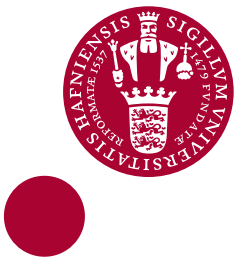

Neuromedin U Does Not Act as a Decretin in Rats

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Lack of decretin action of Neuromedin U in rats

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Summary

Studies on isolated pancreatic islets suggest that Neuromedin U (NMU), a brain and gastrointestinal peptide, act as a *decretin* hormone, inhibiting glucose-stimulated insulin secretion. We investigated whether this effect could be reproduced *in vivo* and in isolated perfused rat pancreas. Unlike the incretin hormone, glucagon-like pepide-1 (GLP-1), intravenous NMU administration had no effects on blood glucose and plasma insulin and glucagon *in vivo*. Moreover, NMU neither changed insulin, glucagon, or somatostatin secretion from isolated perfused rat pancreas, nor affected GLP-1-stimulated insulin and somatostatin secretion. For NMU to act as a decretin hormone, its secretion should increase following glucose ingestion; however, glucose did not affect NMU secretion from isolated perfused rat small intestine, which contained extractable NMU. Furthermore, the two NMU receptors were not detected in endocrine rat or human pancreas. We conclude that NMU does not act as a decretin hormone in rats.

Keywords: Decretin, glucagon-like peptide-1, neuromedin U, insulin secretion, type-2-diabetes.

Introduction

The peptide hormone, Neuromedin U (NMU), expressed in the brain and gastrointestinal tract in rats and humans (Augood et al., 1988; Austin et al., 1995; Ballesta et al., 1988; Domin et al., 1987; Fujii et al., 2000; Honzawa et al., 1990) has been reported to attenuate glucose-stimulated insulin secretion from isolated perfused rat pancreas and from perifused human pancreatic islets (Alfa et al., 2015; Kaczmarek et al., 2009; Kaczmarek et al., 2006). As this effect is opposite of that of the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), NMU was proposed to be a *decretin* hormone. However, the underlying mechanisms responsible for the proposed decretin effect were unclear, and more importantly, it is unknown whether the inhibitory effect of NMU on insulin secretion occurs in a physiological setting. We reasoned that for NMU to act as a decretin hormone during physiological circumstances, it should be secreted from the gut in response to at least glucose ingestion and to circulate in sufficient amounts to influence the endocrine pancreas, but NMU has proven difficult to measure in plasma and is generally considered a locally acting neuropeptide (Greenwood et al., 2011), which would be incompatible with a decretin function of NMU in the strict sense. If NMU can be demonstrated to be secreted from the gastrointestinal tract in response to glucose, it might indeed act as a *decretin* hormone with postprandial insulin secretion being regulated in a balance between stimulatory incretin and inhibitory decretin hormones, but evidence for this is lacking. This question is particularly relevant for the regulation of insulin secretion after gastric bypass surgery, where it is debated whether the postoperative improvement in glucose tolerance is due to prevention of the actions of a duodenal decretin hormone (the upper gut hypothesis) or promotion of the secretion of incretin hormones (the lower gut hypothesis) (Rubino et al., 2006a; Salinari et al., 2017). However, peripheral NMU injections have been reported to stimulate GLP-1 secretion in mice and to improve glucose tolerance and enhance insulin secretion, which would seem incompatible with the proposed decretin action of NMU (Peier et al., 2011). The purpose of the present study was to investigate the potential decretin actions of NMU in a relevant physiological setting. To this end, we carried out *in vivo* studies in rats and supplemented with studies of isolated

perfused rat pancreas and small intestine, which allow assessment of the *direct* effects of NMU on the secretion of glucagon, insulin and somatostatin (SST) from the pancreas (by excluding potential, confounding systemic factors) as well as measurement of a possible effect of NMU on GLP-1 secretion from the small intestine, and determination whether NMU is secreted from the gut upon luminal glucose exposure.

Results

Effects of intravenous NMU on blood glucose and insulin and glucagon secretion in the rat.

To investigate whether NMU has effects on blood glucose and glucagon and insulin secretion in a physiological setting, we challenged anesthetized rats with intravenous glucose (1g/kg) alone or together with NMU (21 nmol/kg), GLP-1 (2 nmol/kg) or NMU+GLP-1.

Blood glucose: Baseline blood glucose concentrations did not differ between groups (glucose = 8.8±0.2, glucose + GLP-1 = 9.1±0.2, glucose + GLP-1 + NMU = 10±0.6, glucose + NMU = 9.4 ± 0.2, P>0.05 for all groups). Intravenous glucose administration increased blood glucose concentrations, peaking at 1 min after administration in all treatment groups. Administration of GLP-1 lowered peak blood glucose and iAUC values (peak value: glucose = 23±1.8 mM vs. glucose + GLP-1 = 19±1.5 mM, P=0.001, $iAUC_{0\text{-}30\ min}$: glucose = 187±21 min×mM vs. glucose + GLP-1 = 122±19 min×mM, P =0.13; P=0.03 by unpaired two-way t-test), Fig. 1A and B). Blood glucose excursions over the entire experiment were similar in the glucose and glucose + NMU groups ($iAUC_{0\text{-}30min}$ = 185±18 min×mM, P>0.99 compared to glucose group, Fig. 1A and B), and NMU had no significant effect on the glucose lowering effects of GLP-1, (glucose+GLP-1+NMU, $iAUC_{0\text{-}30min}$ = 158±24 min×mM, P=0.65 compared to glucose+GLP-1 group, Fig. 1A and B).

Plasma insulin: Plasma insulin concentrations did not differ between groups at baseline (P>0.05). Glucose administration caused an instant and robust increase in plasma insulin, which was potentiated by 200-300% by GLP-1, both with respect to peak concentrations

and overall concentrations ($iAUC_{0\text{-}30min}$: glucose = 84±9.6 min×ng/ml vs. glucose+GLP-1 = 174±29 min×ng/ml, P<0.05, *n* = 10, Fig. 1.C and D). The potentiating effect of GLP-1 on glucose-stimulated insulin secretion was not significantly altered when NMU was co-administered (($iAUC_{0\text{-}30min}$ = 161±20 min×ng/ml glucose+GLP-1+NMU), $P_{Glucose+GLP\text{-}1\ vs.\ Glucose+GLP\text{-}1+NMU}$ =0.97). Similarly, there was no difference between glucose + NMU and glucose alone on plasma insulin levels (iAUC's = 104±22 min x ng/ml, P=0.91 compared to glucose alone, *n* = 10, Fig.1.D).

Glucagon: Plasma glucagon was low at baseline (<4 pM for all groups) and did not change significantly at any time points in any of the groups in response to the treatments (P≥0.29 between all groups) and the concentration profiles over the course were not significantly different either ($iAUCs_{0\text{-}30min}$ glucose = 100±25 min×pM vs. glucose+GLP-1 = 59.7±12 min×pM vs. glucose+GLP-1+NMU = 58.2±9.9 min×pM vs. glucose+NMU = 85.7±15 min×pM, P≥0.29 for all groups vs. glucose alone, *n* = 10, Fig. 1E and F).

Effects of NMU on isolated perfused rat pancreas

As NMU had no detectable effects on the secretion of glucagon or insulin *in vivo,* we next studied the potential effects of NMU on the secretion of these hormones using the isolated perfused rat pancreas. This model has superior sensitivity because the perfusate is collected from the vein draining the preparation meaning that secreted peptides do not reach the liver and systemic circulation were they may be exposed to extensive extraction, degradation and dilution.

Insulin secretion increased approximately 3.5-fold when glucose was elevated from 3.5 to 10 mM (total secretory outputs during 15 min perfusion: 3.5 mM glucose = 1960±165 pmol vs. 10 mM glucose = 6983±1503 pmol, P<0.01, *n* = 7, Fig. 2A.1 and 2A.2). In the same experiments NMU administration (100 nM) neither affected insulin secretion at 3.5 nor at 10 mM glucose concentrations ($P_{baseline\ vs.\ NMU\ response\ at\ 3.5\ mM\ glucose}$ = 0.41, $P_{baseline\ vs.\ NMU\ response\ at\ 10\ mM\ glucose}$ = 0.22, n=7, Fig. 2A.1 and 2A.2). In control experiments, insulin secretion (at 10 mM glucose) approximately doubled in response to GLP-1 (total output at baseline (15 min) = 5602±692 pmol vs. total output during GLP-1 administration (15 min)

= 11581±1526 pmol, P<0.01, Fig. 2B.1 and 2B.2) and when administered twice in the same experiment, the second response was not significantly different from the first (total outputs: 6357±1917 pmol vs. 7250±1957 pmol, P=0.56, Fig. 2B.3). In a separate line of experiments, GLP-1 administration resulted in a comparable increase in insulin secretion ($P_{baseline\ vs.\ response}$<0.05) and the response was unaffected by co-administration of NMU (total outputs during 15 min: GLP-1 = 7367±1124 pmol vs. GLP-1+NMU = 7375±1215 pmol, *n* =7, Fig. 2C.1-2C.3). In the same set of experiments glucagon and SST secretion were also investigated.

Glucagon secretion responded to glucose concentrations in a pattern opposite that of insulin, decreasing by a factor of two in response to glucose elevation (total outputs during 15 min: 449.0±101 vs. 262.6±39.8 pmol, P<0.05, Fig. 3A.1 and 3A.2), but neither GLP-1 nor NMU affected its secretion (Fig. 3B,C). *Somatostatin* secretion increased by approximately 50% in response to increased glucose concentration (total baseline output (during 15 min) = 44.9±5.5 pmol vs. output at GLP-1 infusion (15 min) = 64.6±7.3 pmol, P<0.05, Fig. 3D.1 and D2). GLP-1 approximately doubled SST secretion (total output at baseline (15 min) = 57.7±4.5 pmol vs. total output during GLP-1 administration = 89.5±8.9 pmol, P < 0.01, Fig. 3.E1 and 4.E2) and did so to a comparable extent when administered twice in the same experiment (total outputs = 24.0±4.5 vs. 21±5.7 = P=0.66, Fig. 3.E3). NMU had no effect on glucose- and GLP-1-stimulated SST secretion (P = 0.84, Fig. 3F1-3).

Gastrointestinal expression and secretion for NMU in the rat

NMU was expressed in the duodenum, proximal jejunum, distal ileum and colon in the rat reaching tissue concentrations of ~20-50 pmol/g (sensitivity < 1 pmol/g). NMU was undetectable in extracts of the esophagus and ventricle (Fig. 5A). Despite the relevant amounts of NMU in the extracts of the small intestine, no measurable NMU was detected in venous effluents from the perfused gut, neither at baseline nor in response to infusion of luminal glucose (20% v/w) (data not shown, *n =6*) although the sensitivity would have allowed detection of increases in the effluent concentration of less than 2 pmol/l. In

contrast, GLP-1 was secreted in significant amounts under basal conditions and its secretion increased approximately 8-fold in response to luminal glucose ($P < 0.001$ compared to baselines (Kuhre et al., 2017)).

Control for bioactivity of the applied NMU

To ensure that the lack of effects of NMU on pancreatic hormone secretion was not attributable to lack of activity of the synthetic NMU peptide applied in the different experiments, we tested the ability of the same peptide to activate the rat NMUR1 via the G_q pathway. In COS-7 cells transiently expressing the rat NMUR1, the peptide (rat NMU-23) activated the receptor with a potency of 19 nmol/l ($logEC_{50}$ -7.7±0.11, *n*=3: Fig. 4B).

NMUR1 and NMUR2 expression in the pancreas

For NMU to act directly on the endocrine pancreas, one of the two NMU receptors has to be expressed here. Thus, we investigated the expression of the two NMU receptors, NMUR1 and NMUR2, by qPCR. In both rat and human islets, NMUR1 expression was below detection limit (Ct-value > 33). NMUR2 expression was minimal, but detectable, in human and rat islets and was approximately 25 and 43-folds lower than the respective expression of GLP-1R (Ct-value GLP-1R: human = 27.9±0.55, rats = 22.6±0.12) (Fig. 5C, D). In addition, we investigated NMUR1 and NMUR2 expression in sorted human alpha, beta and delta-cells. Expression levels were extracted from published single cell RNA sequencing databases, originating from 3 different populations (combined n = 26) (Muraro et al., 2016; Segerstolpe et al., 2016; Xin et al., 2016). NMUR1 and NMUR2 were not detected in any of the endocrine cell types, but GLP-1R (positive control) was detected in both beta and delta cells (Fig. 4E).

Consistent with the lack of expression, NMUR1 could not be detected in the rat pancreas by immunohistochemistry, and was also absent islets (Fig. 4E and supplementary figure 1), but the receptor was expressed in the small intestine (Fig. 4F). NMUR2 was also not detected in the pancreatic islets (or the small intestine), but it was detected in the exocrine rat pancreas (Fig. 4E).

No effects of NMU on GLP-1 secretion from isolated perfused rat small intestine

NMU has been reported to stimulate GLP-1 secretion in mice when administered peripherally (Peier et al., 2011). To investigate whether this effect is a direct effect of NMU actions on the gut, we administered NMU-23 to isolated perfused rat small intestine. NMU did not affect GLP-1 secretion (baseline secretion: 233.8 ± 93.5 pmol/15 min vs. NMU = 308.5 ± 123.9 pmol/15 min, P=0.57, *n*=5) although the positive control, taurine-deoxycholic acid, increased secretion 4-fold (from 405.6 ± 151 pmol/15 min to 1391 ± 324 pmol/15 min; Supplementary fig. 2A-C).

Discussion

NMU is a neuropeptide predominately expressed in the brain and small intestine (Augood et al., 1988; Domin et al., 1987). It was reported to attenuate glucose-stimulated insulin secretion from *in situ* perfused rat pancreas and perifused human islets (Alfa et al., 2015; Kaczmarek et al., 2009; Kaczmarek et al., 2006), which led to the proposal that NMU represents a new physiologically relevant inhibitory factor. One of the theories offered to explain the anti-diabetic outcome of gastric bypass surgery is that secretion of an upper intestinal *decretin* factor is eliminated by the bypass (Rubino et al., 2006b). Therefore, it was proposed (Alfa et al., 2015; Kaczmarek et al., 2009; Kaczmarek et al., 2006) that one such factor could be NMU. The studies leading to this proposal were based on *in situ* perfused rat pancreas and perifused isolated human islets. In the perfusion study, the attenuating effect of NMU appeared to be mediated indirectly by NMU-stimulated SST secretion (through activation of NMUR1 on the pancreatic δ-cell)(Kaczmarek et al., 2009), while the study on perifused human islets (Alfa et al., 2015) concluded that the decretin effect of NMU resulted from a direct effect on the β-cell, as in that study, NMUR1 was found to co-localize with insulin but not with glucagon or SST. Besides this apparent inconsistency regarding its mechanism of action, two additional observations question the potential decretin role of NMU: 1) peripheral administration of NMU in mice was reported to stimulate GLP-1 secretion and improve glucose-tolerance after OGTT (Peier et al.,

2011), which must be assumed to counteract a decretin effect of NMU since GLP-1 is a potent enhancer of glucose-stimulated insulin secretion, and 2) the majority of studies regarding NMU-receptors have shown that NMUR1 and -2 couple to $G_q/11$(Raddatz et al., 2000; Shan et al., 2000; Szekeres et al., 2000), meaning that activation of the receptors will result in the production of IP_3 (inositol trisphosphate) and DAG (diacylglycerol) which would be associated with *stimulation* of insulin secretion (Straub and Sharp, 2002). However, most importantly, it remained unknown whether NMU acts as a decretin under physiological conditions. Therefore, we examined the effects of NMU on blood glucose and plasma glucagon and insulin in rats challenged with an intravenous glucose bolus (delivered intravenously in order to avoid secretion of GLP-1 from the gut). In this model, NMU had no effects on any of the parameters when administered alone and did not significantly counteract the glucose lowering effects of GLP-1. We next investigated whether glucose would stimulate the secretion of NMU from its primary site of expression – the intestine. Contradicting a physiological role of NMU as a decretin hormone, we detected no measureable NMU secretion from isolated perfused rat small intestine (both upper and lower small intestine were investigated separately), be it at basal conditions or in response to luminal glucose. In contrast, in the same experiments, luminal glucose stimulated the secretion of GLP-1 approximately 8-fold compared to baseline (to 400-500 fmol/min) (Kuhre et al., 2017). Importantly, the lack of detectable NMU secretion is unlikely to be related to insensitivity of the assay we developed for measuring NMU as this assay reacts with endogenous rat NMU demonstrated by the measurements on gut biopsies and furthermore has a low detection limit (below 2 pmol/l). To unveil any potential direct effects of NMU on pancreatic hormone secretion with greater sensitivity, we also investigated the effects of NMU on glucagon, insulin and SST secretion from the isolated perfused rat pancreas. Consistent with the *in vivo* study, NMU had no effects on the secretion of glucagon, insulin and SST, be it at low or high glucose, and NMU also did not affect the stimulatory effects of GLP-1 on insulin and SST secretion. As NMU-23 has been shown to activate NMUR1 and -2 with comparable potency and efficacy (Brighton et al., 2008), and since the concentration used was well above EC_{50} for both receptors, a clear response was expected, if the receptors were expressed. The reason for the different results

obtained by us and those obtained by (Kaczmarek et al., 2006), where NMU in a comparable experimental model was found to inhibit insulin secretion when administered in the same dose as in our study (100 nM) (Kaczmarek et al., 2006), is difficult to unravel. However, the inhibitory effect (15-25%) elicited by NMU was only clear when the pancreas was perfused at a supra-physiological glucose concentration (26.4 mM), whereas no effect was observed at a physiological glucose concentration (6.6 mM), closer to the glucose concentrations used in our studies (3.5 and 10 mM). The same authors later reported that the inhibitory effects of NMU on insulin secretion might be mediated via secretion of SST (Kaczmarek et al., 2009), but in that study SST secretion was only minimally affected by NMU (10-15% increase), and our data do not support a stimulatory role of NMU on SST secretion. Importantly, the lack of responses to NMU in our experiments is unlikely to reflect that inappropriate NMU concentrations had been used or that the peptide was not bioactive, since NMUR1 was activated by the NMU-23 peptide used for the *in vivo* and perfusion studies with a potency (EC_{50}) of 19 nM (in agreement with previously published results(Raddatz et al., 2000)). In the three studies reporting a decretin effect of NMU, the authors used NMU-25, whereas we used NMU-23. We chose to use NMU-23 rather than NMU-25, since NMU-23 is the molecular form of the peptide produced in rats (whereas NMU-25 is the form produced in humans).

In addition to the negative functional data, we were unable to detect expression of the two NMU-receptors (NMUR1 and -2) in the endocrine pancreas (Islets of Langerhans) by immunohistochemistry. The three studies reporting a decretin effect of NMU did report NMUR1 expression but with striking inconsistency. In the study by Alfa and colleagues, NMUR1 was found expressed in the pancreatic β-cells, whereas Kaczmarek and colleagues located the receptor to the δ-cell (Alfa et al., 2015; Kaczmarek et al., 2009; Kaczmarek et al., 2006). Based on the available data, it is impossible to deduce whether the discrepancies between our results and the results published by Kaczmarek reflect differences in antibody specificity. Unfortunately, in the cited studies neither NMUR1 negative tissue nor tissues from $NMUR1^{-/-}$ mice were investigated. However, the anti-NMUR1 antibody used by Alfa and colleagues appears to be the same as the one we used, but while we find minimal

staining in the rat islets, they found a staining intensity in human beta cells comparable to the intensity of insulin staining. In our study, staining was evident in neurons in the rat brain (positive control), indicating that the negative findings in the pancreas were not due to technical issues. In order to look further into this inconsistency, we investigated NMUR1 and NMUR2 expression in the islet by qPCR. In both rat and human islets, NMUR1 expression was below detection limit whereas NMUR2 expression was low and very close to the detection limit. In support of this, RNA sequencing based NMUR1 and -2 expressions in sorted human α, β and δ-cells, extracted from three public available databases (Muraro et al., 2016; Segerstolpe et al., 2016; Xin et al., 2016), showed no detectable expression of the receptors. In contrast, GLP-1R was, in agreement with other studies (Tornehave et al., 2008) expressed in β-cells but not α-cells. Again, this contrasts to the results presented by Alfa and colleagues, as they reported identification of NMUR1 mRNA in human islets. Their expression data are difficult to assess as primer sequences were not provided. Furthermore, data are presented as delta-delta Ct-values normalized to expression in liver tissue (2.3 fold higher in islets), but the delta-Ct-value for liver expression was not reported which is unfortunate, as NMUR1 expression was previously reported undetectable in human liver tissue (Raddatz et al., 2000). The absolute level of expression in islets reported by Alfa et al. is therefore difficult to evaluate.

Taken together, our study therefore demonstrates that NMU does not as act as a decretin hormone in rats and is unlikely to have any direct effects on the endocrine pancreas in humans.

Author Contributions

REK, NJWA and JJH conceptualized and designed the study. REK, NJWA and BH performed the in vivo experiments. REK and CBC performed and analyzed isolated perfused pancreata. REK, BH and NJWA performed and analyzed *in vivo* experiments. BH, NJWA and PAS developed the NMU RIA assay and assayed gut perfusion and biopsies samples, IMM isolated tissue and quantified NMU. MBNG and MMR performed and analyzed pharmacology experiments. SMG and TMP did qPCR expression profile of

NMUR1, NMUR2 and GLP-1R on rat and human pancreatic islets. NJWA did the analysis of single cell RNA sequencing data from sorted human alpha-, beta- and delta-cells. RA and NJWA performed and analyzed immunofluorescence. REK and NJWA drafted the manuscript; CBC, SMG, MBNG, PAS, IMM, CØ, BH, TMP, RA MMR, BH and JJH critically revised the manuscript for important intellectual content and approved the final version. JJH is responsible for the integrity of the work as a whole.

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Main figure titles and legends

Fig. 1. Effects of GLP-1 and NMU on blood glucose clearance and plasma insulin and glucagon. Data are shown as means±SEM. *A:* Blood glucose (mM), *C:* Plasma insulin (ng/ml), *E:* Plasma glucagon (pM). Response to intravenous injection with glucose (black) glucose+GLP-1(brown), glucose+GLP-1+NMU (blue), and glucose+NMU (grey). *B, D, F*: iAUC's of *A, C* and *E,* respectively. Stars above time points indicate difference between glucose group vs. glucose+GLP-1 group (brown stars) and glucose vs. glucose+GLP-1+NMU group (blue stars). $N = 10$. Statistical significance was tested by One way ANOVA for repeated measurements followed by Bonferroni multiple comparisons test; *$P < 0.05$, ***$P < 0.001$.

Fig. 2. Insulin secretion from isolated perfused rat pancreas is controlled by glucose levels and GLP-1 but not by NMU. Data are shown as means±SEM. *A.1 and 2.* Insulin secretion/output in response to NMU at 3.5 and 10 mM glucose. *B.1-3:* Insulin secretion/output (at 10 mM glucose) in response to GLP-1 (1 nM). *C1-C3:* Insulin secretion (at 10 mM glucose) in response to GLP-1 (1 nM) and NMU (100 nM). Dots show results from respective experiments. Statistical significance was assessed by paired t-test; *P<0.05, **P<0.01, ****P<0.0001, $n = 7$.

Fig. 3. Glucagon and somatostatin secretion from isolated perfused rat pancreas is controlled by glucose levels but not by GLP-1 or NMU. Data are shown as means±SEM. *A.1 and 2.* Glucagon secretion in response to NMU (100 nM) at 3.5 and 10 mM glucose. *B.1-3:* glucagon secretion (at 10 mM glucose) in response to GLP-1 (1 nM). *C1-C3:* glucagon secretion (at 10 mM glucose) in response to GLP-1 and GLP-1 (1 nM) + NMU (100 nM). *D.1 and 2.* Somatostatin secretion in response to NMU (100 nM) at 3.5 and 10 mM glucose. *E.1-3:* Somatostatin secretion (at 10 mM glucose) in response to GLP-1 (1 nM). *F1-C3:* Somatostatin secretion (at 10 mM glucose) in response to GLP-1 and GLP-1 + NMU (100 nM). Dots show results from respective experiments. Statistical significance was assessed by paired t-test *P<0.05, **P<0.01, $n = 7$.

Fig. 4. NMU expression down the rat gastrointestinal tract, NMUR1 receptor activation and NMUR1 and -2 expressions in pancreas and gut. *A:* Extractable NMU (total) concentrations down the gastrointestinal tract in the rat. *B:* NMUR1 receptor activation by synthetic rat NMU-23 in COS-7 cells transiently expressing the rat NMUR1 (measured as IP_3 formation). *C:* NMUR-1, NMUR-2, and GLP-1R expression in human islets. D: NMUR-1, NMUR-2 and GLP-1R expression in rat islets. . E: Expression of NMUR1, NMUR2 and GLP-1R in sorted human alpha-, beta-, and delta-cells islets (by single cell RNA sequencing). *F*: Representative NMUR1 and NMUR2 staining's in rat pancreas and small intestine. Data in A and B are show as means±SEM. Dots in *A* indicate individual data points. "I" in subfigure *E* indicate pancreatic islets. Statistical significance was assessed by One way ANOVA for repeated measurements followed by Bonferroni multiple comparisons test (*A, C and D*), ; *P<0.05, **P<0.01, ****P<0.0001, *n(A) = 7, n(B) = 3, n(C) = 6 n(D) = 5, n(E) = 27 (collected from three different populations), n(F) = 4.*

STAR methods

Contact of reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Jens Juul Holst (jjholst@sund.ku.dk).

Experimental model and subject details

Animal studies

Ethical considerations: Animal studies were conducted with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and approved by the local ethical committee and were conducted in accordance with the EU Directive 2010/63/EU and guidelines of Danish legislation governing animal experimentation (1987) and the NIH (publication number 85-23).

Animals: Male Wistar rats were obtained (~250g) from Janvier (Saint Berthevin Cedex, France) and housed two-a-cage under standard conditions with ad libitum access to chow and water, following a 12:12h light and dark cycle. Rats were allowed to acclimatize for at least one week before the day of study.
Cell line studies: COS-7 cells (non-human primate cell line, isolated from a male *Chlorocebus aethiops* (Green monkey)) were purchased at ATCC (Cat. No. CRL-1651) and cultured at 10% CO_2 and 37°C in Dulbecco's modified Eagle's medium 1885 supplemented with 10% foetal bovine serum, 2 mM glutamine, 180 units/ml penicillin, and 45 g/ml streptomycin.

Method details

In vivo study: At the respective day of study, food was withdrawn just before the feeding period (the dark period, 1700h), meaning that rats were "semi-fasted" at the time of study. Rats were divided into weight matched groups and anesthetized by a subcutaneous injection with Hypnorm/midazolam (0.079 mg fentanyl citrate + 2.5 mg fluanisone + 1.25 mg midazolam/ml: 0.3 ml/100g body weight). The abdominal cavity was opened and a needle inserted into the inferior caval vein, blood (300 µL/time point) was withdrawn through this needle into EDTA coated syringes (cat. no. 03690, Sigma Aldrich) and samples were

transferred to 1.5 ml EDTA-coated tubes (cat. no. 200 K3E, Microvette; Sarstedt, Nümbrecht, Germany). Between sample collections, the needle was flushed with isotonic saline to clean the needle and prevent clot formation (100 µL, room temperature). Samples were drawn at *time* -5, 0, 1, 2, 5, 10, 15, 30 min. In total 2.4 mL blood was withdrawn (corresponding to approximately 10% of the total blood volume). Approximately 0.8 mL isotonic saltwater was administered in total, compensating for approximately half of the fluid loss. Baseline samples were collected 5 min after insertion of the needle (-5min) and immediately before administration of test substance (0 min), which was gently injected through the needle over 5 seconds (total volume 1 mL). The rats received intravenously either 10% (w/v) glucose (1 mL/300g body weight ~ 0.3g in total), 10% glucose + 2 nmol/kg GLP-1, 10% glucose + 2 nmol/kg GLP-1 + 21 nmol/kg NMU or 10% glucose + 63 nmol/kg NMU. Rats from the same cage received different treatments. Blood glucose was measured immediately after collection as described below. The remaining sample was kept on ice, centrifuged within 15 min (1,650 *g*, 4°C, 10 min) and plasma was transferred to fresh Eppendorf tubes and stored at -20°C until hormone quantification. Weight did not differ between experimental groups (mean ± SEM: glucose = 322±4.8g, glucose+GLP-1 = 290±29g, glucose+GLP-1+NMU = 296±23g, glucose+NMU = 316±4.9g, n = 10, $P > 0.05$ for all groups).

Isolated perfused rat pancreas: Male Wistar rats (~350g) were obtained from Janvier and housed and anesthetized as described above. The entire large intestine and the small intestine were removed after tying off the supplying vasculature, leaving only the proximal duodenum connected to the pancreas *in situ.* Furthermore, the spleen and stomach were removed and the kidneys were excluded from the isolation by tying off the renal arteries. The abdominal aorta was tied off just below the diaphragm and immediately after a catheter was inserted retrogradely into the abdominal aorta just proximal to the renal arteries, so that the aorta now exclusively supplied the pancreas (through both the coeliac- and the superior mesenteric artery). The pancreas was perfused with perfusion buffer (5.0 ml/min) which had been heated to 37°C and oxygenated with 95% O_2, 5% CO_2 prior to perfusion (resulting in maximal O_2 saturation and a pH of 7.3-7-4) by use of a UP100 Universal Perfusion

System from Hugo Sachs (Harvard Apparatus, March Hugstetten, Germany). Venous effluent samples were collected each minute from a catheter inserted into the portal vein. Immediately after establishment of perfusion, the rat was euthanized by perforation of the diaphragm. The preparation was allowed to stabilize for approximately 30 min before the experiment was started. Test stimulants consisted of 1 nM GLP-1, 100 nM NMU and 10 mM L-arginine provided by the manufacturers specified below. All stimulants were diluted in perfusion buffer without addition of solubilizers. L-arginine, a well-known stimulator of glucagon, insulin and somatostatin secretion, was infused at the end of all experiments as a positive control for pancreas responsiveness. Perfusion pressure and output were closely monitored throughout the experiment and used as an indication of the wellbeing of the preparation. Both parameters remained stable during the perfusion. Methods are described in more details elsewhere (Christiansen et al., 2015).

Isolated perfused rat small intestine: In brief, the small intestine was isolated and perfused from male Wistar rats (~250g) by a protocol that was similar to the pancreas perfusion protocol. Depending on the study, the entire small intestine was isolated (in the studies were synthetic NMU was administered) or the upper or lower half of the small intestine was isolated (in the studies were luminal glucose was administered). In contrast to the pancreas perfusion studies, the intestine was perfused only through the upper mesenteric artery, which does not supply the stomach, spleen and kidneys – these organs were therefore left untouched. Only the large intestine and either the upper or lower half of the small intestine were removed after tying off the supplying vasculature. A plastic tube was inserted into the lumen of the remaining part of the intestine (either the upper of lower half) and the lumen cautiously flushed with isotonic saline followed by a continuous slow infusion (0.5 ml/min). The gut was perfused vascularly at a rate of 7.5 ml/min and venous effluent was collected each minute from the portal vein. Luminal glucose (20% w/v, in isotonic saline) was administered at an initial rate of 2.5 ml/min for the first three minutes (to quickly replace the saline in the lumen) and hereafter at 0.5 ml/ min for the remaining stimulus period. After stimulation the lumen was flushed with isotonic saline at 2.5 ml/min the first three minutes and then at 0.5 ml/min for the remaining time of the experiment.

Synthetic NMU-23 and tauro-deoxycholic acid (pos. control) was prepared in perfusion buffer and administered intra-arterially through a three-way cock-valve by use of a syringe pump. The method is described in more detail elsewhere (Kuhre et al., 2014b).

Chemicals and peptides: GLP-1 (7-36NH_2) and NMU (amidated rat NMU-23) were purchased from Bachem (Cat. no. 4016663 and 4030663, Weil am Rhein, Germany). D-glucose and L-arginine were purchased from Sigma Aldrich (Cat. no. G9270 and A6969, Brøndby, Denmark). Peptides were initially dissolved in milliQ-water supplemented with 1% (w/v) human serum albumin (HSA) (Cat. No. 12666, Millipore, Copenhagen, Denmark), in both cases to reach a concentration of 1 mM, and then diluted further in either isotonic saline supplemented with 10% glucose and 1% HSA (*in vivo* experiment) or in perfusion buffer (perfusion studies). Perfusion buffer was a modified Krebs-Ringer buffer supplemented with 0.1% (w/v) BSA (Fraction V; cat. no. 1.12018.0500, Merck, Ballerup, Denmark), 5% (w/v) dextran T70 to balance osmolarity (Pharmacosmos, Holbaek, Denmark) and 5 mM pyruvate, fumarate and glutamate and 3.5 or 10 mM glucose as indicated in the result section. The buffer was pH adjusted to 7.4-7.5.

Immunohistochemistry: Antibodies: Alexa Fluor® 488 goat anti-rabbit IgG was from Invitrogen (cat. no. A-11008, Naerum, Denmark). Anti-NMUR1 (cat.no. ab121959) and anti-NMUR2 (cat.no. ab115549) antibodies were obtained from Abcam (Cambridge, United Kingdom). Intestine and pancreas tissue specimens from rats were fixed in 10% (w/v) formalin in 0.1 M phosphate buffer (pH 7.4) for at least 24 hours at 4°C. The tissue was dehydrated in ethanol and xylol and finally embedded in paraffin wax. 3 µm thick paraffin sections were cut for immunofluorescence and placed on Superfrost Plus glass slides. Before immunostaining, specimens were pretreated with 1 mM ethylene-diamine-tetra-acetic acid (EDTA; pH 8.0) for 2 x 10 min at 475 W in a microwave oven. Anti-NMUR1 and 2 were applied to the sections in 1:100 dilution overnight in a cold room. Wash was performed with PBS between each step. The secondary antibody (FITC conjugated) was added in dilution 1:1000 at room temperature for 1 hour, protected from visible light. Immunofluorescence microscopy was performed with a Zeiss confocal microscope 510 equipped with a 63x objective or an inverted Zeiss Axiovert 220 Apotome

system with the same type of objectives. The images were processed using the Axiovision program (Carl Zeiss, Oberkochen, Germany) and the MetaMorph® software.

Protein Assay Kit (cat. no. 23227, Thermo Fisher Scientific, Fremont, USA). Extracts were purified using tc18 cartridges (cat. no. 036810 Waters, Massachusetts, United States) as previously described in details (Kuhre et al., 2014a). Samples were reconstituted in 1 ml of double buffer (phosphate buffer 80mM, 0,1 % Human Serum Albumin (cat. No. 12666, Merck KgaA, Darmstadt, Germany), EDTA 10 mM and Thimerosal 0,6 mM (cat. No. T-5125, Sigma Aldrich), pH 7.5). NMU (total) was quantified described below and normalized to whole wall tissue weight as well as to total protein content (quantified with BCA-kit) as. The distribution pattern of NMU was similar between normalization procedures; data are presented as pmol/g tissue

750.000 and showed no cross-reaction with GLP-1 (7-36NH_2), GLP-2, glucagon, insulin, somatostatin, neurotensin, motilin, PYY (3-36), pancreatic polypeptide, Exendin (9-39) and secretin in concentrations up to 6 nM. The evaluation of the assay specificity was in line with guidelines discussed and demonstrated previously(Albrechtsen, 2017). As standard we used synthetic rat NMU (cat no. 350285, Abbiotec, San Diego, CA) and the tracer was human NMU (cat no. H-5538, Bachem AG, Bubendorf, Switzerland) ^{125}I-labeled using the standard stoichiometric chloramine T method. The assay sensitivity was below 2 pM and the intra assay coefficient of variation was below 10%.

Quantification and statistical analysis: In vivo data are presented as means±SEM for the respective time points and as well incremental (AUC) values ± SEM. Perfusion data are presented as means ± SEM for the respective time points and as either mean outputs ± SEM or incremental output means± SEM as indicated. Incremental AUC/outputs were calculated by subtraction of baseline values, which for the *in vivo* experiments was defined as the means of -5 and 0 min concentrations in each animal. For the perfusion studies incremental outputs were defined as the difference between baseline outputs (for each animal defined as the mean value of the 10 subsequent observations leading up to the first test substance administration) and response outputs (defined as the output in the period from start of test substance application until levels were returned to baseline). Significance between groups was assessed by one-way ANOVA for repeated measurements (*in vivo* experiments) followed by Tukey multiple comparisons test or by two-way paired t-test (perfusions), as also indicated in respective figure legends. $P<0.05$ was considered significant. All raw datasets can be accessed at: *note to editor: will be uploaded to Mendeley upon acceptance of manuscript.*

Figure 1

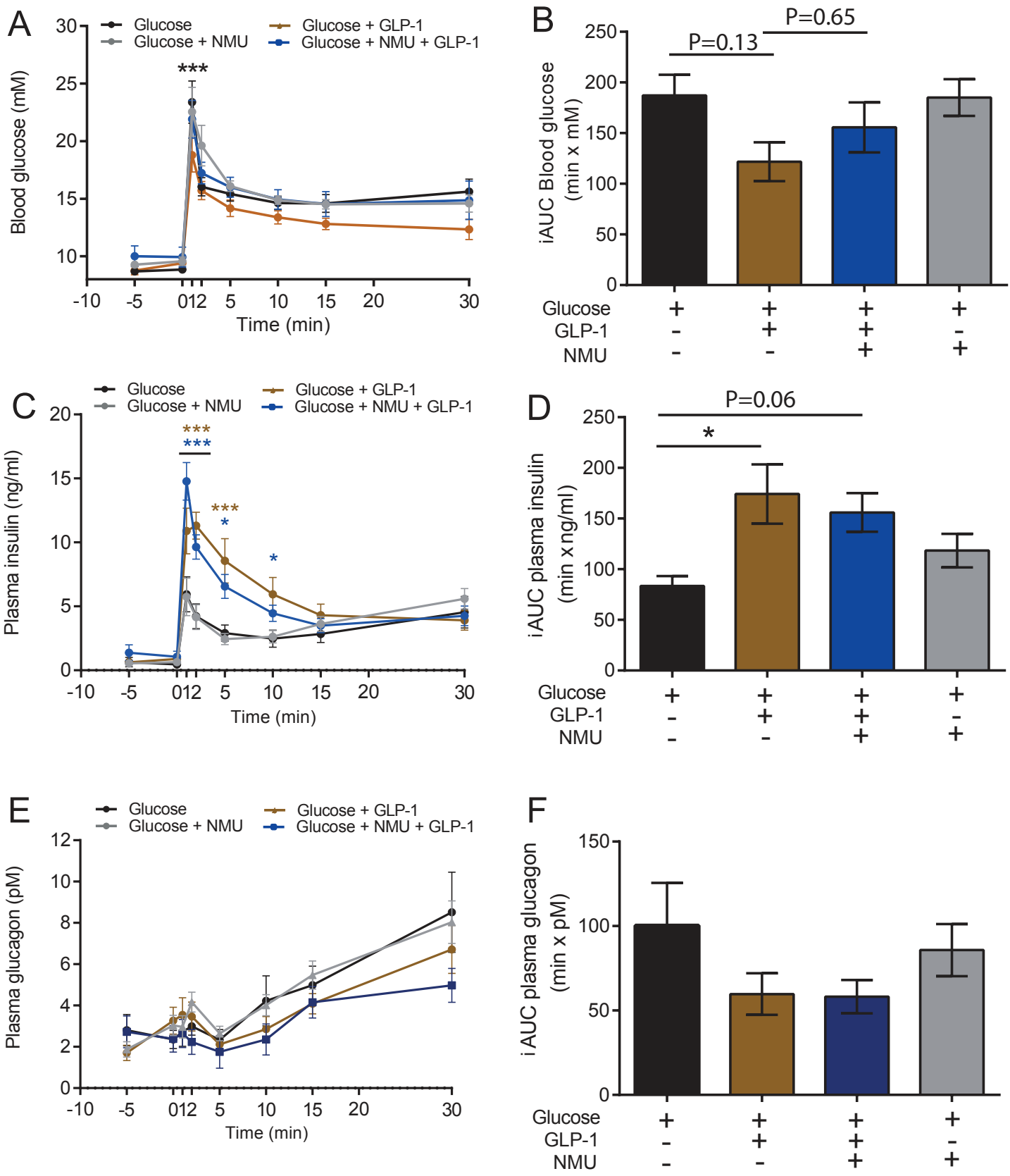

A
Glucose
Glucose + NMU
Glucose + GLP-1
Glucose + NMU + GLP-1
Blood glucose (mM)
Time (min)
***
B
P=0.13
P=0.65
iAUC Blood glucose (min x mM)
Glucose
GLP-1
NMU
C
Plasma insulin (ng/ml)
Time (min)
D
P=0.06
iAUC plasma insulin (min x ng/ml)
E
Plasma glucagon (pM)
Time (min)
F
iAUC plasma glucagon (min x pM)

Figure 2

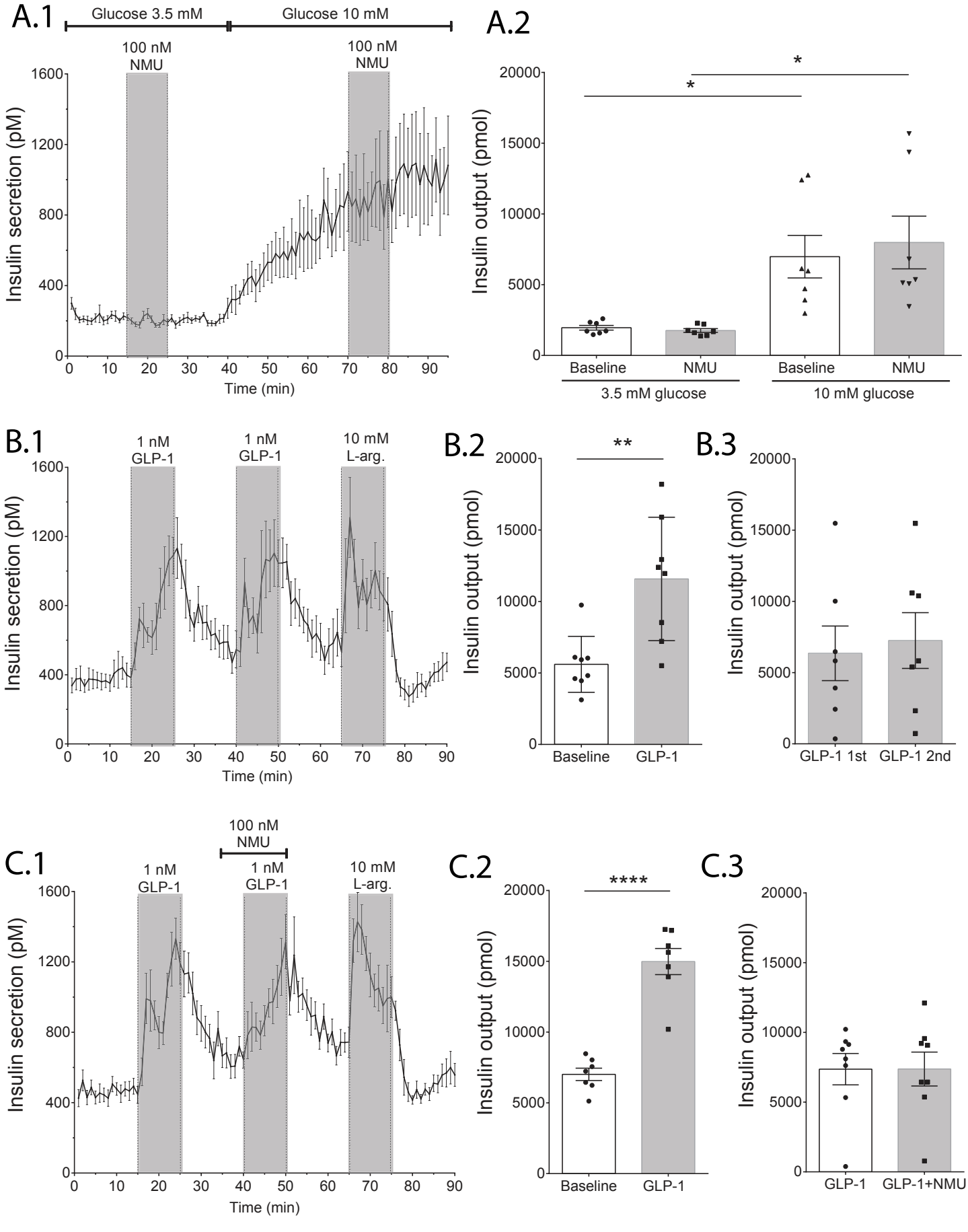

A.1
Glucose 3.5 mM
Glucose 10 mM
100 nM NMU
100 nM NMU
Insulin secretion (pM)
Time (min)
A.2
Insulin output (pmol)
Baseline
NMU
Baseline
NMU
3.5 mM glucose
10 mM glucose
B.1
1 nM GLP-1
1 nM GLP-1
10 mM L-arg.
Insulin secretion (pM)
Time (min)
B.2
Insulin output (pmol)
Baseline
GLP-1
B.3
Insulin output (pmol)
GLP-1 1st
GLP-1 2nd
C.1
100 nM NMU
1 nM GLP-1
1 nM GLP-1
10 mM L-arg.
Insulin secretion (pM)
Time (min)
C.2
Insulin output (pmol)
Baseline
GLP-1
C.3
Insulin output (pmol)
GLP-1
GLP-1+NMU

Figure 3

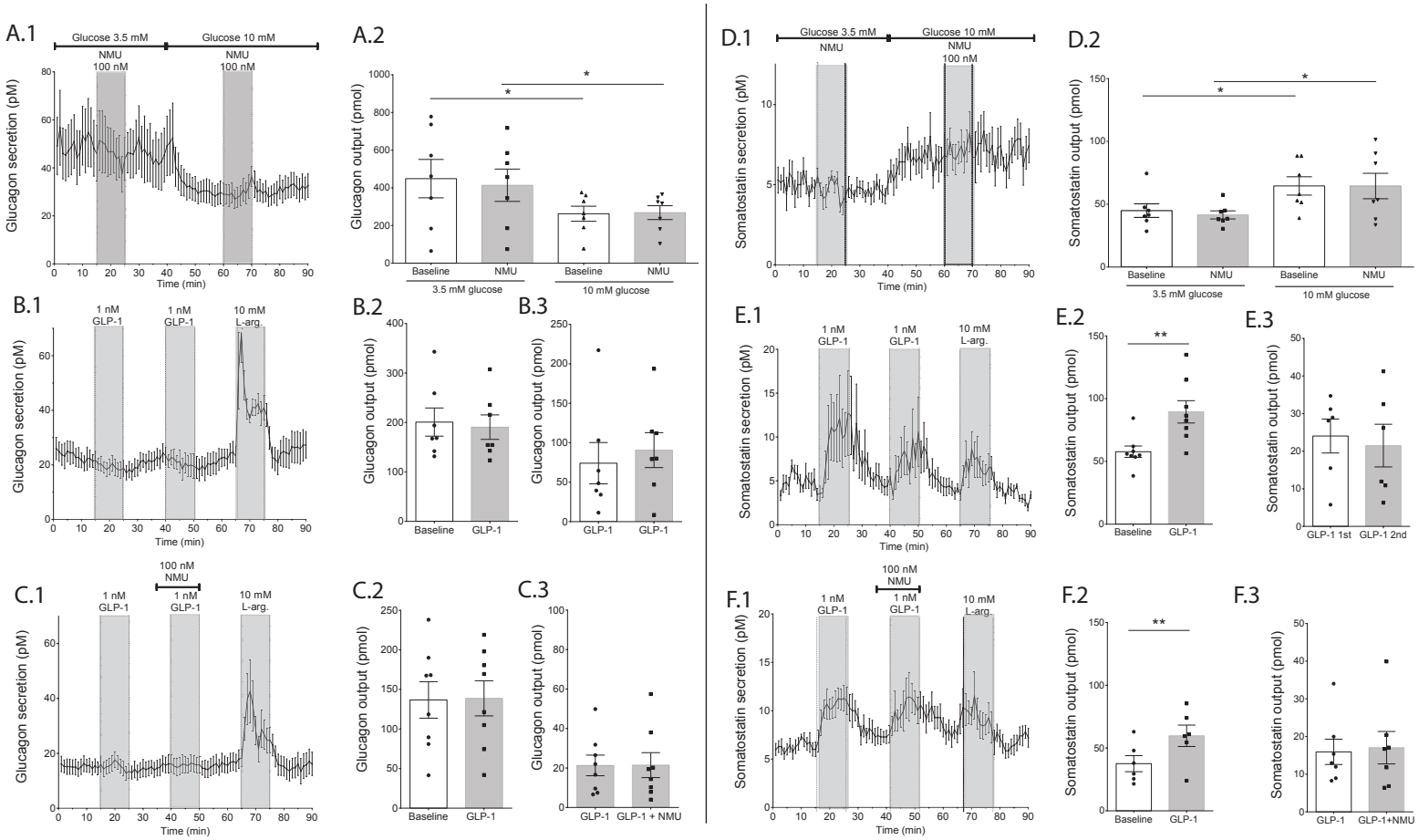
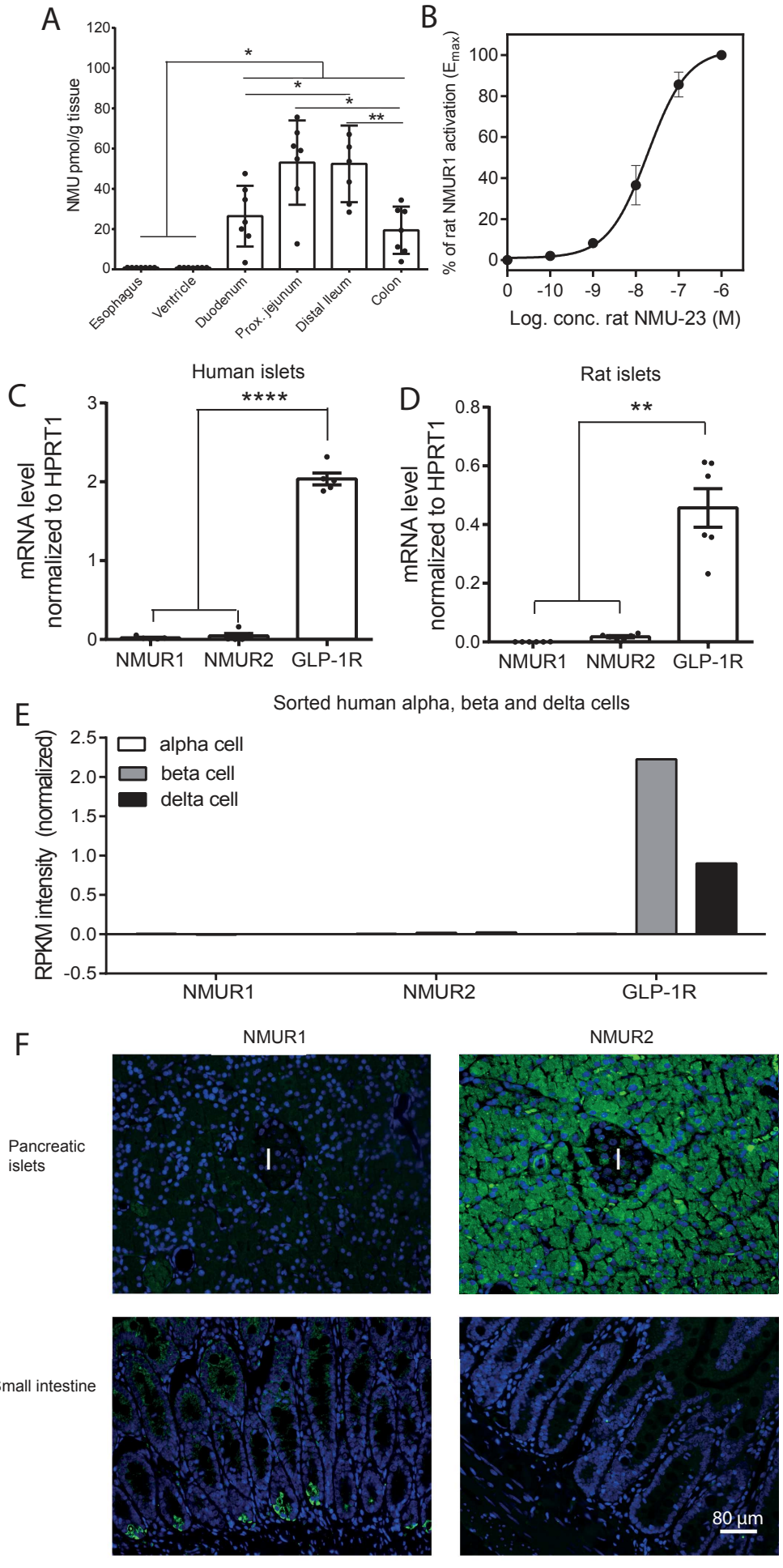

A.1
Glucose 3.5 mM
Glucose 10 mM
NMU 100 nM
NMU 100 nM
Glucagon secretion (pM)
Time (min)
A.2
Glucagon output (pmol)
Baseline
NMU
Baseline
NMU
3.5 mM glucose
10 mM glucose
B.1
1 nM GLP-1
1 nM GLP-1
10 mM L-arg.
Glucagon secretion (pM)
Time (min)
B.2
Glucagon output (pmol)
Baseline
GLP-1
B.3
Glucagon output (pmol)
GLP-1
GLP-1
C.1
100 nM NMU
1 nM GLP-1
1 nM GLP-1
10 mM L-arg.
Glucagon secretion (pM)
Time (min)
C.2
Glucagon output (pmol)
Baseline
GLP-1
C.3
Glucagon output (pmol)
GLP-1
GLP-1 + NMU
D.1
Glucose 3.5 mM
Glucose 10 mM
NMU
NMU 100 nM
Somatostatin secretion (pM)
Time (min)
D.2
Somatostatin output (pmol)
Baseline
NMU
Baseline
NMU
3.5 mM glucose
10 mM glucose
E.1
1 nM GLP-1
1 nM GLP-1
10 mM L-arg.
Somatostatin secretion (pM)
Time (min)
E.2
Somatostatin output (pmol)
Baseline
GLP-1
E.3
Somatostatin output (pmol)
GLP-1 1st
GLP-1 2nd
F.1
100 nM NMU
1 nM GLP-1
1 nM GLP-1
10 mM L-arg.
Somatostatin secretion (pM)
Time (min)
F.2
Somatostatin output (pmol)
Baseline
GLP-1
F.3
Somatostatin output (pmol)
GLP-1
GLP-1+NMU

Figure 4


A
NMU pmol/g tissue
120
100
80
60
40
20
0
*
*
*
**
Esophagus
Ventricle
Duodenum
Prox. jejunum
Distal Ileum
Colon
B
% of rat NMUR1 activation (Emax)
100
80
60
40
20
0
0
-10
-9
-8
-7
-6
Log. conc. rat NMU-23 (M)
C
Human islets
****
mRNA level normalized to HPRT1
3
2
1
0
NMUR1
NMUR2
GLP-1R
D
Rat islets
**
mRNA level normalized to HPRT1
0.8
0.6
0.4
0.2
0.0
NMUR1
NMUR2
GLP-1R
E
Sorted human alpha, beta and delta cells
RPKM intensity (normalized)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
alpha cell
beta cell
delta cell
NMUR1
NMUR2
GLP-1R
F
NMUR1
NMUR2
Pancreatic islets
Small intestine
80 µm

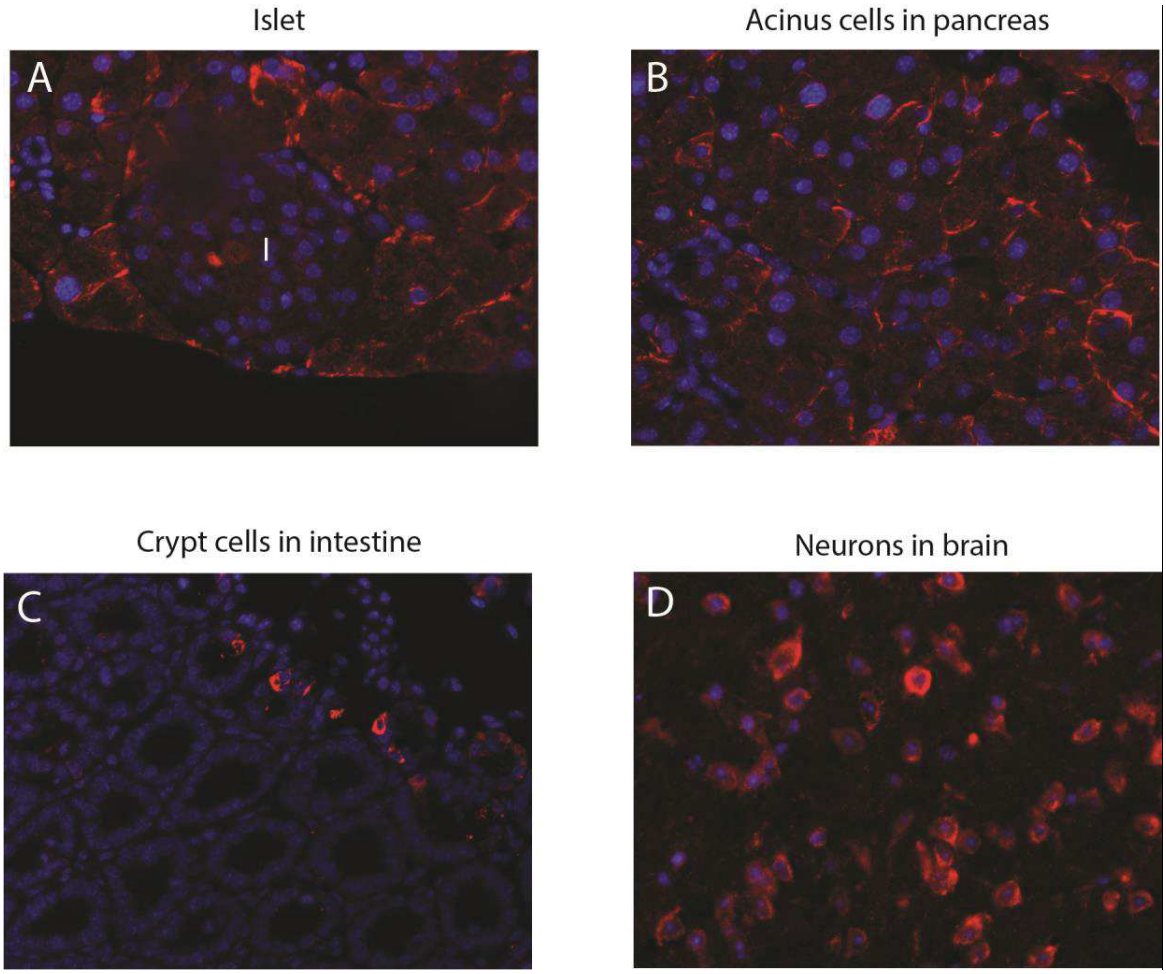
CellPress

KEY RESOURCES TABLE

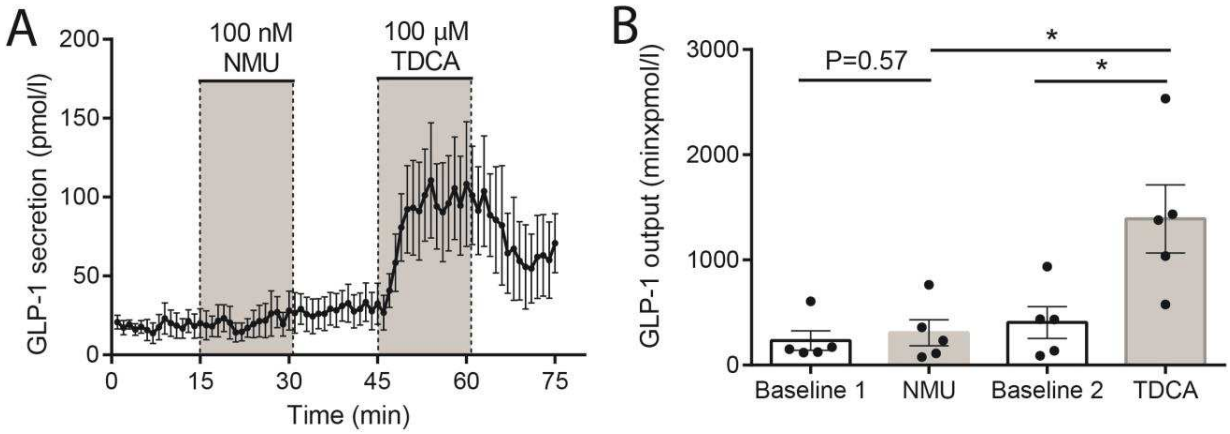
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor® 488 goat anti-rabbit IgG	Invitrogen	Cat # A-11008,
Anti-NMUR1	Abcam	Cat # Ab121959
Anti-NMUR2	Abcam	Cat # Ab115549
Bacterial and Virus Strains		
-	-	-
Biological Samples		
Donated human islets	European Consortium for Islet Transplantation (ECIT), Milan, Italy	N/A
Rat islets	Harvested locally	N/A
Rat gut tissue	Harvested locally	N/A
Chemicals, Peptides, and Recombinant Proteins		
Neuromedin U-23 (amidated)	Bachem	Cat # 4030663
GLP-1 7-36 amide	Bachem	Cat # 4016663
Human Serum Albumin (HSA)	Millipore	Cat # 12666
Bovine Serum Albimin (BSA), Fraction V	Merck	Cat # 1.12018.0500
Dextran T70	Pharmacosmos	Cat # 551000709005
SYBR Green Mastermix	Life Technologies/Thermos Fisher Scientific	Cat# 4472919
Trifluoroacetic acid (TCA, 1%)	Thermo Fisher Scientific	Cat # TS-28904
Critical Commercial Assays		
Rat insulin ELISA	Mercodia	Cat# 10-1250-01
Glucagon ELISA – 10 µL	Mercodia	Cat# 10-1281-01
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat # 23227
Deposited Data		
All raw data	This paper	Will be deposited upon acceptance of manuscript
Single cell RNA sequencing data bases	Muraro et at. 2016 (Pubmed ID: 27693023), Segerstolpe et al. 2016 (Pubmed ID: 27667667), Xin et al. 2016 (Pubmed ID: 27667665).	GSE85241, GSE81608 and E-MTAB-5061.
Experimental Models: Cell Lines		
COS-7 cells (isolated from a male Chlorocebus aethiops (Green monkey))	ATCC	Cat# CRL-1651
Experimental Models: Organisms/Strains		
Rattus narvegicus, Wistar, Male		

Oligonucleotides		
Listed in supplementary table 1	Tag Copenhagen	This paper
Recombinant DNA		
NMUR1 (NM-023100) Rat Untagged Clone	Origene	Cat # RN208112
Software and Algorithms		
GraphPad Prism 7	GraphPad Software, La Jolla, CA 92037, USA	https://www.graphpad.com/
Adobe Illustrator CC 2017	Adobe Systems, San Jose, CA, USA	https://www.adobe.com/products/illustrator.html
MetaMorph Micoscopy Automation and Image Analysis Softwars	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy#gref
Jupyter Notebook	Project Jupyter	http://jupyter.org
Axiovision program	Carl Zeiss,	https://www.zeiss.com/microscopy/int/products/microscope-software/axiovision.html
Other		
-	-	-

Supplementary figure titles and legends



Supplementary figure 1: NMUR1 expression in rat. A: Rat islet (indicated with "I), B: Acinus cells in the pancreas, C: crypt cells in the small intestine, D: neurons in the brain. Blue staining is DAPI (nuclei staining) and red staining is NMUR1. Scale bars: A = 50 µm, B: 70 µm, C = 40 µm, D = 50 µm.



Supplementary figure 2: No effects of NMU-23 on GLP-1 secretion from isolated perfused rat small intestine. GLP-1 (total) secretion from isolated perfused rat small intestine (entire small intestine) in response to intra-arterial administration of NMU-23 (100 nM) and Tauro-deoxycholic acid (TDCA, 100 µM), respectively. A: GLP-1 secretion during the time course of the study, B: Total GLP-1 output during baseline periods and during stimulation with NMU and TDCA, respectively. Dots in B shows results from respective experiments. Data are shown as means±SEM. Statistical significance was assessed by One way ANOVA for repeated measurements; *P<0.05, n =5.

Rat primers

Nmur1	TAACCCGGTGCTCTACAACC	CAGGAGGGTCTGTCTCTTGC
Nmur2	TTCTGGTGTGCATGGTGATT	CAGTCTCGAAGAGGGCTGTC
Glp-1r	ATCCACCTGAACCTGTTTGC	GCAGTATTGCATGAGCAGGA
Hprt1	GCAGACTTTGCTTTCCTT	CCGCTGTCTTTTAGGCTT

Human primers

Nmur1	TCATGAGCGTGCTCTACCTG	ACGACCAGGACAAACAGCAT
Nmur2	ACTTCTTCCTCCCCGTGTCT	GCGCCACATCTCATAGACCT
Glp-1r	GTTCCCCTGCTGTTTGTTGT	CTTGGCAAGTCTGCATTTGA
Hprt1	ATGCTGAGGATTTGGAAAGG	TAATCCAGCAGGTCAGCAAA

1	F	22	22.8
2	F	52	20.4
3	M	64	27.5
4	F	60	23.6
5	M	34	23.1
6	M	50	24.8

Supplementary table 2: Anthropometric data from human pancreatic donors.