

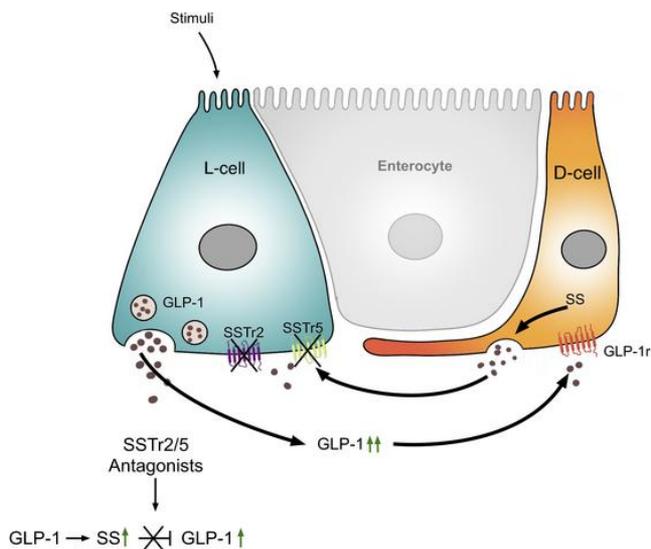
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Antagonizing somatostatin receptor subtype 2 and 5 reduces blood glucose in a gut- and GLP-1R-dependent manner

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

Abstract

Somatostatin (SS) inhibits glucagon-like peptide-1 (GLP-1) secretion in a paracrine manner. We hypothesized that blocking somatostatin subtype receptor 2 (SSTR2) and 5 (SSTR5) would improve glycaemia by enhancing GLP-1 secretion.

In the perfused mouse small intestine the selective SSTR5 antagonist (SSTR5a) stimulated glucose-induced GLP-1 secretion to a larger degree than the SSTR2 antagonist (SSTR2a). In parallel, mice lacking the SSTR5R showed increased glucose-induced GLP-1 secretion. Both antagonists improved glycaemia in vivo in a GLP-1 receptor (GLP-1R) dependent manner, as the glycaemic improvements were absent in mice with impaired GLP-1R signalling and in mice treated with a GLP-1R specific antagonist. SSTR5a had no direct effect on insulin secretion in the perfused pancreas whereas SSTR2a increased insulin secretion in a GLP-1R independent manner. Adding a dipeptidyl peptidase 4 inhibitor (DPP-4i) in vivo resulted in additive effects on glycaemia, however, when glucose was administered intraperitoneally the antagonists was incapable of lowering blood glucose. Oral administration of SSTR5a, but not SSTR2a lowered blood glucose in diet induced obese mice.

In summary, we demonstrate that selective SSTR antagonists can improve glucose control primarily through the intestinal GLP-1 system in mice.

Introduction

Hormones secreted from the gut are known to be of importance for the regulation of glucose metabolism (1-3), with the incretin hormone glucagon-like peptide-1 (GLP-1) in particular, contributing to enhance postprandial insulin secretion (4-8). Patients with type 2 diabetes have significantly impaired incretin function (9-12), and treatment with stable GLP-1 receptor agonists (GLP-1RAs) or enhancing endogenous levels of active GLP-1 using dipeptidyl peptidase 4 inhibitors (DPP-4is) effectively lowers blood glucose levels (13, 14). GLP-1 secretion is regulated by complex mechanism(s). We, and others, have shown that GLP-1 secretion is under paracrine influence from somatostatin (SS) receptor signalling (15-18). Five somatostatin receptor subtypes, denoted SSTR1-5 exist and prior studies, including our own, have found that SSTR5 in particular is enriched in GLP-1 producing cells (17, 19-21). Furthermore, we found that SSTR5 is a powerful tonic inhibitor of GLP-1 secretion (17). SSTR antagonism may therefore improve glycaemic control, and targeting the SSTR5 in vivo as a means to improve glucose tolerance has been suggested (22-25). It is still debated whether the effect of SSTR antagonism on glucose tolerance is mediated by the gut or if there is a direct effect on the endocrine pancreas in which SSTRs are also expressed (22-24, 26-28).

We hypothesize that especially SSTR5 and to some degree SSTR2 maintain glucose homeostasis through paracrine regulation of intestinal GLP-1. To address this, we first studied the secretion of glucose-induced GLP-1 secretion in response to blocking either SSTR5, or the less expressed SSTR2 (17, 29), in the isolated perfused mouse intestine. Using different mouse models, we assessed whether SSTR2 and SSTR5 antagonists (SSTR2a and SSTR5a, respectively) improve whole body glucose metabolism and we investigated the underlying mechanism(s), including GLP-1 receptor (GLP-1R) signalling. We also combined the SSTR antagonists with a DPP-4i to elucidate whether a further improvement in glycaemic

control could be obtained if the secreted GLP-1 was protected from degradation. To show that the antagonists acted through gut-derived mechanisms, i.e. through the SS-GLP-1 axis leading to increased endogenous GLP-1 secretion, and not by direct stimulation of the pancreas, we used the isolated perfused mouse pancreas and performed intraperitoneal glucose tolerance tests (IPGTT) *in vivo*.

Results

SSTR5 and SSTR2 antagonism increases glucose-induced GLP-1 secretion with different magnitudes in the isolated perfused WT mouse small intestine.

The luminal glucose stimulation with vascular SSTR2a tended to increase GLP-1 output in venous effluents compared to glucose stimulation alone (luminal glucose: 67 ± 10 fmol/20 min vs luminal glucose + SSTR2a: 111 ± 30 fmol/20 min, $p=0.15$) (Figure 1A and B). SS secretion followed the same pattern as GLP-1 during SSTR2a infusion, and we found a correlation between SS and GLP-1 concentrations of $R^2=0.67$, based on average output each minute from 1 min to 100 min (Supplementary Figure S1A). In the case of SS secretion, the observed increase when SSTR2a was combined with luminal glucose was significantly higher compared to glucose administration alone (luminal glucose: 13 ± 5 fmol/min vs luminal glucose + SSTR2a: 55 ± 11 fmol/min $p=0.02$) (Figure. 1C and D). Intra-arterial infusions of the SSTR5a increased the GLP-1 response to luminal glucose about 3-fold (luminal glucose: 98 ± 17 fmol/20 min vs luminal glucose + SSTR5a: 310 ± 43 fmol/20 min, $p=0.0052$) (Figure. 1E and F). Based on the fold-changes, calculated for the baseline-subtracted glucose-induced mean GLP-1 output vs the mean GLP-1 output after glucose + SSTR2a or SSTR5a stimulation, we found that SSTR5a increased glucose-induced GLP-1 release more than SSTR2a (fold-change for SSTR2a+glucose vs glucose: 1.75 ± 0.5 ; fold-change for SSTR5a+glucose vs glucose 3.6 ± 0.6 , $p=0.03$). SS again followed the same pattern as GLP-1, where we found a correlation between SS and GLP-1 secretion, $R^2=0.89$ (Supplementary Figure S1B), and the increase in SS output was significantly higher when SSTR5a was combined with luminal glucose compared to glucose administration alone (luminal glucose: 28 ± 6 fmol/min vs luminal glucose + SSTR5a: 190 ± 37 fmol/min $p=0.0045$) (Figure 1G and H).

We then investigated whether glucose-induced GLP-1 secretion would be increased in a mouse model lacking SSTR5 (*Sstr5*^{-/-} mice). Glucose-induced GLP-1 output in *Sstr5*^{-/-} mice increased more than 3-fold compared to that of WT littermates (*Sstr5*^{+/+} mice: 51±10 fmol/min vs *Sstr5*^{-/-} mice: 184±13 fmol/min, p<0.0001) (Figure 1I and J). Somatostatin outputs were likewise significantly higher upon glucose stimulation in *Sstr5*^{-/-} mice compared to *Sstr5*^{+/+} (*Sstr5*^{+/+}: 41.3±15 fmol/20 min, *Sstr5*^{-/-} mice: 329.2±91 fmol/20 min, p=0.0017) and correlated with the GLP-1 response (*Sstr5*^{+/+} R²=0.51, *Sstr5*^{-/-} R²=0.83) (Supplementary Figure S1C and D).

SSTR5 and SSTR2 antagonism lower blood glucose during an OGTT via a gut-dependent mechanism

To investigate whether the increase in glucose-induced GLP-1 secretion, caused by SSTR2a and SSTR5a in the perfused mouse intestine, would affect glucose tolerance during an OGTT in vivo, the antagonists were administered subcutaneously (sc) 15 min before an oral glucose load in male mice. Similar experiments were carried out in female mice, revealing no gender-specific variations (Supplementary Figure S2A, B and C). Both SSTR2a and SSTR5a significantly lowered glucose concentrations compared to vehicle (iAUC_{0-90min} vehicle 794 ± 55 mmol/l x min vs SSTR2a 508 ± 69 mmol/l x min, p=0.01) (vehicle vs SSTR5a 579 ± 73 mmol/l x min, p=0.03) (Figure 2A). When comparing the iAUC₀₋₃₀ there was no overall effect on insulin levels of either SSTR2a or SSTR5a compared to vehicle (vehicle vs SSTR2a, p=0.6 and vehicle vs SSTR5a, p=0.9), although, SSTR2a did increase insulin levels significantly after 15 min of administration (at time 0 min) compared to vehicle (p=0.002) (Figure 2B). Total GLP-1 levels were significantly higher in SSTR5a treated mice compared to vehicle at time 0 min (p<0.0001), 15 min (p<0.0008) and 30 min (p<0.004) (Figure 2C) whereas no significant effect was seen in SSTR2a treated mice (p=0.2).

We then investigated whether an additional improvement in glucose tolerance could be obtained by combining SSTR2a and SSTR5a during an OGTT. When combining the two antagonists, an additive improvement of glucose tolerance compared to vehicle was observed, and the combination both improved blood glucose levels and insulin secretion significantly compared to vehicle (Supplementary Figure S2D and E).

To investigate direct pancreatic effects of SSTR5a and SSTR2a, we used the isolated perfused pancreas model in male mice and infused the antagonists by intra-arterial infusion at low (3.5 mM) and high (15 mM) glucose.

SSTR2a did not affect mean insulin output compared to the mean preceding baseline at low glucose ($p=0.5$), whereas an increase from the preceding baseline period was observed at high glucose ($p=0.005$) (Figure 2D). This increase in insulin was unchanged when the SSTR2a was combined with the GLP-1R antagonist Exendin9-39 (Ex9-39), where no difference between the two peaks was found based on iAUC ($p=0.16$) (Figure 2E). SSTR2a significantly increased mean glucagon output at low glucose ($p=0.005$) and significant, albeit minor, increase was observed at high glucose ($p=0.04$) (Figure 2F). SSTR2a significantly increased mean SS output both at low and high glucose levels ($p=0.0007$ and $p=0.02$ respectively) (Figure 2G).

SSTR5a did not affect insulin secretion at low or high glucose ($p=0.4$ and $p=0.9$, respectively) (Figure 2H). A small significant decrease in glucagon secretion was observed at low glucose ($p=0.02$), however the drop was not meaningful, decreasing from 12 ± 1 fmol/min to 10 ± 1 fmol/min, while no effect was observed on glucagon secretion at high glucose ($p=0.5$) (Figure 2I). Compared to the preceding

baseline period, there was no effect of the antagonist on SS levels at either low or high glucose ($p=0.6$ and $p=0.5$, respectively) (Figure 2J).

To corroborate our hypothesis, that especially SSTR5 antagonism lowers blood glucose through gut-derived mechanisms and not through direct stimulation of pancreatic hormones, we administered the SSTR2a and SSTR5a by sc injections 15 min before ip injection of glucose or PBS, thereby avoiding stimulating intestinally derived GLP-1. When glucose was administered ip instead of orally, neither antagonists had any effect on blood glucose levels compared to the control group (iAUC_{0-90min} sc PBS/ip glucose 627 ± 83 mmol/l x min. vs sc SSTR2a/ip glucose 570 ± 103 mmol/l x min, $p=0.5$, and sc. PBS/ip glucose vs sc SSTR5a/ip glucose 735 ± 67 mmol/l x min, $p=0.4$) (Figure 2K and L).

SSTR5a and SSTR2a lower blood glucose in a GLP-1R dependent manner and combining the antagonists with a DPP-4i further improved glucose tolerance

To evaluate whether the improved glucose tolerance observed when SSTR2 and SSTR5 are antagonized is GLP-1R dependent, we combined them with Ex9-39, a well characterized GLP-1R antagonist. The presence of Ex9-39 abolished the glucose-lowering effect of both SSTR2a and SSTR5a during an OGTT (iAUC_{0-60min} of SSTR2a: 295 ± 177 mmol/l x min vs SSTR2a + Ex9-39: 717 ± 80 mmol/l x min $p<0.0001$. SSTR5a: 361 ± 82 mmol/l x min vs SSTR5a + Ex9-39: 624 ± 69 mmol/l x min $p=0.013$) (Figure 3A and B).

In line with the effect of GLP-1R antagonism, similar findings were observed in *Glp-1r*^{-/-} mice in which the lowering of blood glucose by SSTR2a and SSTR5a was significantly diminished compared to that observed in *Glp-1r*^{+/+} animals (iAUC_{0-120min} SSTR2 in *Glp-1r*^{+/+} 177.8 ± 28 mmol/l x min vs SSTR2 in *Glp-1r*^{-/-} 421 ± 78 mmol/l x min, $p=0.01$. SSTR5 in *Glp-1r*^{+/+} 265 ± 29 mmol/l x min vs

SSTR5 in *Glp-1r^{-/-}* 435±63 mmol/l x min, p=0.04) (Figure 3C and D). In this subset of experiments, no significant difference between the SSTR5a and vehicle group in *Glp-1r^{+/+}* was observed (p=0.4). This could be due to the rather low dose of the antagonist that was used (4 mg/kg), resulting in variability of the antagonistic effect. In subsequent follow-up studies, we observed that increasing the dose as well as changing the route of administration to oral gavage markedly increased the effect of the SSTR5a on blood glucose (Supplementary Figure S3).

As *Sstr5^{-/-}* mice had increased glucose-induced GLP-1 release compared to WT littermates when their intestine was perfused (Figure 1I), we investigated whether they might have improved glucose control in vivo, and whether this could be abolished by adding Ex9-39 during an OGTT. No significant difference in blood glucose levels were observed comparing *Sstr5^{-/-}* to *Sstr5^{+/+}* (iAUC_{0-90min} vehicle *Sstr5^{+/+}* 424.4 ± 118 mmol/l x min vs vehicle *Sstr5^{-/-}* 269.6 ± 51 mmol/l x min p=0.28) (Figure 3E), which otherwise have been observed by Farb et al. (23). That only a numerical difference is observed here is probably due to the low number of mice available in the present study. When the two groups received Ex9-39, thus removing the effect of GLP-1, blood glucose levels were significantly impaired compared to their respective vehicle (iAUC_{0-90min} *Sstr5^{+/+}* vehicle: 424.4 ± 118 mmol/l x min vs *Sstr5^{+/+}* + Ex9-39: 820.1 ± 144 mmol/l x min, p=0.03. *Sstr5^{-/-}* vehicle: 269.6 ± 51 mmol/l x min vs *Sstr5^{-/-}* + Ex9-39: 763.3 ± 82.7 mmol/l x min, p=0.006) (Figure 3E).

Having shown that SSTR2a and SSTR5a lower blood glucose by potentiating glucose induced GLP-1 secretion (Figure 3 A-D), we investigated whether an improved glycaemic control could be obtained when GLP-1 degradation is prevented, namely by combining the SSTR antagonists with the DPP-4i. Animals receiving the combination of SSTR2a and DPP-4i showed an additive effect on glucose levels compared to vehicle (iAUC_{0-90min}: vehicle: 606 ± 76 mmol/l x min vs DPP-4i: 323 ± 58 mmol/l x min,

p=0.003, vehicle vs SSTR2a: 321 ± 38 mmol/l x min, p=0.04. Vehicle vs SSTR2a + DPP-4i: 144 ± 30 mmol/l x min, p<0.0001) (Figure 3F). The combination of SSTR5a and DPP-4i likewise improved blood glucose levels in an additive manner compared to vehicle (iAUC_{0-90min}: vehicle: 380 ± 52 mmol/l x min vs DPP-4i: 176 ± 36 mmol/l x min, p=0.002, vehicle vs SSTR5a: 217 ± 28 mmol/l x min, p=0.012. Vehicle vs SSTR5 + DPP-4i: 146.2 ± 26.8 mmol/l x min, p=0.0009) (Figure 3G). However, no difference in iAUC was observed between SSTR5a alone and SSTR5a + DPP-4i (p=0.3), but upon mixed effects analysis, a significant difference between the two groups was observed at time 15 min (p=0.004).

SSTR5a is more effective to stimulate glucose-induced GLP-1 secretion in the perfused intestine as well as to improve glucose tolerance in vivo than SSTR2a in DIO and control mice

Using diet induced obese (DIO) mice, we evaluated whether the effect of antagonizing SSTR5 and SSTR2 also translate in a model of disease. The effect of antagonizing SSTR2a and SSTR5a on glucose-induced GLP-1 and SS secretion was investigated in the proximal perfused mouse intestine and their effect on glucose tolerance was evaluated in vivo.

Intra-arterial infusions of SSTR2a did not significantly increase glucose-induced GLP-1 secretion in control mice (luminal glucose: 55.5 ± 20.3 fmol/20 min vs luminal glucose + SSTR2a: 80.6 ± 18.7 fmol/20 min, p=0.26). In DIO mice, SSTR2a infusion led to a 2-fold increase in glucose-induced GLP-1 secretion (luminal glucose: 57.3 ± 11 fmol/20 min vs luminal glucose + SSTR2a: 112 ± 26.6 fmol/20 min, p=0.029) (Figure 4A-C). SS secretion increased during the intra-arterial infusions of SSTR2a compared to luminal glucose infusion alone (luminal glucose vs SSTR2a + glucose p=0.01) (Figure 4D-F). SSTR5a again potentiated glucose-induced GLP-1 secretion the most, with a 4-fold increase in

control mice (luminal glucose: 60.9 ± 14.9 fmol/20 min vs luminal glucose + SSTR5a: 246.5 ± 39.61 fmol/20 min, $p=0.0061$), and a 5-fold increase in DIO mice (luminal glucose: 65.9 ± 16.7 fmol/20 min vs luminal glucose + SSTR5a: 344.1 ± 31.54 fmol/20 min, $p<0.0001$) (Figure 4 G-I). During intra-arterial infusions of SSTR5a, SS secretion increased compared to glucose infusion alone (glucose vs SSTR5a + glucose $p=0.005$) (Figure 4 J-L).

The impact of the SSTR2a and SSTR5a on blood glucose during an OGTT in DIO and control mice was evaluated by sc injection of the antagonists, 15 min before an oral glucose load. However, there was no effect on glucose tolerance after SSTR2a and SSTR5a in DIO mice whereas an effect was seen in control mice (Supplementary Figure S4). This might be due to the route of administration and we therefore used oral administration of the antagonists in the subsequent experiments. Surprisingly, the SSTR2a did not affect glucose tolerance compared to vehicle in DIO or control mice ($iAUC_{0-120min}$ control: vehicle vs SSTR2a $p=0.9$. DIO: vehicle vs SSTR2 $p=0.7$) (Figure 4M and N). In DIO mice, SSTR5a significantly improved glucose tolerance during an OGTT with a trend ($p=0.07$) being observed in control mice ($iAUC_{0-120min}$ control: Vehicle 561 ± 113 mmol/l x min, $n=8$ vs SSTR5a 167 ± 32 mmol/l x min, $n=5$. DIO: Vehicle 1141 ± 106 mmol/l x min, $n=9$, vs SSTR5a 654 ± 106 mmol/l x min, $n=9$, $p=0.02$) (Figure 4M and N).

Discussion

We and others have documented *Sstr2* and *Sstr5* expression on the GLP-1 secreting L-cells, where *Sstr5* expression clearly exceeded the level of *Sstr2* expression (17, 29). Expression of these receptors has also been reported in the islets of the pancreas, both on alpha and beta-cells (26, 27, 30, 31) and previous studies have shown that SSTR5 antagonism can improve blood glucose levels (22-24). Whether this is due to a gut-mediated effect, a direct effect on the pancreas or a combination was unclear. Most studies have focused on the effect of SSTR5 whereas the effect of SSTR2 antagonism on glucose tolerance barely has been touched upon. In this study we demonstrate that antagonising SSTR2 and SSTR5 pharmacologically improved glucose tolerance by potentiating glucose-induced GLP-1 from the small intestine.

We used the isolated perfused mouse proximal intestine to investigate the paracrine actions of SSTRs on glucose induced GLP-1 secretion. The perfused organ models preserve the circulatory system and the paracrine relationships, they minimize degradation of hormones, eliminate interference from other organs and circulating factors, and therefore allow accurate studies of the local secretory patterns of hormone secretion, precisely as they occur in vivo, but where such measurements cannot be performed due to degradation of hormones in the circulation and limited amount of plasma (32).

In the perfused mouse proximal intestine, glucose-induced GLP-1 secretion tended to be increased by SSTR2a, but the SSTR5a was by far the most effective stimulus resulting in a 3-fold increase in GLP-1 secretion in C57/Bl6 mice, and a similar, enhanced secretion of GLP-1 was observed in *Sstr5*^{-/-} mice when glucose was infused intraluminally. Common to all perfusion studies, SS secretion followed a similar pattern as GLP-1 secretion, which is in line with our previous finding that SS secretion is

dependent on GLP-1 stimulation (17), since adding the GLP-1R antagonist (Ex9-39) together with SSTR5a completely abolished the secretion of SS. This, therefore, suggests that the increased SS secretion when antagonising the SSTR5, is brought about by an effect of the antagonists on the L-cell rather than disruption of an autocrine feedback on the D-cell (17). We expect that the same applies to the SSTR2a but this has not yet been tested.

Based on these studies, we expected that the SSTR5a would improve glucose tolerance in a gut-derived, GLP-1 dependent manner during an OGTT in vivo, whereas the SSTR2a would be less effective. Surprisingly, we found that SSTR5a *and* SSTR2a enhanced glucose tolerance equally during an OGTT in WT male and female mice.

Measuring total GLP-1 levels in vivo revealed that the effect elicited by the SSTR5a on glucose tolerance most likely is due to increases in GLP-1 secretion, which is in line with the findings by Farb et al. (23). Even before glucose was administered (at time 0 min), GLP-1 levels were elevated, confirming the strong tonic inhibitory effect exerted by the SSTR5 on GLP-1 secretion even in the basal state (16, 17). No effect on circulating GLP-1 levels was seen for SSTR2a. It should be noted that we were unable to detect increases in GLP-1 levels in the vehicle group after glucose administration in male mice and surprisingly, we observed a drop from -15 to 0 min, whereas an increase was observed in female mice. Additionally, we saw a lower basal secretion of GLP-1 in response to SSTR5a in male mice compared to female mice and there was difference in the response to glucose. Accurate estimation of low levels of endogenous GLP-1 secretion in mice has proven to be challenging due to the pronounced activity of neutral endopeptidase (NEP) 24.11 (33, 34), and there is currently no way of providing more accurate measurements of GLP-1 secretion in mice in vivo. With the applied sandwich ELISA (the most sensitive available) it is most likely only a small fraction of the true GLP-1 release

that is detected. However, it cannot be excluded that there is a genuine sex-difference which need further investigation.

We have previously characterized the antagonistic properties of SSTR2a used for this study, which turned out to be less specific than first anticipated, i.e. antagonizing also SSTR1, 3 and 4 to a variable degree (17), furthermore a study by Hocart et al. showed that the SSTR2a additionally can bind to the SSTR5 (35), however, we did not find any inhibition of SSTR2a on SSTR5 in our previous study. The SSTR5a on the other hand was found to be selective to its receptor (17). The relative non-selectivity of the applied SSTR2a, possibly targeting multiple glucose regulating pathways besides the GLP-1-producing L-cells, could explain why SSTR2a decreases blood glucose in vivo (17). Thus, SSTR2a may cause stimulation of pancreatic hormone release in vivo since expression of *Sstr1*, *Sstr2* and *Sstr3* has been found in the pancreatic islets (36, 37). Expression of *Sstr5* has likewise been reported in rodent beta cells and SSTR5 has been suggested to be the main regulator of insulin secretion in mice (27, 28, 30, 38). However, in our study in the perfused mouse pancreas, there was no effect of SSTR5 antagonism on insulin secretion whether at low or high glucose levels, in agreement with a study involving isolated mouse islets (23). In contrast, SSTR2 antagonism increased insulin during hyperglycaemia as well as increased glucagon secretion during hypoglycaemia. An effect of SSTR2a on glucagon secretion during hypoglycaemia has previously been shown in Streptozotocin-induced diabetic rats and has been suggested as a target to restore the glucagon response to hypoglycaemia in type 1 diabetes (39-42). The effect of SSTR2a on insulin secretion during hyperglycaemia has been studied in SSTR2 knockout mice and in isolated cells, but revealed only weak effects on insulin secretion (30, 43). In contrast, our results from the perfused pancreas demonstrated that SSTR2a can increase insulin secretion during hyperglycaemia.

Secretion of alpha cell-derived GLP-1 has been suggested to play a role in glucose homeostasis in mice (44). GLP-1 is a product of posttranslational processing of proglucagon, which is produced in intestinal L-cells as well as in pancreatic alpha cells. Since the SSTR2a elicited a minor increase in glucagon concentrations during hyperglycaemia in the perfused pancreas, it could be speculated that alpha-cell derived GLP-1 would be released in parallel, which could stimulate insulin release. However, the increase in insulin secretion by the SSTR2a was still evident when combined with Ex9-39, suggesting that the effects of SSTR2a on insulin levels do not involve the GLP-1R in the isolated perfused pancreas.

Surprisingly, our *in vivo* data show that when SSTR2a is given in combination with *ip* glucose (circumventing intestinal hormone release), the effect of SSTR2a on glucose tolerance was completely blunted. The same was observed for the SSTR5a, suggesting that both antagonists improve glucose tolerance *in vivo* through a gut-derived mechanism and not by directly affecting pancreatic secretion. It should be noted that even though GLP-1 levels were modestly elevated after SSTR5a injection during fasting (time 0 min, Fig 2C), this was not associated with any improvement in glycaemic control at time 0 min during the IPGTT experiments. A possible explanation could be that the elevation by SSTR5a in the fasting state is too small to significantly influence insulin/glucagon secretion and thereby glucose tolerance during the IPGTT. Furthermore, we also observed increased levels of insulin during an OGTT at fasting when SSTR2a was injected. Again, however, blood glucose levels during the IPGTT at time 0 min were not affected. Insulin levels during the IPGTT were measured (data not shown), but did not show any elevations after SSTR2a injection. The lack of insulin response could be related to a stress reaction to handling, since activation of the sympathetic nerves is known to markedly inhibit glucose-induced insulin release (45, 46) .

The GLP-1 dependency of the improvement of glucose tolerance by SSTR2 and SSTR5 antagonism in vivo was also evident when WT mice received the SSTR antagonists together with Ex9-39, which completely abolished their beneficial effect on glucose tolerance. Conversely, blocking GLP-1R with the same antagonist in *Sstr5^{-/-}* mice, worsened their otherwise improved glucose tolerance which seemed more pronounced in *Sstr5^{-/-}* mice receiving Ex9-39 than the *Sstr5^{+/+}* mice receiving Ex9-39, and in *Glp-1r^{-/-}* mice neither SSTR2a nor SSTR5a influenced blood glucose levels.

Consistent with the notion that SSTR2a and SSTR5a act through GLP-1, we combined the SSTR2a and SSTR5a with a DPP-4i and saw an additional improvement of glucose tolerance during an OGTT, in agreement with findings previously reported by Farb *et al* regarding SSTR5a (23). It should be noted that when DPP-4 is inhibited we cannot exclude the contribution of other DPP-4 substrates, such as GIP or Peptide YY (14, 47), however, how SSTR2 and 5 antagonism affect these hormones are still unknown.

Having demonstrated that application of SSTR2a and SSTR5a improves glucose tolerance in WT mice, we next investigated their effect in DIO mice. In perfused intestine from these animals (and controls), both SSTR2a and SSTR5a increased glucose-induced GLP-1 secretion. When the antagonists were applied by sc injection in vivo they improved glucose tolerance in WT mice, whereas no effect was seen in DIO mice. We changed the route of administration from sc to oral gavage where after the SSTR5a significantly improved glucose tolerance in both control and DIO mice, supporting findings from earlier studies (22, 23). However, the SSTR2a was incapable of decreasing blood glucose in control and DIO mice. Since the SSTR2a did work in control mice by sc injection it could suggest that this particular SSTR2a is poorly orally available, however, this needs further investigation.

Overall, our findings would suggest that clinical development of SSTR5 antagonism rather than SSTR2 antagonism may be attractive to achieve improved glycaemic control. However, one should be careful when directly comparing two antagonists based on one single dose in vivo. Some of our follow-up studies using higher doses of the SSTR5a and changing the route of application to oral instead of sc administration showed even clearer effects of the SSTR5a on blood glucose. Thus, a more detailed characterization of the two antagonists in vivo, as carried out in the perfusion studies (17), should be performed. However, the wide expression of SSTR2 argues against antagonism for this receptor for future pharmacological development since this may influence many systems; importantly, the *Sstr2* has been found to be highly expressed in certain types of cancers (48-50). SSTR5, on the other hand, is much more discretely expressed with particularly high expression levels in the GLP-1 secreting L-cells in the gut, hopefully limiting any untoward side effects.

When translating animal studies into clinical studies, certain species-specific variations should be considered. It has been reported, that SSTR5 is much more abundant in beta cells from humans than from rodents (23, 51, 52). In line with that, a human study using the SS agonist pasireotide, with a high affinity for SSTR5, resulted in both decreased insulin and GLP-1 levels (53). Targeting both GLP-1 and insulin secretion with a SSTR5 antagonist could perhaps be an advantage in the treatment of hyperglycaemia in humans.

Methods

Animals

Male and female C57BL/6JRj mice (9-12 weeks, males: 24-30 g, females: 18-23 g) were purchased from Janvier, Saint Berthevin Cedex, France. Male GLP-1R knockout (*Glp-1r^{-/-}*) and wild type (WT) littermates (*Glp-1r^{+/+}*) (9-16 weeks, 24-30 g) were developed and characterized previously in our laboratory (54). Male diet induced obese (DIO) mice and controls (27-31 weeks, DIO: 46-59 g, controls: 30-36 g) were purchased from Taconic (Hudson, NY). Male SSTR5 knockout mice (*Sstr5^{-/-}*) and *Sstr5^{+/+}* littermates (18 weeks, 34-40 g) were a kind gift from Eli Lilly Research Laboratories (Eli Lilly and Company, Indianapolis, Indiana), originally obtained from Taconic. C57BL/6JRj and *Glp-1r^{-/-}* mice were housed 2–8 mice per cage, whereas the *Sstr5^{-/-}* mice and *Sstr5^{+/+}* were housed 1-3 per cage. The DIO and control mice were housed 4-6 mice per cage. All mice were under a 12 h light/dark cycle with free access to standard rodent chow and water. The DIO mice had free access to high fat diet (Cat. no. D12492, 60 % kcal% fat, Research Diets, Inc. New Brunswick, USA) instead of standard chow.

Compounds

The following compounds were purchased from Bachem, Germany; SSTR2 antagonist, (PRL-2915 cat. no. H-6056), Exendin 9-39 (Ex9-39) (cat. no H-8740), GLP-1 (cat. no H-6795) and Bombesin (cat.no. H-2155). Dimethyl sulfoxide (DMSO, CAS no 67-68-5), 2-Hydroxypropyl beta-cyclodextrin (HPCD, cat. no H107) and Arginine (cat. no. A5131) were purchased from Sigma Aldrich. The SSTR5 antagonist (Compound B, 6-[[1-[(4-Chloro-3,5-diethoxyphenyl)methyl]-4-piperidinyl]amino]-3-pyridinecarboxylic acid; hydrochloride) was a kind gift from F. Hoffmann-La Roche Ltd., Basel,

Switzerland (22). The DPP-4i valine pyrrolidide was a kind gift from Novo Nordisk A/S, Måløv Denmark.

Perfusion of the proximal small intestine and pancreas in mice

Male C57BL/6JRj, DIO/control mice or *Sstr5*^{-/-} / *Sstr5*^{+/+} were anaesthetized with intraperitoneal (ip) injection of Ketamine/Xylazine (0.1 ml/20 g) (Ketamine 90 mg/kg (Ketaminol Vet.; MSD Animal Health, Madison, NJ, USA), Xylazine 10 mg/kg (Rompun Vet.; Bayer Animal Health, Leverkusen, Germany). The perfusion setup and procedures have been described before (17, 31, 55). In short, the colon and the distal part of the intestine were removed leaving 12-15 cm of the proximal small intestine for perfusions. When performing the pancreas perfusion, the entire intestine was removed, except for the most proximal part of the duodenum, which shares vessels with the pancreas. Next, for both operations, the spleen and stomach were tied off and removed and the kidneys were ligated. For the intestinal perfusions, a tube was placed in the proximal opening of the intestine, allowing luminal perfusion of 37 °C isotonic saline (0.9 %) NaCl, at a rate of 0.035 ml/min. Hereafter a catheter (BD Insyte Autoguard, 24 GA 0.75 IN, 0.7 x 19 mm, BD, Denmark) was placed in the abdominal part of the aorta for vascular perfusion of the intestine and pancreas via the celiac and the superior mesenteric arteries. A similar catheter was placed in the portal vein and the effluent was collected every minute using a fraction collector. When the catheters were in place, the mice were euthanized by perforation of the diaphragm. The intestine was perfused at a flow rate of 2.5 ml/min and the pancreas with 1.5 ml/min using a modified Krebs Ringer bicarbonate buffer. The buffer was pH adjusted to ~7.5 and contained 0.1 % BSA (Merck KGaA), 5 % Dextran T-70 (Dextran Products Limited, Scarborough, Canada), 3.5 mmol/l glucose and 5 mmol/l each of pyruvate, fumarate and glutamate and for gut perfusions 10 µmol/l 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, cat. no. 5879) and 5

mmol/l Vamin (a mixture of essential and non-essential amino acids; Fresenius Kabi, Copenhagen, Denmark). In a subset of the pancreas perfusions, the concentration of glucose in the buffer was increased from 3.5 to 15 mM after 40 min. During the experiment, the perfusion medium was heated to 37 °C and gassed with a 95 % O₂/5 % CO₂ mixture. After an equilibration period of 30 min, the experiment was initiated.

The perfused intestine was stimulated with 20 % (w/v) glucose from the luminal side alone or in combination with intra-arterial infusions of 1 μM SSTR2a, SSTR5a or Ex9-39. The chosen concentration was based on *in vitro* data showing, for both antagonists, half maximal inhibitory concentrations (IC₅₀) in the nano molar range for their respective receptors (IC₅₀ for SSTR2a 355 nM, 80 nM for SSTR5a and 12 nM for the GLP-1R), as well as on dose-response experiments carried out in the perfused proximal mouse intestine, published previously (17, 56).

Oral glucose tolerance tests

Male and female C57BL/6JRj, male *Glp-1r^{-/-}*, *Glp-1r^{+/+}*, male *Sstr5^{-/-}*, *Sstr5^{+/+}* and male DIO and control mice were fasted for 5-6 hours (8:00-13:00/14:00) with free access to water. The following compounds were administered by subcutaneous (sc) injection 15 min before an oral glucose load (0.004 ml/g, 2 g/kg body weight, 50 % w/v dissolved in 0.9 % NaCl). SSTR5a dissolved in PBS + 1.5 % DMSO was given in a dose of 4 mg/kg (or 8 mg/kg for the combination study with DPP-4i), SSTR2a dissolved in PBS + 1.5 % DMSO in a dose of 4 mg/kg, exendin 9-39 (Ex9-39) dissolved in milliQ water + 1.5 % DMSO in a dose of 4 mg/kg, valine pyrrolidide dissolved in PBS 120 mg/kg and GLP-1 dissolved in milliQ water + 1 % Bovine Serum Albumin (BSA) in a dose of 30 nmol/kg. Vehicle groups received PBS + 1.5 % DMSO. In DIO and control mice, the SSTR2 and SSTR5

antagonists were administered by oral gavage at a dose of 50 mg/kg, dissolved in a 30 % HPCD and 70 % milli-Q water solution. Oral gavage of the antagonists was applied 30 min before the oral glucose load. Blood glucose concentrations were measured in tail blood (~2 µl) with a handheld glucometer (Accu-Chek Mobile, catalog no. 05874149001; Roche Diagnostics, Mannheim, Germany). For hormone measurements, larger blood samples (~75 µl) were collected from the retro bulbar plexus using EDTA-coated capillary tubes (Micro Haematocrit Tubes, Ref. no. 167313 Vitrex Medical A/S, Herlev, Denmark) at times 0, 15, 30 and 60 min or -15, 0, 15 and 30 min. Blood was transferred to Eppendorf tubes and centrifuged at 3000 g for 10 min. Plasma was transferred to PCR tubes and stored at -80 °C until analysis.

Biochemical measurements for perfusion studies

Effluent samples from the perfused intestine and pancreas were measured by validated in-house radioimmunoassays (RIAs) using antibodies targeting GLP-1, glucagon, insulin and SS. The GLP-1 antibody (ab 89390) targets the amidated C-terminus of the peptide, thereby quantifying total GLP-1 (intact as well as N-terminally truncated forms of GLP-1) (57). Glucagon was measured using a C-terminally directed antibody (ab 4305) (58) and insulin was measured using an antibody raised against porcine insulin, which cross-reacts strongly with mouse, human and rat insulin (ab 2006-3) (59). SS was measured with an antibody targeting both isoforms of SS (SS-14 and SS-28, ab 1758) (60).

Biochemical measurements for in vivo studies

Plasma concentrations of insulin and GLP-1 were quantified using enzyme-linked immunosorbent assays (ELISAs) from Mercodia, Uppsala, Sweden (cat. no 10-1247-01 and 10-1278-01, respectively) and carried out according to the manufacturer's protocols.

Statistical analysis

All data are presented as mean \pm SEM and differences resulting in $p < 0.05$ were considered significant. In the perfusion studies, changes were evaluated based on either the mean incremental hormone output (subtraction of the preceding mean 5 min baseline output from the mean stimulation period plus 5-min post-stimulation period, due to the often delayed response previously observed (17)) or on total mean output. When data from two consecutive stimulations in the same animal were compared, significance was tested by paired t-test, and by unpaired t-test when testing responses from two different types of mice (i.e. WT versus knockout mice). Correlation analysis was based on the average output each minute from 1 min to 100 min or from 1 min to 40 min. Statistical evaluations in all in vivo studies are based on the incremental area under the curve (iAUC) from oral glucose gavage or ip administration (time 0 min) until the end of experiment. Significance was evaluated by one-way ANOVA followed by the Holm-Sidak post hoc analysis to correct for multiple testing. Time-dependent changes in two groups were evaluated by two-way ANOVA followed by Tukey post hoc analysis to correct for multiple testing. Calculations and graphs were made in GraphPad Prism 6 (La Jolla, CA, USA).

Study approval

All mice were used and kept in accordance with the recommendations of the National Institutes of Health (publication number 85-23), and experiments were carried out with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833 and 2018-15-0201-01397).

Author contribution

S.L.J., N.J.W.A., J.P., C.F.D., and J.J.H. conceived and designed research. S.L.J., J.A.W, K.D.G and J.E.H. performed in vivo experiments. S.L.J. performed and designed perfusion experiments. S.L.J. N.J.W.A, J.A.W, K.D.G, J.E.H. T.B.F, H.K. and R.E.M. analysed and interpreted results and S.L.J. drafted manuscript. N.J.W.A., J.A.W, K.D.G., J.E.H., T.B.F, H.K., J.P., C.F.D., R.E.M., and J.J.H. edited and revised manuscript. All authors approved final version of manuscript.

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Figures and Figure Legends

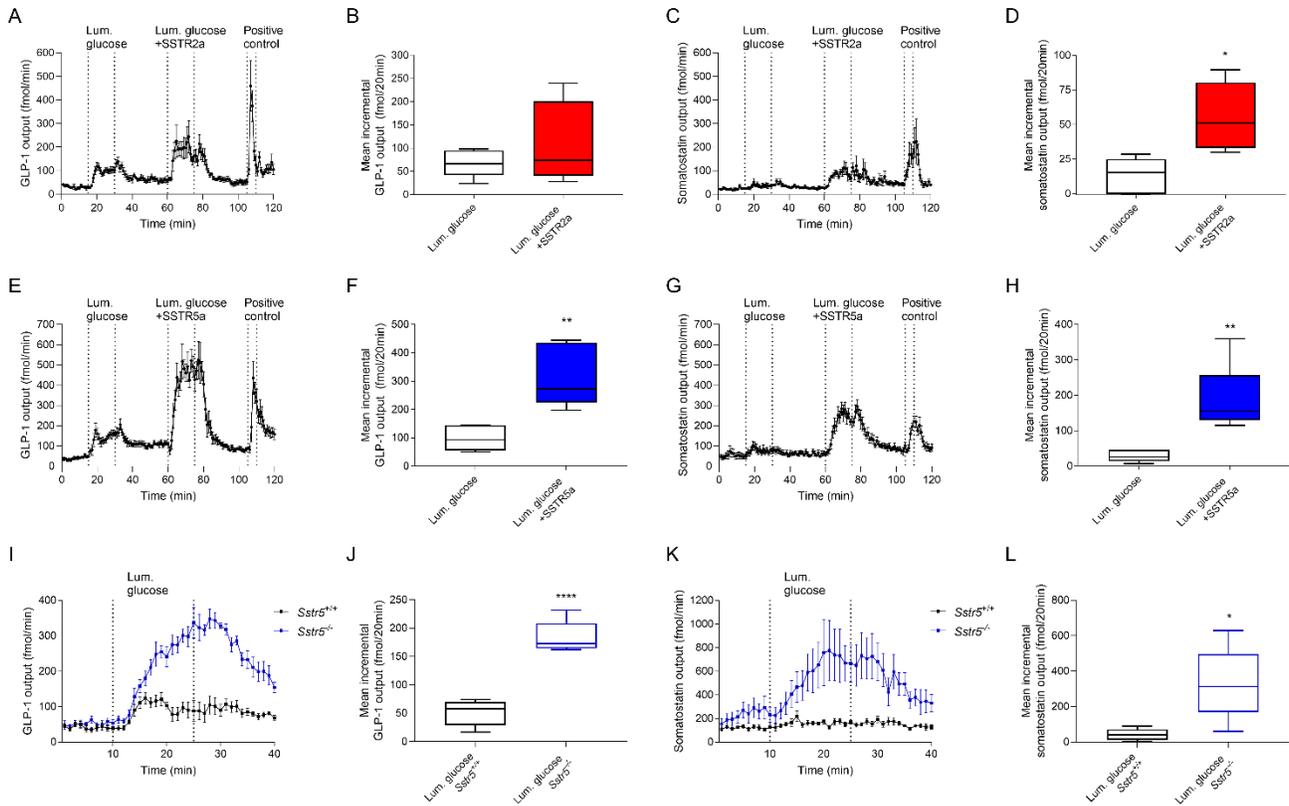


Figure 1. SSTR5a enhances glucose-induced GLP-1 secretion more than SSTR2a in the perfused mouse proximal small intestine. GLP-1 and somatostatin levels in the effluent from the perfused proximal small intestine of non-fasted C57BL/6JRj (A-H) or *Sstr5*^{-/-} and *Sstr5*^{+/+} male mice (I-L). The intestine preparations were stimulated with luminal glucose (20 % w/v) alone or in combination with a simultaneous intra-arterial infusion of either 1 μ M SSTR2a or SSTR5a, where after GLP-1 and SS were measured. A-D) GLP-1 and SS output (fmol/min) or mean incremental output (fmol/20 min) in response to glucose and glucose + SSTR2a in C57BL/6JRj mice, GLP-1: n=8, SS: n=5. E-H) GLP-1 and SS output (fmol/min) or mean incremental output (fmol/20 min) in response to glucose and glucose + SSTR5a in C57BL/6JRj mice, GLP-1 and SS: n=6. I-L) GLP-1 or SS output (fmol/min) or mean incremental output (fmol/20 min) after luminal infusion of glucose in male *Sstr5*^{-/-} (blue) or *Sstr5*^{+/+} mice (black), n=5. Bombesin was used as the positive control. Data are presented as means \pm SEM. Statistical significance was tested by paired t-test (B, D, F and H) or unpaired t-test (J and L). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. The box plots show the median and 25th and 75th percentiles, and the whiskers represent the smallest and highest value.

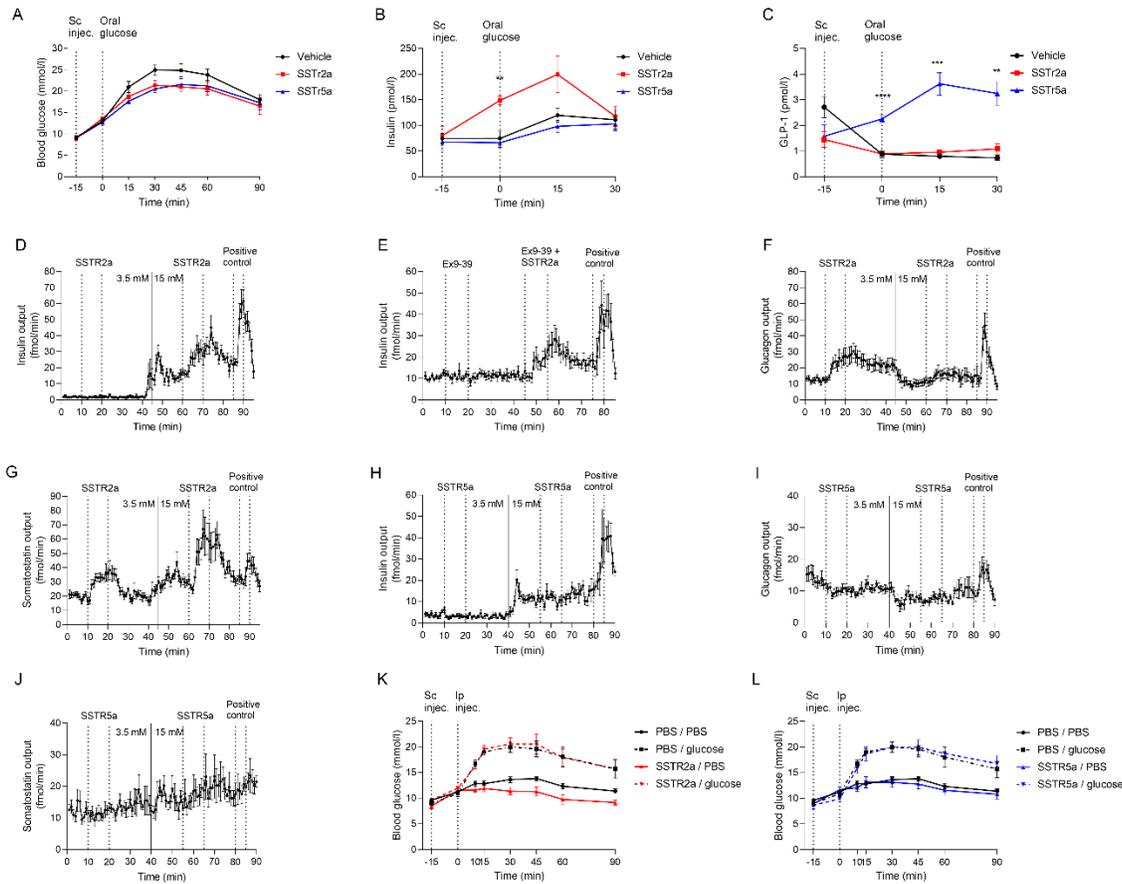


Figure 2. SSTR2a and SSTR5a lower blood glucose in vivo. During hyperglycaemia, in the perfused pancreas, SSTR2a increases insulin whereas SSTR5a does not. A-C) Plasma blood glucose (mmol/l), insulin levels (pmol/l) and GLP-1 levels (pmol/l) after male C57BL/6JRj mice received vehicle (black), 4 mg/kg SSTR2a (red) or SSTR5a (blue) by sc injection 15 min before an oral glucose load, n=8. D-J) Insulin, glucagon and insulin levels after the pancreas was perfused with a perfusion buffer at low glucose concentration (3.5 mM) from 0-40 min, whereafter the buffer was exchanged to a high glucose containing buffer (15 mM) for the rest of the experiment (D, F-J), or at a constant concentration of 15 mM (E). 1 μ M of SSTR2a, SSTR5a or Ex9-39 were added to the arterial perfusate via a side-arm. 10 mM arginine was used as positive control at the end of each perfusion experiment, n=6. K-L) Male mice received sc injections of vehicle (PBS), 4 mg/kg SSTR2a or SSTR5a at time -15 min and at time 0 min they received ip injection of glucose or PBS. K) Blood glucose (mmol/l) levels after the following injections: PBS sc at -15 and ip PBS at 0 min (black line), sc PBS at time -15 min and ip glucose at 0 min (black dashed line), sc SSTR2a at -15 min and ip PBS at 0 min (red line), or sc SSTR2a at -15 min and ip glucose at 0 min (red dashed line), n=8. L) The same as K, but with SSTR5a, represented in blue. SSTR5a sc at -15 min and ip PBS (blue line), ip glucose at 0 min and sc SSTR5a at -15 min (blue dashed line), n=8. Data are presented as means \pm SEM. Statistical significance at specific time points was assessed by two-way ANOVA followed by Tukey post hoc analysis to correct for multiple testing in vivo and by paired t-test in the perfusion experiments. **p<0.01, ***p<0.001 and ****p<0.0001.

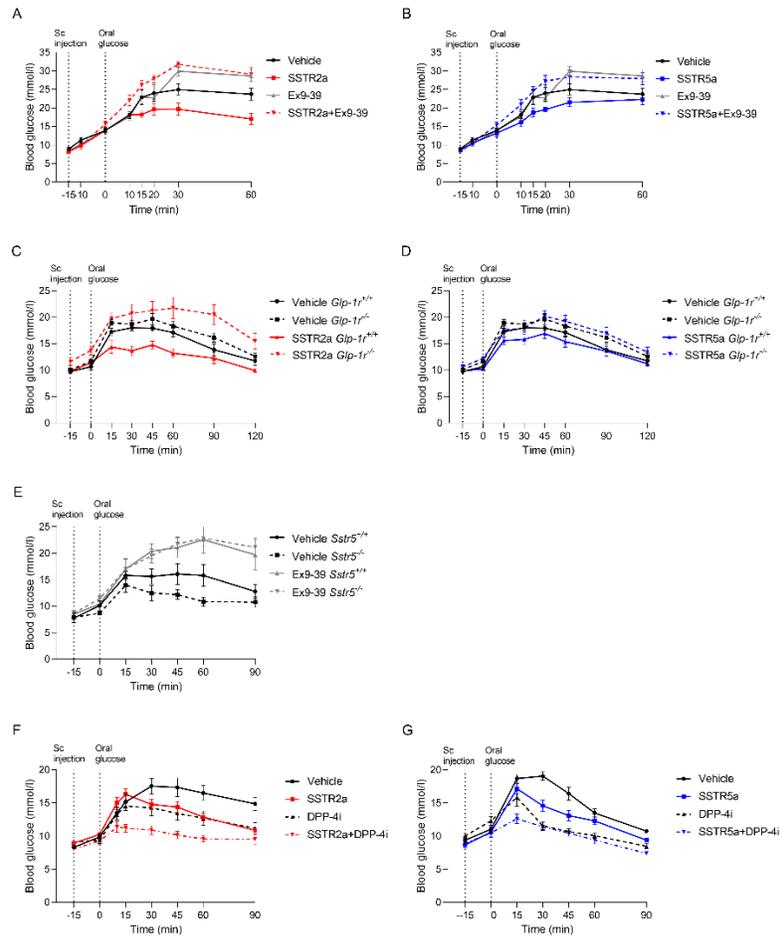


Figure 3. The glucose lowering effect of SSTR2a and SSTR5a is GLP-1R dependent. A) Blood glucose levels (mmol/l) in C57BL/6JRj mice receiving vehicle (black line), 4 mg/kg SSTR2a (red line), 4 mg/kg Ex9-39 (grey line) or SSTR2a+Ex9-39 (dashed red line) sc 15 min before an oral glucose load, n=5-8. B) The same as A, but for 4 mg/kg SSTR5a (blue), 4 mg/kg SSTR5a + 4 mg/kg Ex9-39 (dashed blue line), n=5-8. C) Blood glucose levels (mmol/l) in *Glp-1r*^{-/-} or *Glp-1r*^{+/+} mice after vehicle or 4 mg/kg SSTR2a. *Glp-1r*^{+/+} receiving vehicle (black line), *Glp-1r*^{-/-} receiving vehicle (black dashed line), *Glp-1r*^{+/+} receiving 4 mg/kg SSTR2a (red line), *Glp-1r*^{-/-} receiving 4 mg/kg SSTR2a (dashed red line), n=8-13. D) The same as C but for 4 mg/kg SSTR5a indicated with blue. *Glp-1r*^{+/+} receiving 4 mg/kg SSTR5a (blue line), *Glp-1r*^{-/-} receiving 4 mg/kg SSTR5a (dashed blue line), n=10-13. E) Blood glucose levels (mmol/l) in *Sstr5*^{-/-} or *Sstr5*^{+/+} mice receiving vehicle or 4 mg/kg Ex9-39. *Sstr5*^{+/+} receiving vehicle (black line), *Sstr5*^{-/-} receiving vehicle (black dashed line), *Sstr5*^{+/+} receiving 4 mg/kg Ex9-39 (grey line), *Sstr5*^{-/-} receiving 4 mg/kg Ex9-39 (grey dashed line), n=4-5. F) Blood glucose levels (mmol/l) after administration of vehicle (black line), 4 mg/kg SSTR2a (red line), 120 mg/kg DPP-4 (dashed black line) and a combination of SSTR2a and DPP-4i (red dashed line), n=8. G) The same as F but for 8 mg/kg SSTR5a (blue line and SSTR5+ DPP-4i (blue dashed line), n=6-8. Data are shown as means±SEM and significance was evaluated based on iAUC by one-way ANOVA followed by the Holm-Sidak post hoc analysis to correct for multiple testing.

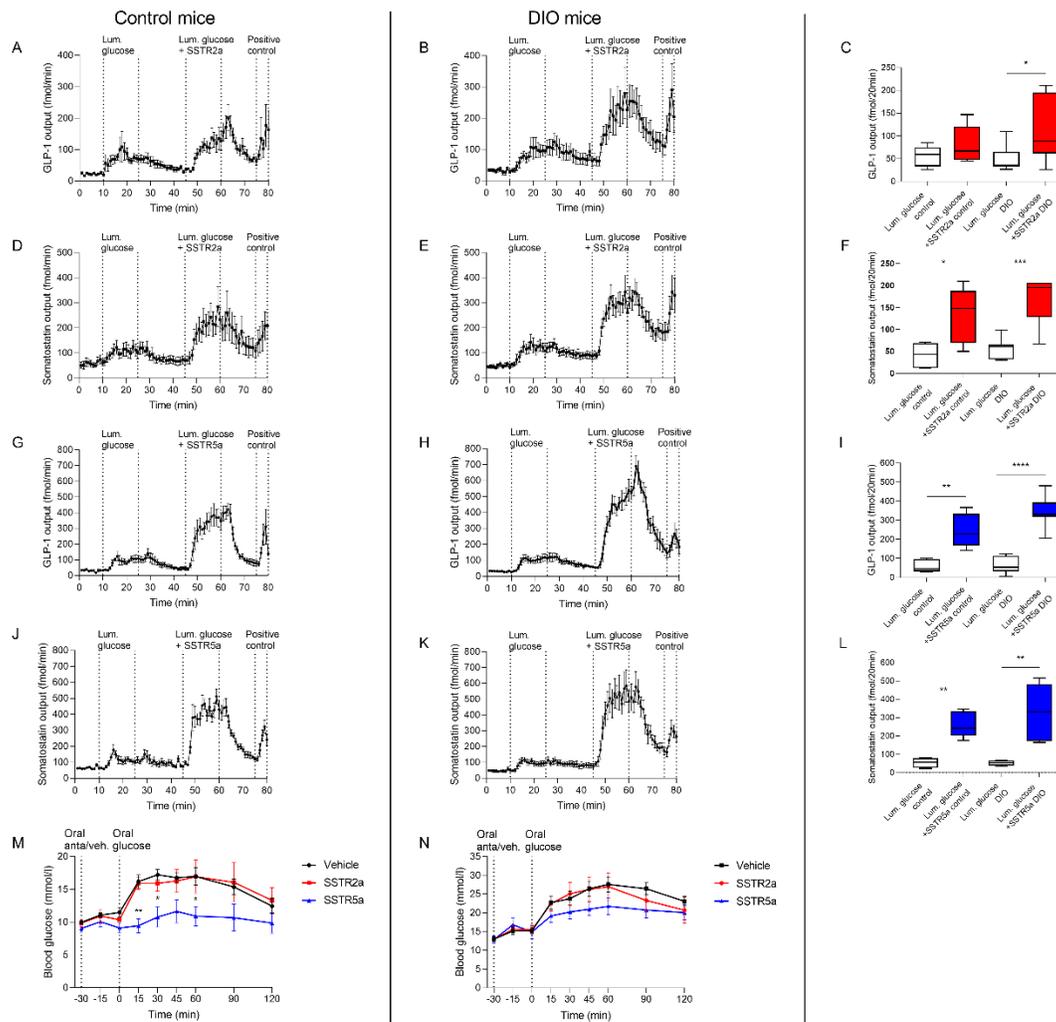


Figure 4. In proximal perfused intestine of DIO and control mice, SSTR5a stimulates glucose-induced GLP-1 secretion more than SSTR2a, and SSTR5a improves glucose tolerance when applied orally in DIO mice. A-L) GLP-1 and SS output (fmol/min) or mean incremental output (fmol/20 min) in proximal intestinal perfusions of control and DIO mice. The intestine was stimulated with luminal glucose alone or in combination with simultaneous intra-arterial infusion of 1 μ M SSTR2a or SSTR5a. A: n=7, B: n=5, D: n=7, E: n=6, G: n=7, H: n=5, J: n=7, K: n=4. M-O) In vivo studies in control and DIO mice undergoing an OGTT after oral administration of vehicle (black), 50 mg/kg SSTR2a (red) or SSTR5a (blue) 30 min before oral glucose. M) Blood glucose levels (mmol/l), n=5-6, control mice. N) Same as M but in DIO mice, n=4. Data are presented as means \pm SEM. Statistical significance at specific time points was assessed by two-way ANOVA followed by Tukey post hoc analysis to correct for multiple testing (M) and by paired t-test (C, F, I and L), * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001. The box plots show the median and 25th and 75th percentiles, and the whiskers represent the smallest and highest value.

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