

Gut-specific Neprilysin Deletion Protects Against Fat-induced Insulin Secretory Dysfunction in Male Mice

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Abstract

Neprilysin is a ubiquitous peptidase that can modulate glucose homeostasis by cleaving insulinotropic peptides. While global deletion of neprilysin protects mice against high-fat diet (HFD)-induced insulin secretory dysfunction, strategies to ablate neprilysin in a tissue-specific manner are favored to limit off-target effects. Since insulinotropic peptides are produced in the gut, we sought to determine whether gut-specific neprilysin deletion confers beneficial effects on insulin secretion similar to that of global neprilysin deletion in mice fed a HFD. Mice with conditional deletion of neprilysin in enterocytes (NEP^{Gut-/-}) were generated by crossing Vil-Cre and floxed neprilysin mice. Neprilysin activity was almost abolished throughout the gut in NEP^{Gut-/-} mice, and was similar in plasma, pancreas, and kidney in NEP^{Gut-/-} vs control mice. An oral glucose tolerance test was performed at baseline and following 14 weeks of HFD feeding, during which glucose tolerance and glucose-stimulated insulin secretion (GSIS) were assessed. Despite similar body weight gain at 14 weeks, NEP^{Gut-/-} displayed lower fasting plasma glucose levels, improved glucose tolerance, and increased GSIS compared to control mice. In conclusion, gut-specific neprilysin deletion recapitulates the enhanced GSIS seen with global neprilysin deletion in HFD-fed mice. Thus, strategies to inhibit neprilysin specifically in the gut may protect against fat-induced glucose intolerance and beta-cell dysfunction.

Key Words: neprilysin, gut, insulin secretion, oral glucose tolerance test, mouse

Abbreviations: CCK, cholecystokinin-8; DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GSIS, glucose-stimulated insulin secretion; HFD, high-fat diet; OGTT, oral glucose tolerance test.

Type 2 diabetes is associated with obesity, insulin resistance, and insulin secretory dysfunction; the latter is required for the development of hyperglycemia (1). Identifying new approaches to prevent impaired insulin secretion in diabetes is therefore of critical importance.

Neprilysin is a ubiquitous peptidase that has recently gained attention as a therapeutic target in type 2 diabetes (2). Its activity is increased in obesity and type 2 diabetes (3, 4). Pharmacological inhibition of neprilysin in individuals with obesity and/or type 2 diabetes has been associated with improved glycemic control (5, 6) and insulin sensitivity (7) when combined with an angiotensin receptor blocker for several weeks/years. We previously reported that in a mouse model of beta-cell dysfunction, pharmacological neprilysin inhibition was also associated with improved insulin secretion (8, 9). Further, we showed that global genetic deletion of neprilysin in mice protected against high-fat diet (HFD)-induced insulin secretory dysfunction (10), improved glycemic status and insulin sensitivity (3), and reduced hepatic gluconeogenesis (11).

Since neprilysin has several substrates, its inhibition via a tissue-specific strategy might be favored to limit off-target and deleterious effects that could occur with systemic inhibition [eg, elevated angiotensin II levels (12)]. Neprilysin is expressed in the gut (13) where it can cleave locally produced insulinotropic

peptides, such as glucagon-like peptide-1 (GLP-1) (14, 15). We previously found that in lean mice, acute inhibition of intestinal neprilysin enhanced glucose-stimulated insulin secretion (GSIS) under physiological conditions (16). However, it is unknown whether such a beneficial effect on GSIS also occurs in the setting of increased dietary fat.

Here we generated mice with gut-specific neprilysin deletion (NEP^{Gut-/-}) and evaluated *in vivo* whether these mice had improved GSIS compared to their littermate controls when fed a HFD.

Materials and Methods

Mouse Generation and Housing

Floxed neprilysin (NEP^{fl/fl}) mice on a C57BL/6N background were generated at Cyagen (Santa Clara, CA, USA), wherein exons 5 to 7 of the mouse neprilysin gene were selected as the conditional knockout region. In the targeting vector, a Neo cassette flanked by Rox sites and a “SA-IRES-EGFP-pA” cassette in the reverse orientation were cloned downstream of exons 5 to 7. Exons 5 to 7 and the “SA-IRES-EGFP-pA” cassette were flanked with LoxP and Lox2272 sites (Fig. 1A). B6.Cg-Tg(Vil1-cre)1000Gum/J (Vil-Cre) mice were obtained from the Jackson Laboratory (#021504; also known as

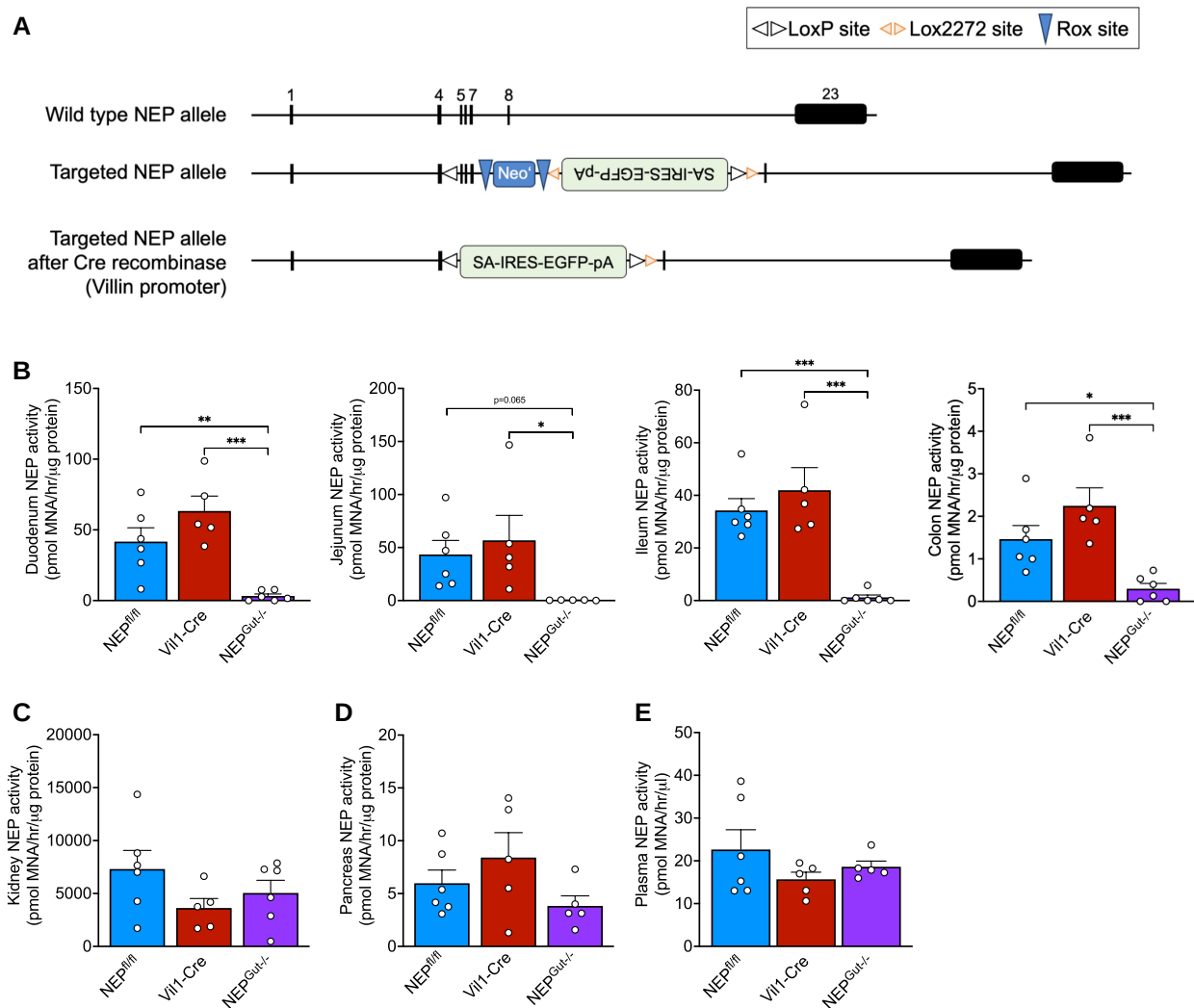


Figure 1. Intestinal, but not kidney, pancreas or plasma neprilysin activity is markedly reduced in NEP^{Gut-/-} mice. (A) Schematic of Cre-LoxP targeting strategy used to generate mice with conditional deletion of neprilysin in enterocytes (NEP^{Gut-/-}). Neprilysin activity was measured in protein extracts from (B) duodenum, jejunum, ileum, colon (C), kidney, and (D) pancreas and (E) in plasma collected from chow-fed male NEP^{Gut-/-} mice and their littermate control mice (NEP^{fl/fl} and Vil1-Cre mice) at ~12 weeks of age (n = 5-6/group). Data are mean ± SEM. *P < .05, **P < .01, ***P < .001.

Vil1-Cre 1000). These Vil1-Cre mice express Cre recombinase in villus and crypt epithelial cells of the small and large intestines. NEP^{fl/fl} and Vil1-Cre mice were crossed to generate mice with conditional deletion of neprilysin in enterocytes (NEP^{Gut-/-}). All mice were born at the expected Mendelian ratios, appeared healthy, and were genotyped for recombination of the Cre and floxed alleles.

Mice were housed up to 5 per cage with a 12 hours light/12 hours dark cycle and maintained on chow diet (PicoLab Rodent Diet 20 #5058, LabDiet, St. Louis, MO, USA) with ad lib access to food and water. All experiments were performed in male NEP^{Gut-/-} mice and age-matched littermate controls. The study was approved by the VA Puget Sound Health Care System Institutional Animal Care and Use Committee.

Mouse Cohorts for Verification of Neprilysin Deletion and HFD Feeding

A cohort of male 12-week-old NEP^{Gut-/-} mice and age-matched littermate controls was used to confirm conditional deletion of neprilysin in NEP^{Gut-/-} mice. Neprilysin activity was measured

in 4 parts of the intestine (duodenum, jejunum, ileum, and colon). To rule out off-target effects, neprilysin activity was also determined in kidney, pancreas, and plasma from NEP^{Gut-/-} and control mice. Kidney was selected due to its high expression of neprilysin (17) and pancreas and plasma due to previously recognized effects of neprilysin within these sites to modulate insulin secretion and/or glucose homeostasis (8, 9, 18).

In a separate cohort, 11-week-old male mice were fed a HFD (60% kcal fat, D12492; Research Diets Inc; New Brunswick, NJ, USA) for 14 weeks. Body weight was assessed and oral glucose tolerance test (OGTT) performed at baseline and at the end of the 14-week feeding period as described later.

Tissue Protein Extraction

For neprilysin activity measures, sections of duodenum, jejunum, ileum, colon, kidney, and pancreas were homogenized in protein lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 0.5% Triton X-100, 25 mmol/L ZnCl₂, pH 7.4) containing EDTA-free protease inhibitor cocktail (cOmplete™ ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor

Cocktail, Cat #05892791001, Roche, Basel, Switzerland) and phosphatase inhibitors (PhosSTOP™, Cat #4906845001 Roche). For intestinal active GLP-1 measures, sections of distal small intestine (including ileum) were homogenized in 100 mmol/L HCl/70% ethanol. Homogenization was done using a Bullet Blender Tissue Homogenizer (NextAdvance, Troy, NY, USA). The protein concentration of each extract was quantified via the bicinchoninic acid assay (Pierce, Waltham, MA, USA).

Plasma and Tissue Neprilysin Activity Assay

Neprilysin activity was assessed in plasma (5 μ L) and tissue extracts (40 μ g) by an established fluorometric enzyme method, as previously described (3, 16, 18). Briefly, glutaryl-alala-phe-4-methoxy-2-naphthylamine was broken down by neprilysin in samples to phe-4-methoxy-2-naphthylamine and then the fluorescent product methoxy-2-naphthylamine by aminopeptidase M. Each sample was assayed both in the absence or presence of the neprilysin inhibitor, thiorphan, to differentiate neprilysin activity from nonspecific endopeptidase activity. Fluorescence was compared against a methoxy-2-naphthylamine standard curve.

OGTT

Before and following the 14-week HFD feeding period, OGTTs were performed in conscious mice fasted for 6 hours. Tail vein blood was collected at baseline and at 10, 20, 30, 60, 90, and 120 minutes after oral glucose (2 g/kg) administration for measurement of plasma glucose. Incremental area under the curve for glucose from 0 to 120 minutes was calculated to determine glucose tolerance. Retro-orbital blood was collected in heparinized tubes at baseline for plasma insulin measurement and at 10 minutes post glucose bolus for plasma insulin and active GLP-1 measurement. Aprotinin (500 kU/mL), EDTA (3.2 mmol/L), and diprotin A (0.01 mmol/L) were added to the blood collected for GLP-1 measurement to avoid its degradation. Plasma samples were stored at -30°C (for glucose and insulin) or -80°C (for GLP-1) prior to assay. The quantitative insulin sensitivity check index (QUICKI) was calculated as $1/[\log(I0) + \log(G0)]$, where I0 and G0 are fasting plasma insulin ($\mu\text{U/mL}$) and blood glucose (mg/dL) levels. Glucose-stimulated insulin secretion was assessed as the ratio of the change in insulin to the change in glucose ($\Delta\text{insulin}/\Delta\text{glucose}$) from 0 to 10 minutes.

Plasma Glucose, Insulin and Active GLP-1 Measurements

Plasma glucose and insulin levels were determined using the glucose oxidase method and a mouse ultrasensitive Insulin ELISA (Alpco, Salem, NH, USA; Cat #80-INSMSU-E01; RRID: AB_2792981), respectively. Plasma and intestinal active GLP-1 concentrations were measured using the active GLP-1 immunoassay (Meso Scale Discovery, Rockville, MD, USA; Cat #K1503OD, RRID:AB_2935695).

Statistical Analyses

Data are presented as mean $\pm$ SEM. Bar graphs also show individual values. Statistical significance was determined using one-way ANOVA or two-way ANOVA/mixed-effects model (with time and genotypes being the 2 variables) with Holm-Sidak's post hoc analysis. A $P < .05$ was considered statistically

significant. Statistical calculations and graphs were made using GraphPad Prism (v. 10.1.0 for Mac; GraphPad Software, La Jolla, CA, USA).

Results

NEP^{Gut-/-} Mice Display Marked Reduction in Intestinal Neprilysin Activity

To verify the loss of intestinal neprilysin in NEP^{Gut-/-} mice, neprilysin activity was assessed in extracts from different segments of the intestine (duodenum, jejunum, ileum, and colon) and from kidney and pancreas, as well as in plasma collected from all groups of 12-week-old chow-fed male mice (NEP^{fl/fl}, Vil-Cre, and NEP^{Gut-/-} mice). As expected, neprilysin activity was almost completely abolished in the 4 segments of the intestine (Fig. 1B) in NEP^{Gut-/-} vs littermate control NEP^{fl/fl} and Vil-Cre mice. In contrast, neprilysin activity in kidney (Fig. 1C), pancreas (Fig. 1D), and plasma (Fig. 1E) did not significantly differ among the 3 groups of mice.

HFD-fed NEP^{Gut-/-} Mice Exhibit Lower Fasting Glucose Than Control Mice

To determine whether NEP^{Gut-/-} mice were protected against HFD-induced weight gain and hyperglycemia, a separate cohort of 11-week-old male mice were fed a 60% fat diet for 14 weeks. At week 0, body weight, fasting plasma glucose and insulin levels were similar in all groups of mice (Table 1). All mice significantly gained weight on a HFD, with no difference observed in body weight at the end of the 14-week HFD feeding period between groups (Table 1). After 14 weeks of HFD, fasting plasma glucose levels were significantly elevated in control mice but not in NEP^{Gut-/-} mice when compared to baseline (Table 1). Also, fasting plasma glucose at week 14 remained significantly lower in NEP^{Gut-/-} vs Vil-Cre control mice (Table 1). To determine whether NEP^{Gut-/-} mice were protected against HFD-induced insulin resistance, fasting plasma insulin levels were measured, and the surrogate index of insulin sensitivity, QUICKI, was then calculated using fasting insulin and glucose values. Fasting plasma insulin levels significantly increased and QUICKI significantly decreased in all groups of mice on the HFD, with no significant differences amongst groups at the end of the 14-week HFD period (Table 1).

HFD-fed NEP^{Gut-/-} Mice Display Improved Glucose Tolerance and Higher GSIS Than Control Mice

To determine whether NEP^{Gut-/-} mice were protected against HFD-induced insulin secretory dysfunction, OGTTs were performed before and at the end of the 14 weeks of a HFD. At week 0, glucose levels during the OGTT were similar in all 3 groups of mice (Fig. 2A). In contrast, at week 14, glucose levels during the OGTT were significantly lower in NEP^{Gut-/-} vs control mice (Fig. 2B). As expected, the incremental area under the curve for glucose from 0 to 120 minutes was significantly increased in all groups of mice at week 14 vs week 0 (Fig. 2C) but remained lower at week 14 in NEP^{Gut-/-} vs NEP^{fl/fl} and Vil-Cre mice (Fig. 2C). Further, while insulin release in response to oral glucose did not differ among groups at baseline (Fig. 2D), it significantly increased in NEP^{Gut-/-} vs Vil-Cre and NEP^{fl/fl} control mice at 14 weeks (Fig. 2E), even after accounting for the prevailing glucose concentration during the OGTT (Fig. 2F). Of note, in contrast to control mice,

Table 1. Body weight, fasting plasma glucose levels, fasting plasma insulin levels, and QUICKI measured before and at the end of the 14-week high-fat diet period

	Week 0			Week 14		
	NEP ^{fl/fl} (n = 7)	Vil-Cre (n = 6)	NEP ^{Gut-/-} (n = 15)	NEP ^{fl/fl} (n = 7)	Vil-Cre (n = 6)	NEP ^{Gut-/-} (n = 15)
Body weight (g)	26.1 ± 1.0	25.1 ± 1.0	28.4 ± 1.4	48.5 ± 2.0 ^a	49.7 ± 1.5 ^a	51.5 ± 1.7 ^a
Fasting plasma glucose (mmol/L)	10.4 ± 1.1	10.6 ± 1.0	11.11 ± 0.5	14.6 ± 1.0 ^a	17.0 ± 1.4 ^a	12.7 ± 0.6 ^b
Fasting plasma insulin (pmol/L)	309 ± 45	243 ± 48	354 ± 76	1410 ± 174 ^a	1887 ± 625 ^a	1505 ± 204 ^a
QUICKI	0.26 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.21 ± 0.01 ^a	0.21 ± 0.01 ^a	0.22 ± 0.01 ^a

Data are displayed as mean ± SEM. The QUICKI was calculated as $1/[\log(I0) + \log(G0)]$, where I0 and G0 are fasting plasma insulin (μU/mL) and blood glucose (mg/dL) levels, respectively n = 7, 6, and 15 in NEP^{fl/fl} mice, Vil-Cre mice, and NEP^{Gut-/-} mice, respectively. Abbreviations: QUICKI, quantitative insulin sensitivity check index. ^a*P* < .05 vs week 0 of the same genotype. ^b*P* < .01 vs Vil-Cre at week 14.

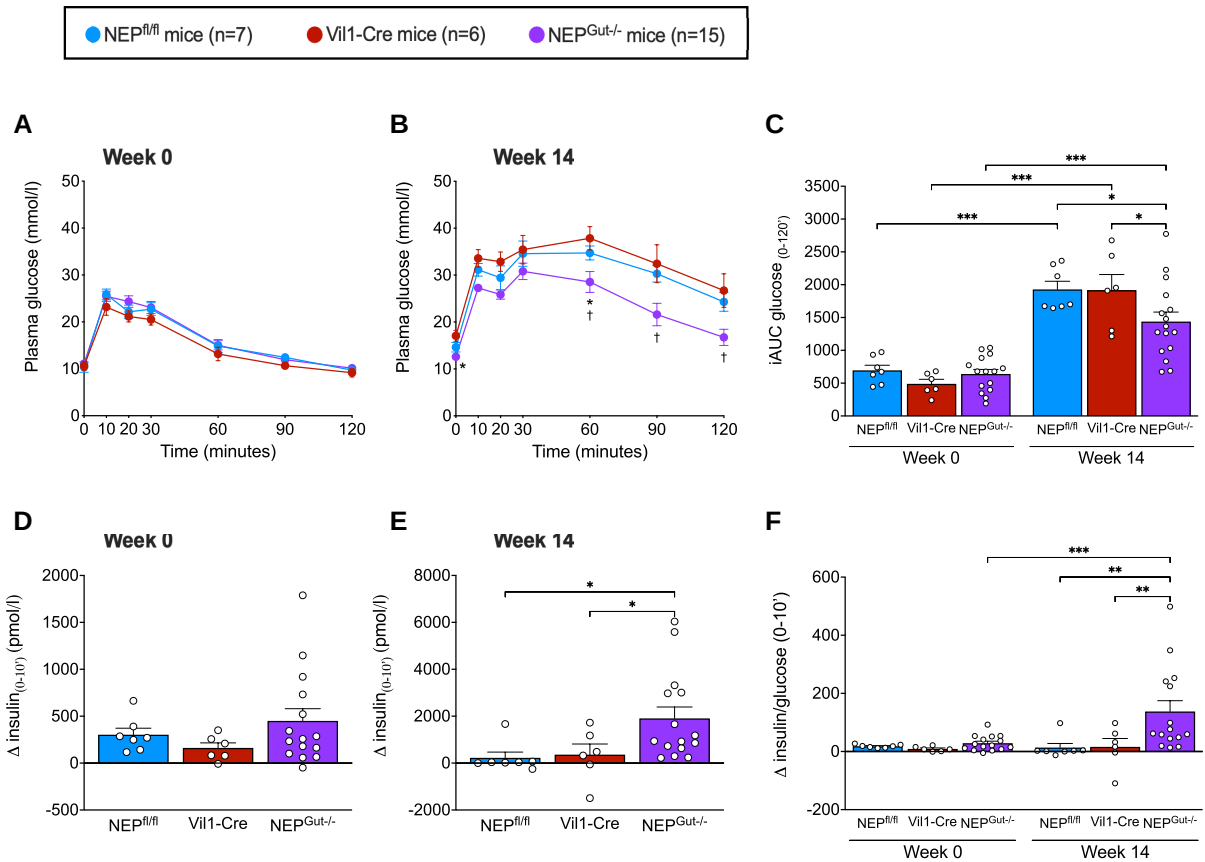


Figure 2. High-fat-fed NEP^{Gut-/-} mice display better glucose tolerance and higher insulin release in response to glucose than control mice. Eleven-week old NEP^{Gut-/-} mice (n = 7) and their littermate control Vil-Cre (n = 6) and NEP^{fl/fl} (n = 15) mice were fed a HFD for 14 weeks. Plasma glucose profile during the OGTT (2 g glucose/kg) performed (A) before (week 0) and (B) at the end (week 14) of the HFD feeding period. **P* < .05 NEP^{Gut-/-} vs Vil-Cre mice; †*P* < .05 NEP^{Gut-/-} vs NEP^{fl/fl} mice. (C) Incremental area under the curve for glucose from 0 to 120 minutes. Insulin release during the oral glucose tolerance test assessed as (D, E) Δinsulin from 0 to 10 minutes and (F) ratio of Δinsulin from 0 to 10 minutes over Δglucose from 0 to 10 minutes. Data are mean ± SEM. **P* < .05, ***P* < .01, ****P* < .001. Abbreviations: HFD, high-fat diet; OGTT, oral glucose tolerance test.

GSIS was also significantly increased in NEP^{Gut-/-} after the 14 weeks of HFD feeding vs baseline (Fig. 2F).

NEP^{Gut-/-} Mice Display Comparable Plasma and Intestinal Active GLP-1 Levels to Control Mice

Since we previously observed increased plasma active GLP-1 levels in globally deficient neprilysin knockout mice fed a

HFD (3), we measured active GLP-1 levels in plasma collected 10 minutes after the oral glucose challenge in NEP^{Gut-/-} and control mice before and after 14 weeks of a HFD. Plasma active GLP-1 levels at baseline and after 14 weeks of HFD feeding were similar and did not differ among genotypes both at week 0 and week 14 (Fig. 3A). Further, active GLP-1 levels measured in distal small intestine at the end of the 14-week

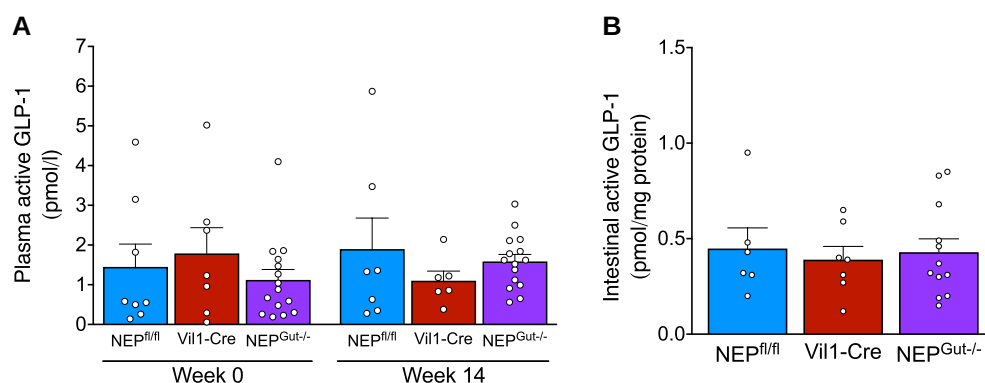


Figure 3. Active GLP-1 levels do not differ in plasma or intestine from NEP^{Gut-/-} vs control mice. (A) Active GLP-1 levels measured in plasma collected 10 minutes after the oral glucose challenge in NEP^{fl/fl}, Vil1-Cre, and NEP^{Gut-/-} mice before (week 0) and at the end (week 14) of the HFD feeding period. (B) Active GLP-1 levels measured in extracts of distal small intestine from NEP^{fl/fl}, Vil1-Cre, and NEP^{Gut-/-} mice fed a HFD for 14 weeks. Data are mean $\pm$ SEM. $n = 6-15$ /group.

Abbreviations: GLP-1, glucagon-like peptide-1; HFD, high-fat diet.

HFD feeding period were similar among the 3 groups of mice (Fig. 3B).

Discussion

In this study, by generating a new tissue-specific knockout mouse model, we show deletion of neprilysin in the gut decreases fasting plasma glucose, improves glucose tolerance, and increases glucose-stimulated insulin secretion in the setting of increased dietary fat. These findings emphasize a glucoregulatory role for intestinal neprilysin via the enteroinsular axis.

In clinical practice, a neprilysin inhibitor (sacubitril) is used in combination with a renin angiotensin system blocker (valsartan) for treatment of chronic heart failure. Evidence from clinical trials of sacubitril/valsartan indicates a beneficial glycemic impact of neprilysin inhibition in individuals with heart failure and type 2 diabetes (5, 6). In obese and/or diabetic mice, genetic (3, 10) or pharmacologic (8, 9) inhibition of neprilysin was associated with improved glucose tolerance and beta-cell function. Neprilysin is a peptidase that plays a pivotal role in degrading numerous peptides, including some with glucoregulatory properties as well as those involved in regulation of the cardiovascular system, neural signaling, and immune responses (2). Its ubiquitous distribution and broad substrate specificity underscore the necessity for targeted strategies to mitigate potential off-target effects and/or side effects associated with systemic inhibition. In clinical practice, sacubitril is combined with valsartan largely due to a need to control effects of elevated angiotensin II levels induced by systemic neprilysin inhibition (12). However, a recent study revealed that the acute administration of sacubitril/valsartan to individuals with obesity and type 2 diabetes resulted in impaired rather than improved glucose tolerance during a meal tolerance test (19). This effect was attributed to hyperglucagonemia induced by systemic neprilysin inhibition (19). Therefore, by employing tissue-specific strategies, a more precise modulation of neprilysin activity could be achieved, preserving essential physiological functions while effectively targeting pathological processes in specific tissues/organs.

At the level of specific tissues, we previously suggested the intestinal compartment per se participates in the insulinotropic effect of neprilysin inhibition. We observed that acute

and localized pharmacological inhibition of intestinal neprilysin in mice enhanced GSIS under physiological conditions (16). For the current study, we generated a mouse model with conditional deletion of neprilysin specifically in the enterocytes (NEP^{Gut-/-} mice) using Cre recombinase driven by the villin 1 promoter. Our validation of this model demonstrated near complete ablation of neprilysin activity throughout the gut, while its activity remained unaffected in the plasma. Notably, neprilysin activity in other tissues where it is abundantly expressed, namely kidney, or where its expression has been shown to affect GSIS, namely pancreas, was also not altered in NEP^{Gut-/-} mice. We previously reported that islets lacking neprilysin are protected against palmitate-induced reduction of GSIS (10). While we did not directly measure neprilysin activity in the islets of NEP^{Gut-/-} mice, we feel that depletion of intestinal neprilysin using a Cre recombinase targeted to epithelial cells of the intestine likely does not affect neprilysin activity in islets since it does not impact neprilysin activity in the whole pancreas (including after 14-week HFD feeding; data not shown). To assess whether intestinal-specific neprilysin deletion could be beneficial for GSIS in the context of increased dietary fat, NEP^{Gut-/-} and littermate control mice were fed a HFD for 14 weeks and insulin responses to oral glucose were determined. As expected, all mice developed impaired glucose tolerance on a HFD; however, NEP^{Gut-/-} displayed improvements in both glucose tolerance and GSIS compared to control mice, confirming a role for the intestinal compartment in the insulinotropic effect of neprilysin inhibition under HFD conditions.

A potential mechanism underlying the enhancement of GSIS with inhibition of intestinal neprilysin in HFD-fed mice may be through the incretin GLP-1, a substrate of neprilysin (14, 15). Improvements in glycemia and beta-cell function in HFD-fed mice with global neprilysin deficiency have been associated with increased active GLP-1 levels (3). Even though plasma active GLP-1 levels were not increased in our NEP^{Gut-/-} mice fed a HFD, it is possible GLP-1 receptor (GLP-1R) signaling plays a role in the beneficial insulinotropic effects observed. Indeed, acute inhibition of neprilysin specifically in the gut improves GSIS in a GLP-1R-dependent manner despite no increase in systemic active GLP-1 levels (16), suggesting that a localized reduction in GLP-1 degradation increases GLP-1R activation in the gut that then triggers insulin

secretion via neural signaling (20). We measured active GLP-1 levels in distal intestine, where there is typically a high density of GLP-1-producing L cells, to determine whether lack of neprilysin activity at this site is associated with higher concentrations of active GLP-1. We found that active GLP-1 levels were comparable between $NEP^{Gut-/-}$ and control mice, suggesting no preservation of local active GLP-1 with intestinal neprilysin deficiency. That said, these data do not rule out that mesenteric or portal active GLP-1 levels may be higher in $NEP^{Gut-/-}$ mice and that activation of GLP-1Rs in the hepatoportal region could be mediating the beneficial effects of selective intestinal neprilysin deficiency. Additionally, data from a very recent paper has highlighted inherent difficulties in reliably measuring plasma active GLP-1 levels during an OGTT in mice and that in vivo inhibition of the other GLP-1 degrading enzyme, namely dipeptidyl peptidase-4 (DPP-4), is required to stabilize GLP-1 (21). Importantly, inhibition of DPP-4 influences levels of glucose and insulin; thus it cannot be applied in studies such as ours where a primary outcome is to evaluate insulin secretion. Of note, our current data contrast with a previous study that reports that ablation of DPP-4 in enterocytes did not improve glucose tolerance when mice were fed a HFD (22). The authors concluded that DPP-4 in the villin⁺ gut epithelium is dispensable for incretin-mediated glucose regulation (22). We also cannot exclude that other insulinotropic peptides produced in the gut and known substrates of neprilysin, such as gastric inhibitory peptide (14), cholecystokinin-8 [CCK (23, 24)], and gastrin (24), may have contributed to the beneficial effects on GSIS and glucose tolerance observed in our $Nep^{Gut-/-}$ mice. In fact, we previously demonstrated that neprilysin inhibition in intestinal STC-1 cells cultured in high glucose/fat conditions increases CCK bioactivity/levels and that CCK bioactivity/levels are elevated in HFD-fed mice with global neprilysin deficiency (11). Further exploration is warranted to fully elucidate the intricate mechanisms underlying the impact of intestinal neprilysin inhibition on GSIS and to identify mediators involved in this effect.

In addition to impairment in glucose tolerance, control mice fed a HFD developed fasting hyperglycemia and fasting hyperinsulinemia. HFD-fed $NEP^{Gut-/-}$ mice also displayed fasting hyperinsulinemia but did not exhibit fasting hyperglycemia. The latter did not seem to be explained by changes in insulin sensitivity given that the QUICKI was similar among all groups of mice. Alternatively, it is possible that the absence of neprilysin in the gut of $NEP^{Gut-/-}$ mice reduced hepatic glucose production through preservation of gut-derived factors known to suppress gluconeogenesis, such as GLP-1 and CCK, thereby impacting fasting glucose levels. This would be in accordance with our recent data reporting suppression of gluconeogenesis in mice with either gut-selective or whole-body inhibition of neprilysin (11).

In conclusion, our findings identify a specific role for neprilysin in enterocytes in modulating glucose homeostasis and beta-cell function under HFD conditions and suggest that strategies targeting neprilysin specifically in the gut may protect against fat-induced insulin-secretory dysfunction and hyperglycemia.

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Author Contributions

N.E. conceived and designed the study, performed experiments, analyzed and interpreted data, and wrote the manuscript. S.M.M. and B.M.B. designed the study, performed experiments, analyzed and interpreted data, and edited the manuscript. S.Z. conceived and designed the study, analyzed and interpreted data, and edited the manuscript. All authors approved submission of the manuscript. S.Z. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosures

The authors have nothing to disclose.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

References

- Esser N, Utzschneider KM, Kahn SE. Early beta cell dysfunction vs insulin hypersecretion as the primary event in the pathogenesis of dysglycaemia. *Diabetologia*. 2020;63(10):2007-2021.
- Esser N, Zraika S. Neprilysin inhibition: a new therapeutic option for type 2 diabetes? *Diabetologia*. 2019;62(7):1113-1122.
- Willard JR, Barrow BM, Zraika S. Improved glycaemia in high-fat-fed neprilysin-deficient mice is associated with reduced DPP-4 activity and increased active GLP-1 levels. *Diabetologia*. 2017;60(4):701-708.
- Kjeldsen SAS, Gluud LL, Werge MP, et al. Neprilysin activity is increased in metabolic dysfunction-associated steatotic liver disease and normalizes after bariatric surgery or GLP-1 therapy. *iScience*. 2023;26(11):108190.
- Seferovic JP, Claggett B, Seidelmann SB, et al. Effect of sacubitril/valsartan versus enalapril on glycaemic control in patients with heart failure and diabetes: a post-hoc analysis from the PARADIGM-HF trial. *Lancet Diabetes Endocrinol*. 2017;5(5):333-340.
- Wijkman MO, Claggett B, Vaduganathan M, et al. Effects of sacubitril/valsartan on glycemia in patients with diabetes and heart

- failure: the PARAGON-HF and PARADIGM-HF trials. *Cardiovasc Diabetol.* 2022;21(1):110.
7. Jordan J, Stinkens R, Jax T, *et al.* Improved insulin sensitivity with angiotensin receptor neprilysin inhibition in individuals with obesity and hypertension. *Clin Pharmacol Ther.* 2017;101(2):254-263.
 8. Esser N, Mongovin SM, Parilla J, *et al.* Neprilysin inhibition improves intravenous but not oral glucose-mediated insulin secretion via GLP-1R signaling in mice with β -cell dysfunction. *Am J Physiol Endocrinol Metab.* 2022;322(3):E307-E318.
 9. Esser N, Schmidt C, Barrow BM, *et al.* Insulinotropic effects of neprilysin and/or angiotensin receptor inhibition in mice. *Front Endocrinol (Lausanne).* 2022;13:888867.
 10. Zraika S, Koh D-S, Barrow BM, Lu B, Kahn SE, Andrikopoulos S. Neprilysin deficiency protects against fat-induced insulin secretory dysfunction by maintaining calcium influx. *Diabetes.* 2013;62(5):1593-1601.
 11. Esser N, Mongovin SM, Mundinger TO, Barrow BM, Zraika S. Neprilysin deficiency reduces hepatic gluconeogenesis in high fat-fed mice. *Peptides.* 2023;168:171076.
 12. Campbell DJ, Anastasopoulos F, Duncan AM, James GM, Kladis A, Briscoe TA. Effects of neutral endopeptidase inhibition and combined angiotensin converting enzyme and neutral endopeptidase inhibition on angiotensin and bradykinin peptides in rats. *J Pharmacol Exp Ther.* 1998;287(2):567-577.
 13. Bunnett NW, Wu V, Sternini C, *et al.* Distribution and abundance of neutral endopeptidase (EC 3.4.24.11) in the alimentary tract of the rat. *Am J Physiol.* 1993;264(3 Pt 1):G497-G508.
 14. Hupe-Sodmann K, McGregor GP, Bridenbaugh R, *et al.* Characterisation of the processing by human neutral endopeptidase 24.11 of GLP-1(7-36) amide and comparison of the substrate specificity of the enzyme for other glucagon-like peptides. *Regul Pept.* 1995;58(3):149-156.
 15. Windelov JA, Wewer Albrechtsen NJ, Kuhre RE, *et al.* Why is it so difficult to measure glucagon-like peptide-1 in a mouse? *Diabetologia.* 2017;60(10):2066-2075.
 16. Esser N, Mundinger TO, Barrow BM, Zraika S. Acute inhibition of intestinal neprilysin enhances insulin secretion via GLP-1 receptor signaling in male mice. *Endocrinology.* 2023;164(5):bqad055.
 17. Gee NS, Bowes MA, Buck P, Kenny AJ. An immunoradiometric assay for endopeptidase-24.11 shows it to be a widely distributed enzyme in pig tissues. *Biochem J.* 1985;228(1):119-126.
 18. Esser N, Barrow BM, Choung E, Shen NJ, Zraika S. Neprilysin inhibition in mouse islets enhances insulin secretion in a GLP-1 receptor dependent manner. *Islets.* 2018;10(5):175-180.
 19. Wewer Albrechtsen NJ, Møller A, Martinussen C, *et al.* Acute effects on glucose tolerance by neprilysin inhibition in patients with type 2 diabetes. *Diabetes Obes Metab.* 2022;24(10):2017-2026.
 20. Burcelin R, Serino M, Cabou C. A role for the gut-to-brain GLP-1-dependent axis in the control of metabolism. *Curr Opin Pharmacol.* 2009;9(6):744-752.
 21. Smits MM, Galsgaard KD, Jepsen SL, Albrechtsen NW, Hartmann B, Holst JJ. In vivo inhibition of dipeptidyl peptidase 4 allows measurement of GLP-1 secretion in mice. *Diabetes.* 2024;73(5):671-681.
 22. Mulvihill EE, Varin EM, Gladanac B, *et al.* Cellular sites and mechanisms linking reduction of dipeptidyl peptidase-4 activity to control of incretin hormone action and glucose homeostasis. *Cell Metab.* 2017;25(1):152-165.
 23. Deschodt-Lanckman M, Strosberg AD. In vitro degradation of the C-terminal octapeptide of cholecystokinin by 'enkephalinase A'. *FEBS Lett.* 1983;152(1):109-113.
 24. Andersen U, Terzic D, Wewer Albrechtsen NJ, *et al.* Sacubitril/valsartan increases postprandial gastrin and cholecystokinin in plasma. *Endocr Connect.* 2020;9(5):438-444.