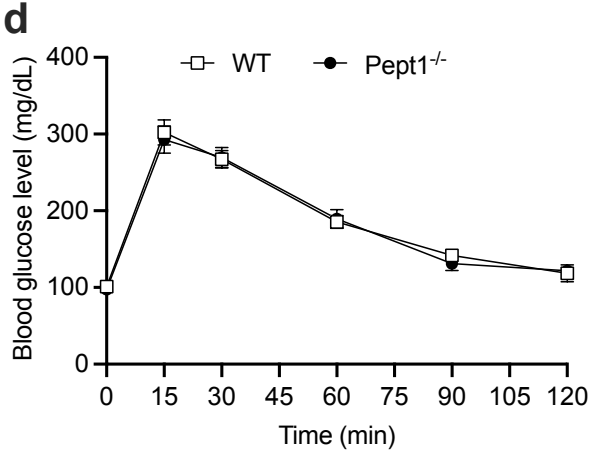
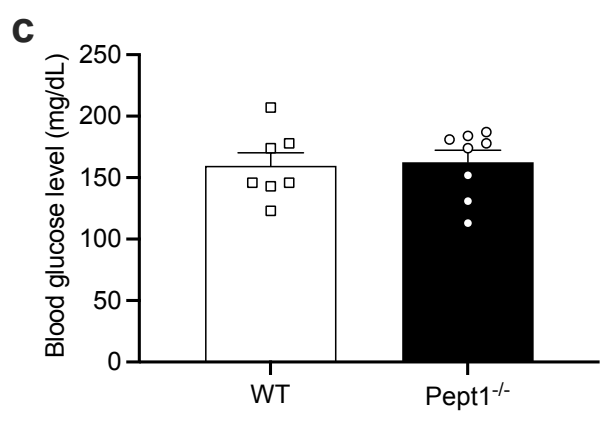
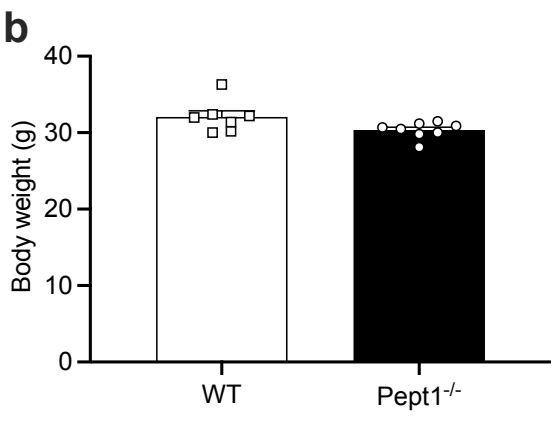
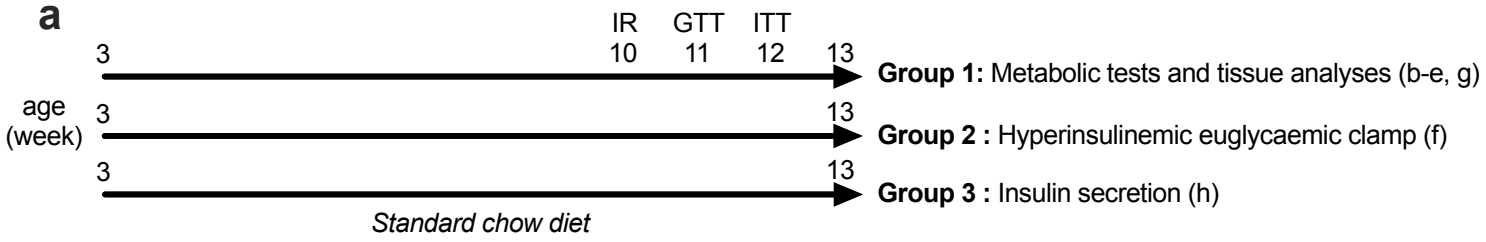
(a) Timeline of experiments performed on the different groups of mice **(b)** insulin (mean \pm SEM, n=6-8) and **(c)** glucose tolerance test (mean \pm SEM, n=11-16), **(d)** Blood glucose level after 6-h fasting and **(e)** Body weight (mean \pm SEM, n=8). Multiple t-test (b, c) or Student t-test (d, e) was performed as a statistical analysis. *p < 0.05, **p < 0.01 versus WT mice.

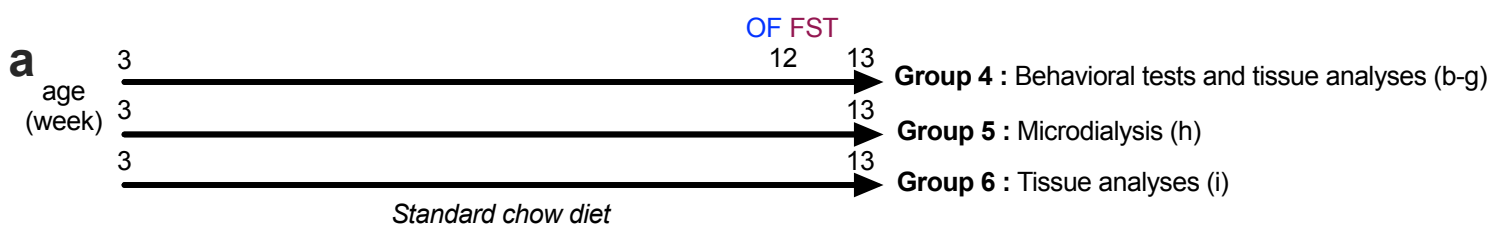
Figure 4 : Nutritional challenge induced by a hypercaloric diet accentuates the anxiety/depressive-like phenotype of PEPT1-deficient mice.

(a) Timeline of experiments performed on the different groups of mice (OFT : open field test, SPT : sucrose preference test, FST : forced swim test) **(b-e)** Travelled distance (b), Immobility (c), number of entries (d) and time spent (e) in the hypercenter during an OFT in WT and *Pept1*^{-/-} mice fed HF-HS diet (mean ± SEM, n=20-23) **(f-g)** Latency to first immobilization (f) and time spent immobile (g) during an FST in WT and *Pept1*^{-/-} mice fed HF-HS diet (mean ± SEM, n=18-20) **(h)** Consumption of water or sucrose-water during a SPT (mean ± SEM, n=15) **(b-h)** Student t-test was performed as a statistical analysis. *p < 0.05, ***p < 0.001 *versus* WT mice (b-g) or *versus* Water consumption (h).

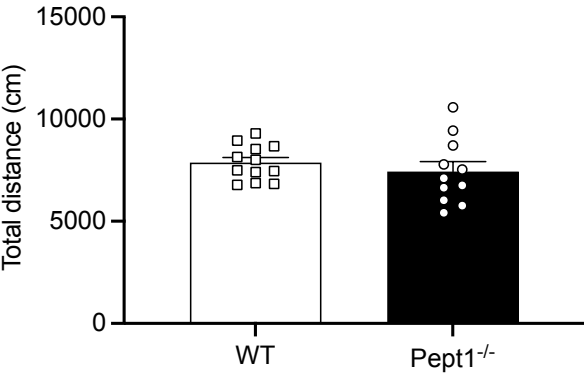
Figure 5 : The phenotype of Pept1^{-/-} mice does not depend on intestinal gluconeogenesis but seems to be associated with defects in insulin signaling in the hippocampus.

(a) Intestinal Glucose-6-phosphatase activity of WT and *Pept1*^{-/-} mice fed either a chow or a protein-enriched diet (mean ± SEM, n=3-4) **(b)** Plasma GLP1 concentration of WT and *Pept1*^{-/-} mice (mean ± SEM, n=5-6) **(c)** Plasma Branched-Chain Amino Acid (BCAA) levels of WT and *Pept1*^{-/-} mice fed HF-HS diet **(d)** Relative protein expression levels of phosphorylated forms of AKT and GSK3 (mean ± SEM, n=5-8). The tissue analyses presented in panels b-d were performed on Group 7 (see Fig. 3a). Student t-test was performed as a statistical analysis. *p < 0.05, ***p < 0.001 *versus* mice fed a standard diet (a) or WT mice (b-d).

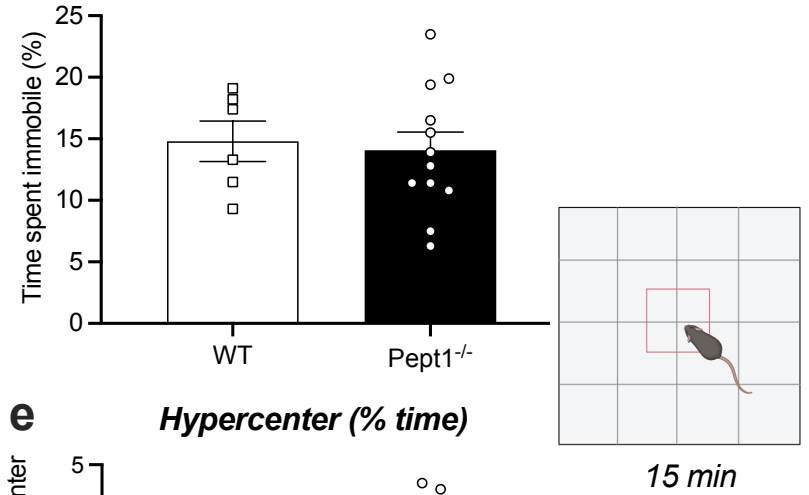




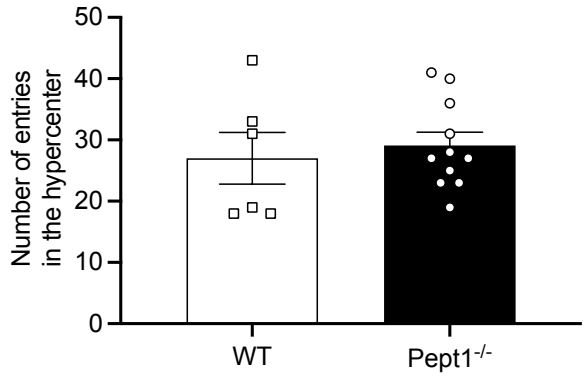
b *Travelled distance*



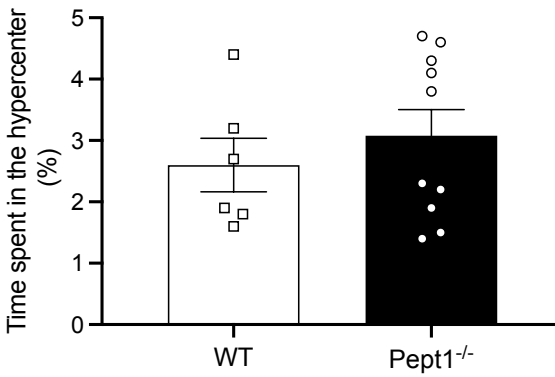
c *Immobility*



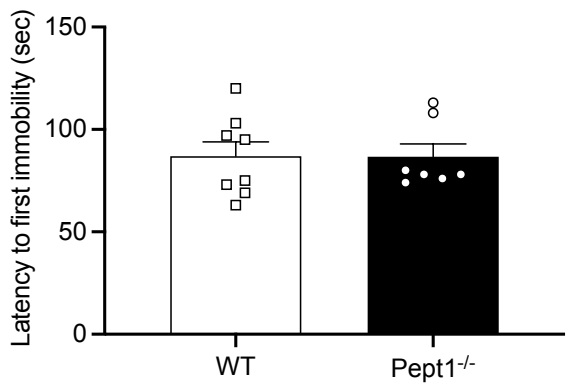
d *Hypercenter (number of entries)*



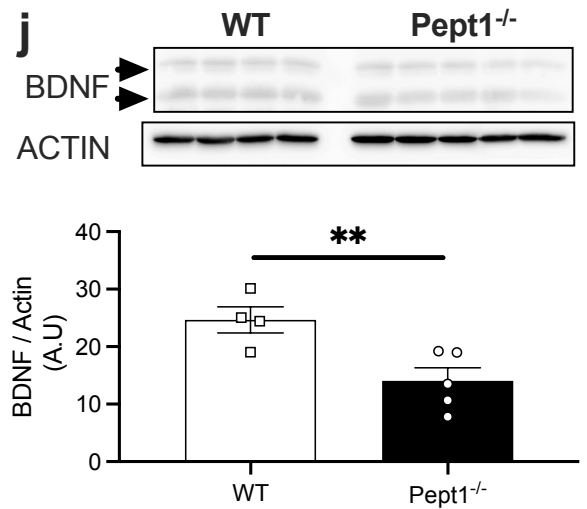
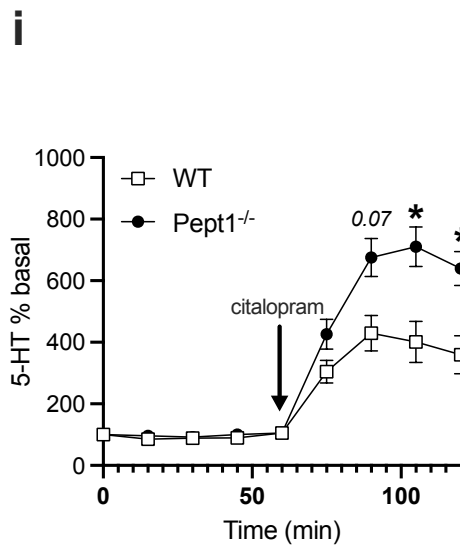
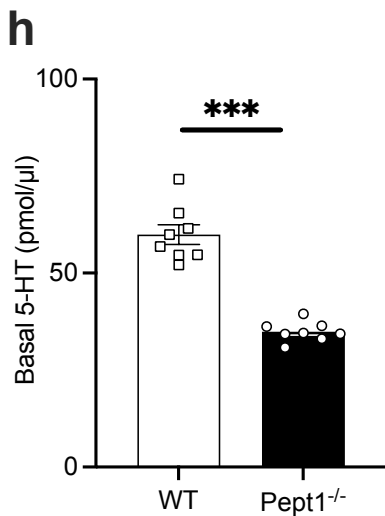
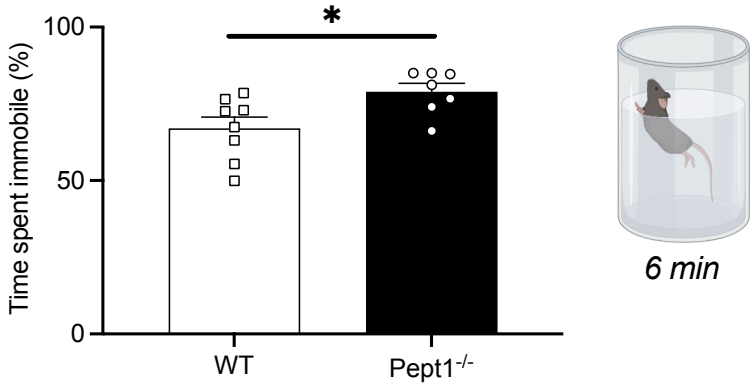
e *Hypercenter (% time)*

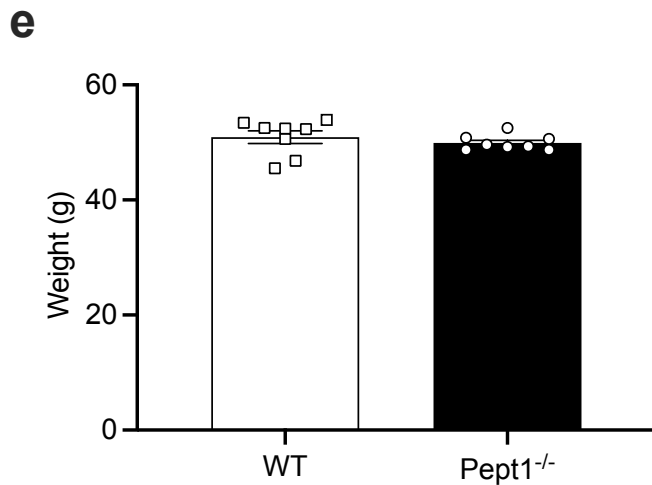
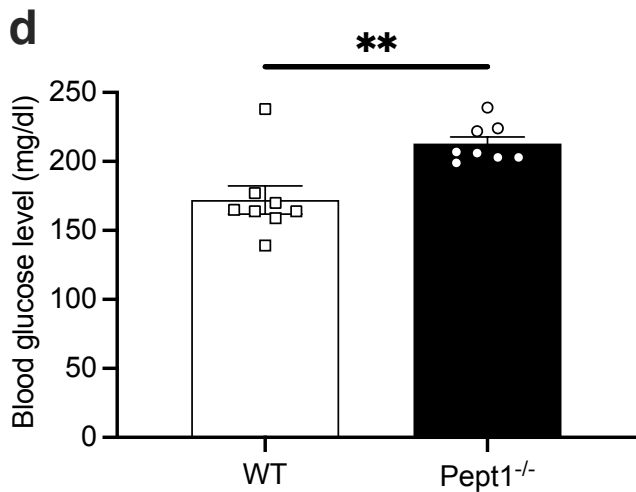
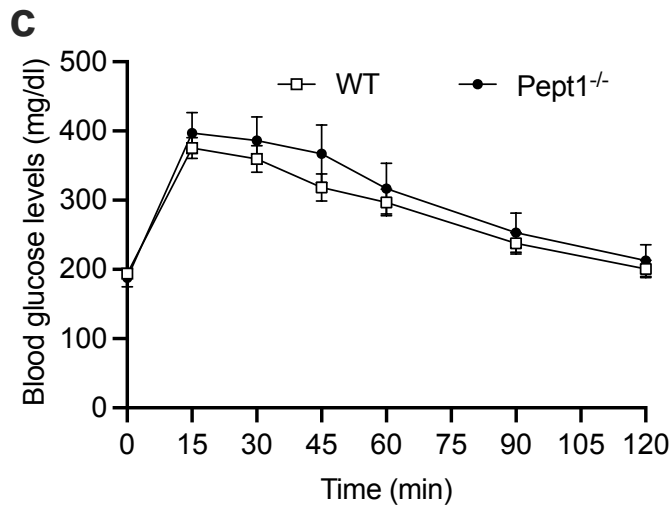
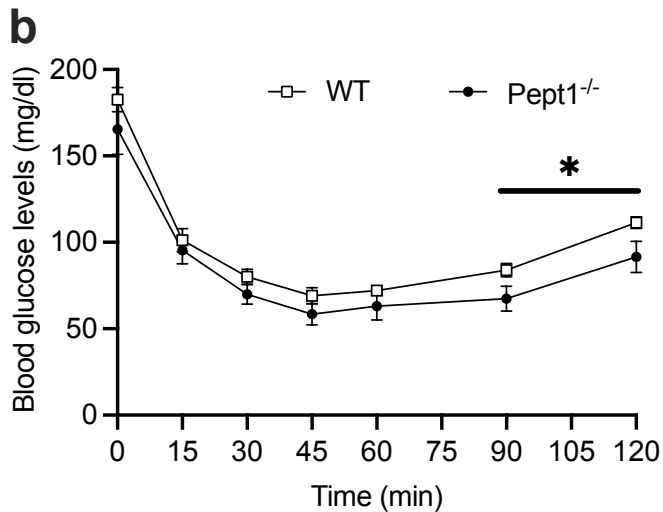


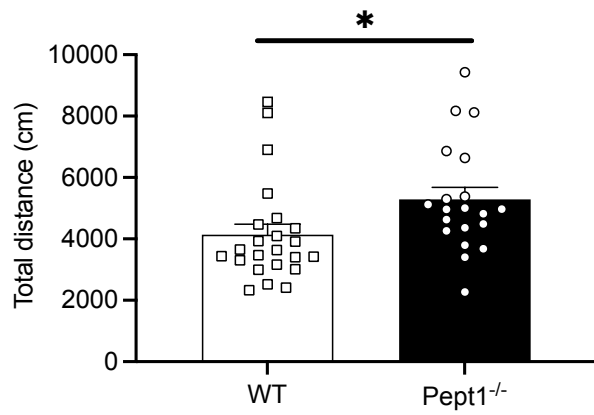
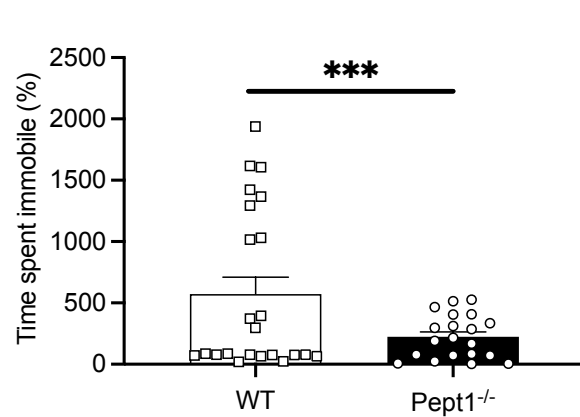
f *Latency to first immobilization*



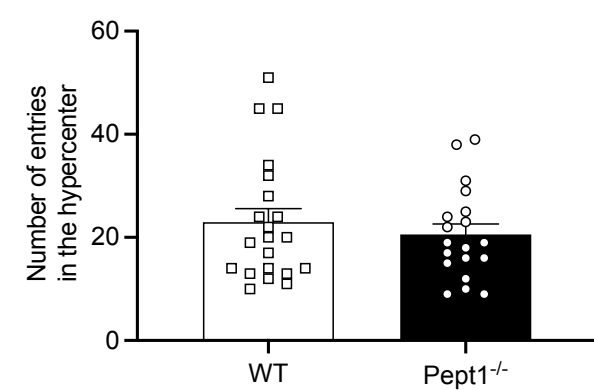
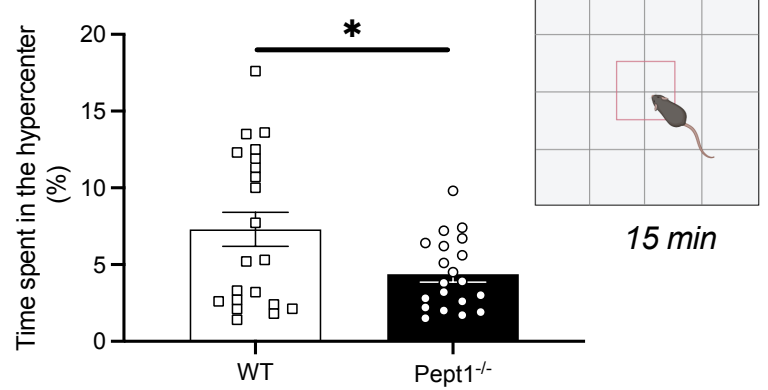
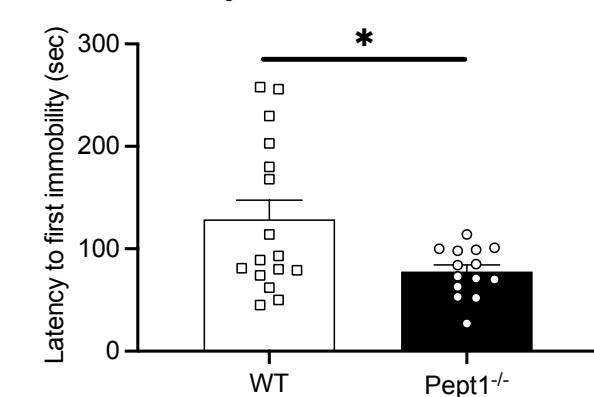
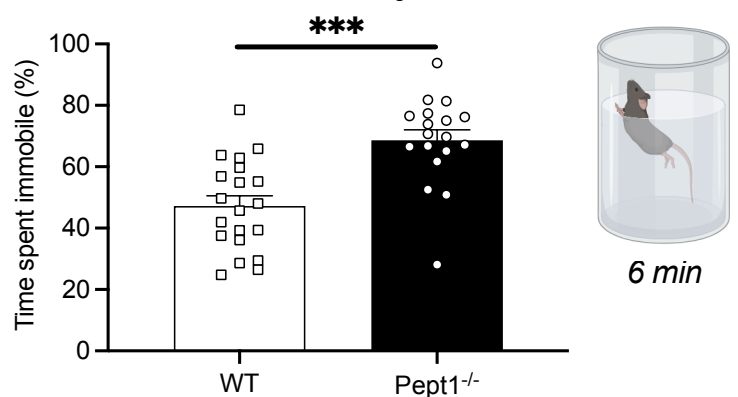
g *Immobility*



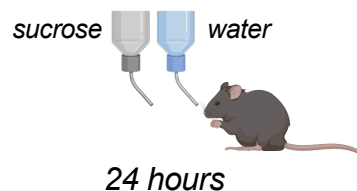
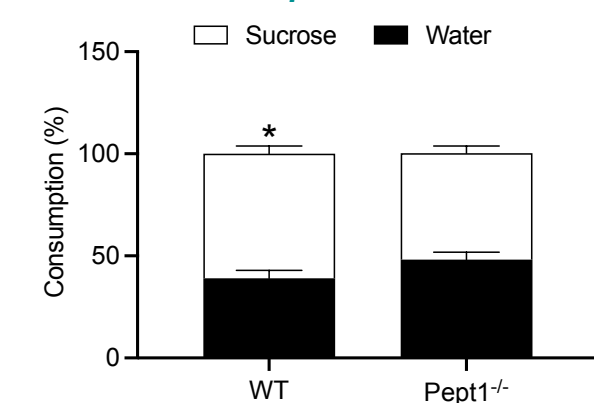


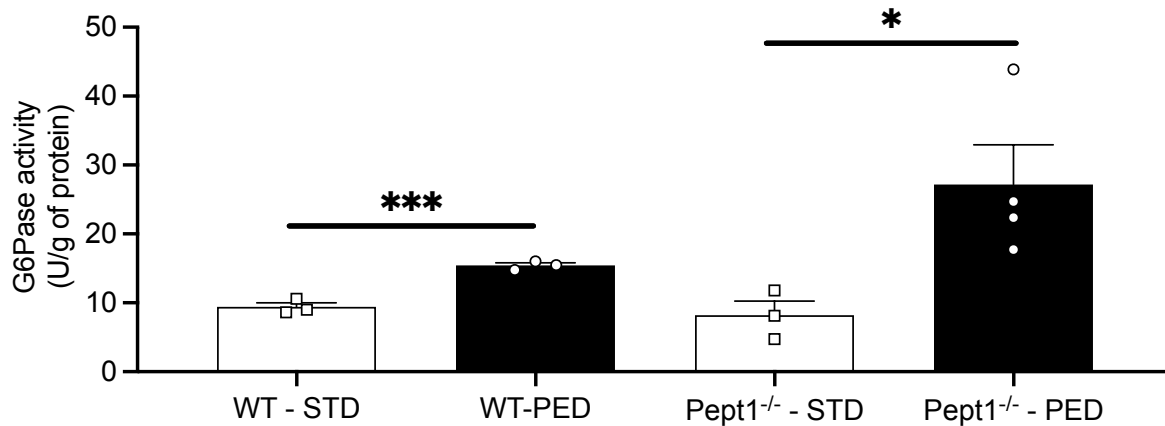
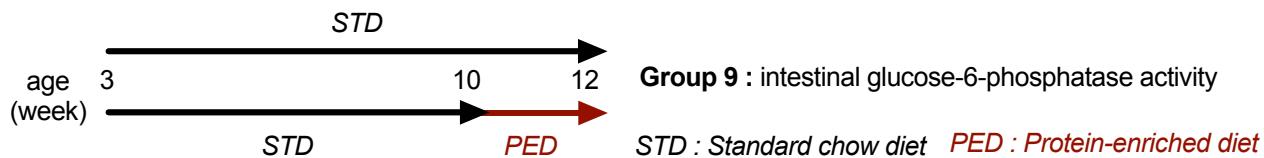
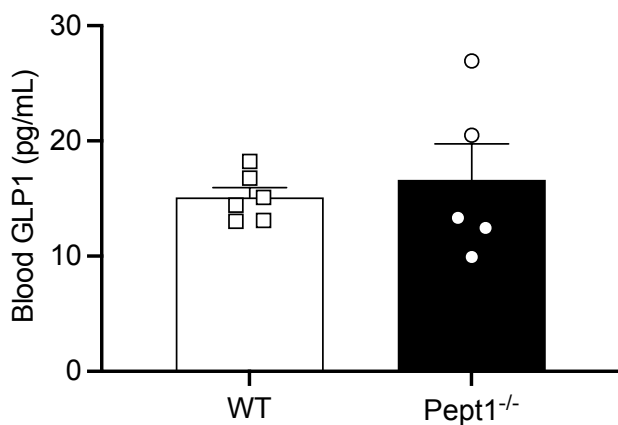
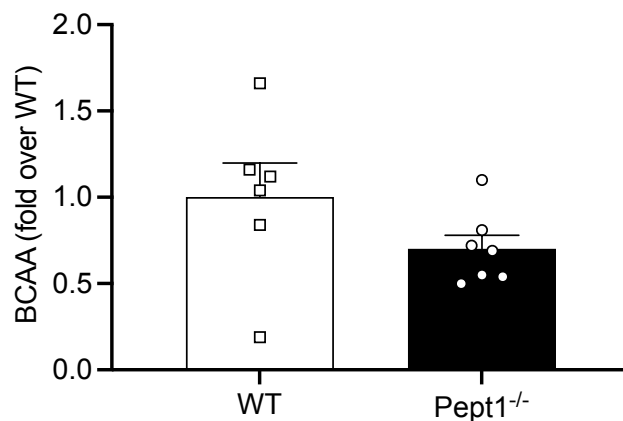
a**b****Travelled distance****c****Immobility**

OPEN FIELD TEST

d**Hypercenter (number of entries)****e****Hypercenter (% time)****f****Latency to first immobilization****g****Immobility**

FORCED SWIM TEST

h**Sucrose preference test**

a**b****c****d**