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Research Article

Locally produced xenin and the neurotensinergic system in pancreatic islet function and β-cell survival

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Abstract

Modulation of neuropeptide receptors is important for pancreatic β-cell function. Here, islet distribution and effects of the neurotensin (NT) receptor modulators, xenin and NT, was examined. Xenin, but not NT, significantly improved glucose disposal and insulin secretion, in mice. However, both peptides stimulated insulin secretion from rodent β-cells at 5.6 mM glucose, with xenin having similar insulinotropic actions at 16.7 mM glucose. In contrast, NT inhibited glucose-induced insulin secretion. Similar observations were made in human 1.1B4 β-cells and isolated mouse islets. Interestingly, similar xenin levels were recorded in pancreatic and small intestinal tissue. Arginine and glucose stimulated xenin release from islets. Streptozotocin treatment decreased and hydrocortisone treatment increased β-cell mass in mice. Xenin co-localisation with glucagon was increased by streptozotocin, but unaltered in hydrocortisone mice. This corresponded to elevated plasma xenin levels in streptozotocin mice. In addition, co-localisation of xenin with insulin was increased by hydrocortisone, and decreased by streptozotocin. Further in vitro investigations revealed that xenin and NT protected β-cells against streptozotocin-induced cytotoxicity. Xenin augmented rodent and human β-cell proliferation, whereas NT displayed proliferative actions only in human β-cells. These data highlight the involvement of NT signalling pathways for the possible modulation of β-cell function.

Keywords: apoptosis, β-cell, COPA, diabetes, islets, neurotensin (NT), neurotensin receptor (NTSR), xenin.
Introduction

Emerging evidence indicates the physiological significance of non-classical islet peptides and neuropeptides for the regulation of insulin secretion and β-cell mass. Such peptides found within islets and studied recently include glucagon-like peptide-1 (GLP-1), glucose-dependent insulino tropic polypeptide (GIP), Peptide YY (PYY), ghrelin pancreatic polypeptide and neuropeptide Y (Fujita et al., 2010; Moffett et al., 2013; 2014; Vasu et al., 2014; Wireup et al., 2014; Khan et al., 2016, 2017). Cholinergic nerve fibres that innervate pancreatic islets also play a role in insulin secretion, implicating involvement of peptidergic as well as classical neurotransmitters (Ahren et al., 2006). Neurotensin (NT), is an interesting candidate in this respect, being a 13 amino acid neuropeptide secreted by pancreatic nerve and enteroendocrine cells in response to a meal (Béraud-Dufour et al., 2010). A predominant focus of NT research to date has been linked to effects on energy balance (Brown et al., 2015). Indeed, a recent study has associated elevated NT levels with the development of obesity (Li et al., 2016). Thus, NT is known to be a satiety hormone, acting in conjunction with leptin (Leinninger et al., 2011). However, the expression of immunoreactive NT, and all major NT receptors (NTSRs), within the endocrine pancreas (Fernstrom et al., 1981), suggests that NT could be fundamentally involved in the regulation of pancreatic β-cell function. Moreover, xenin, an NT-related peptide which is secreted from the intestinal K-cells along with GIP (Anlauf et al., 2000) and shows homology to the biologically active C-terminus of NT (Feurle et al., 1992), has been evidenced in the pancreas (Hamscher et al., 1995), and is thought to be involved in pancreatic endocrine function and regulation of glucose homeostasis (Taylor et al., 2010; Gault et al., 2015; Parthsarathy et al., 2016). Interestingly, similar to NT, xenin has notable satiety effects particularly at elevated doses (Taylor et al., 2010).

The biological effects of NT are mediated via interaction with three well characterised receptors, namely NTSR1, NTSR2 and NTSR3/sortilin (Kalafatakis and Triantafyllou, 2011; Kim et al., 2015). NTSR1 and NTSR2 are classical seven transmembrane spanning G-protein coupled receptors (GPCRs), whereas NTSR3/sortilin is type I single transmembrane domain receptor (Petersen et al., 1997). In terms of β-cell secretory function, NT has been suggested to stimulate insulin secretion at basal glucose concentrations, and to inhibit glucose-stimulated insulin secretion (GSIS) (Dolais-Kitabgi et al., 1979), likely through modulation of protein kinase C activity (Béraud-Dufour et al., 2010). Further studies have also implied a
β-cell protective effect for NT, that could be mediated via NTSR2 (Coppola et al., 2008). Xenin has been suggested to activate NTSR1 (Feurle et al., 2002), however there is evidence for non-NTSR mediated biological actions of xenin (Heuser et al., 2002). Thus, some of the pancreatic actions of xenin could be facilitated by cellular pathways independent of the neurotensinergic system (Mazalla et al., 2012). Taken together, it is clear that NTSR signalling may play an important role in the endocrine pancreas, however potential impact and actions on β-cell secretory function and survival still need to be fully elucidated.

In the present study we demonstrated expression of all major NTSRs in rodent BRIN BD11 and human 1.1B4 β-cells, as well as isolated mouse islets. We then evaluated the presence of NT and xenin in islets in normal, insulin-deficient and insulin-resistant states together with the effects of the two peptides on β-cell proliferation and apoptosis. The results suggest that locally produced NT and xenin play a potential role in the regulation of β-cell function and islet cell adaptations to stress.

Results

Expression of NTSRs by rodent and human β-cell lines as well as NT and COPA by isolated mouse islets

Expression of NTSR1 was markedly (P<0.001) elevated in BRIN BD11 cells when compared to GIPR (Figure 1A). In contrast, mRNA expression of NTSR1 was similar in 1.1B4 cells and relatively lower (P<0.01) in isolated mouse islets (Figure 1B,C). NTSR2 expression was similar to GIPR expression in BRIN BD11 β-cells and mouse islets (Figure 1A,B), but lower (P<0.01) in human 1.1B4 β-cells (Figure 1C). Finally, NTSR3 mRNA levels were higher (P<0.05 to P<0.001) in BRIN BD11 cells, 1.1B4 cells and mouse islets (Figure 1A-C). Interestingly, when compared to respective levels of the housekeeping gene β-actin, GIPR transcripts were approximately 200- and 1000-fold higher in mouse islets than in the BRIN BD11 and 1.1B4 β-cells, respectively (Figure 1A-C). Similarly, NTSR2 mRNA expression was approximately 300- and 1500-fold greater in mouse islets when compared to BRIN BD11 and 1.1B4 β-cells, respectively (Figure 1A-C). In addition, NTSR3 transcript levels were around 50-fold higher in mouse islets than in both cultures β-cell lines, when compared to respective β-actin levels (Figure 1A-C). As shown by Figure 1D, mouse islets expressed both NT and the gene responsible for synthesis of xenin, COPA, at levels 20-40% of that of...
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insulin. These data reveal that transcripts for COPA, insulin, NT and NTSR’s1-3 are present in islets.

**Effects of NT and xenin on insulin release from rodent and human β-cell lines, isolated mouse islets and secretion of xenin from islets**

As expected, KCl (30 mM), alanine (10 mM) and GLP-1 (10^{-6} M and 10^{-8} M) significantly \((P<0.001)\) increased insulin release from BRIN BD11 (Figure 2A,B) and 1.1B4 (Figure 2C) β-cells at either 5.6 or 16.7 mM glucose levels. NT and xenin also significantly \((P<0.001)\) stimulated insulin release at 5.6 mM glucose from BRIN BD11 cells at the highest peptide concentration \((10^{-6} M)\) examined (Figure 2A). As would be expected, rodent BRIN BD11 and human 1.1B4 β-cells possessed clear and significant \((P<0.001)\) glucose responsive insulin secretory actions following exposure to 16.7 mM glucose (Figure 2B,C). However, whilst xenin had similar \((P<0.001)\) insulinotropic actions at 16.7 mM glucose in BRIN BD11 cells (Figure 2B), NT failed to potentiate GSIS and actually significantly \((P<0.05 \text{ to } P<0.001)\) reduced the insulin secretory response evoked by 16.7 mM glucose at concentrations of 10^{-7} M and above (Figure 2B). However, insulin secretion in response to NT at 16.7 mM glucose was still elevated \((P<0.05 \text{ to } P<0.001)\) when compared to 5.6 mM glucose alone (Figure 2B). Similar insulinostatic \((P<0.05)\) and insulinotropic \((P<0.001)\) effects of NT and xenin, respectively, were observed in human 1.1B4 β-cells (Figure 2C). In addition, although concentrations of 10^{-8} and 10^{-7} M NT and xenin had no effect on the insulin secretory function of isolated mouse islets, at 10^{-6} M NT significantly inhibited \((P<0.01)\) and xenin augmented \((P<0.01)\) insulin secretory responses at 16.7 mM glucose (Figure 2D), in complete harmony with findings from BRIN BD11 and 1.1B4 β-cells (Figure 2B,C). To elucidate NTSR involvement, BRIN BD11 cells were incubated at 16.7 mM glucose with NT or xenin \((10^{-8} \text{ and } 10^{-6} M)\) alone or in the presence of the NTSR antagonist, SR142948A (Figure 2E). SR14294p8A partially reversed the insulinotropic action of 10^{-6} M xenin (Figure 2E). In addition, the insulinostatic effect of NT at 10^{-8} and 10^{-6} M was fully inhibited by SR142948A (Figure 2E). Interestingly, the murine NTSR2 agonist levocabastine, although partially inhibiting 16.7 mM glucose induced insulin release, completely reversed the insulinostatic effect of 10^{-6} M NT (Figure 2G). Moreover, in the presence of xenin, levocabastine, also moderately reversed the insulin releasing actions of xenin (Figure 2G). Furthermore, xenin secretion from isolated mouse islets was examined, with glucose (20 mM) and arginine (30 mM) inducing a small but significant \((P<0.05 \text{ and } P<0.01; \text{ respectively})\) increase in xenin
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Neurotensin, xenin and β-cells release (Figure 2F). Total islet xenin content was also assessed (50-80 islets, n=6), and was 12.9 ± 1.7 pg/50 islets. These data reveal that xenin, including that released from islets, may have functional effects on insulin secretion.

Effects of NT and xenin on membrane potential and intracellular Ca²⁺ in rodent BRIN BD11 cells

KCl (15 mM) and alanine (10 mM) significantly (P<0.001) depolarised BRIN BD11 cells and augmented [Ca²⁺]i, respectively, at 5.6 mM glucose (Figure 3A-D). NT had no effect on cell membrane potential and [Ca²⁺]i (Figure 3A-D). Xenin also had no effect on [Ca²⁺]i (Figure 3C,D), but increased (P<0.05) membrane potential over the 300 s observation period (Figure 3A,B). Xenin-induced increases in BRIN BD11 cell membrane potential were markedly (P<0.001) less than that evoked by KCl (Figure 3A,B). These data suggest that xenin modulates insulin release by mechanisms other than membrane potential and [Ca²⁺]i.

Effects of NT and xenin on glucose tolerance in mice

Administration of 25 nmol/kg NT in combination with glucose had no effect on individual or overall blood glucose levels and corresponding plasma insulin concentrations in mice (Figure 4A-D). However, the same dose of xenin significantly (P<0.001) lowered blood glucose levels at 15 and 30 min post-injection (Figure 4A), culminating in a significantly (P<0.05) reduced overall glycaemic excursion when compared to glucose alone (Figure 4B). In agreement, corresponding overall glucose-stimulated plasma insulin concentrations were significantly (P<0.05) increased by xenin (Figure 4D). Such data indicate that xenin exerts incretin-like actions when administered parenterally.

Effects of streptozotocin and hydrocortisone induced insulin-deficiency and insulin-resistance on islet expression and localisation of NT and xenin

As would be expected, streptozotocin reduced (P<0.01) β-cell area, and hydrocortisone increased (P<0.001) β-cell area compared to control mice (β-cell areas were 2221±283, 1240±1260 and 7085±687 µm², respectively). Streptozotocin mice also presented with increased (P<0.001) α and PP cell areas, whereas α and PP cell area were not altered in hydrocortisone-treated mice when compared to controls (α-cell areas were 3961±436, 2322±211 and 2044±226 µm², and PP cell areas 3954±412, 1694±140 and 1918±195 µm² in streptozotocin, hydrocortisone and control mice, respectively). Representative islets images
Neurotensin, xenin and β-cells showing immunoreactive xenin or NT in islets of control, streptozotocin and hydrocortisone treated mice are shown in Figure 5A-C. Streptozotocin treatment significantly increased ($P<0.05$) co-localisation of xenin with glucagon (Figure 5A,D), while hydrocortisone did not alter xenin islet distribution (Figure 5A,D). In addition, streptozotocin mice had significantly elevated ($P<0.05$) plasma xenin levels when compared to control and hydrocortisone treated mice (Figure 5E). Interestingly, xenin immunoreactivity was observed in β-cells (Figure 5B) and streptozotocin treatment significantly decreased ($P<0.05$) whilst hydrocortisone increased ($P<0.05$), co-localisation of xenin with insulin (Figure 5F). None of the treatment interventions affected pancreatic xenin levels, and intriguingly pancreatic and intestinal xenin levels were more or less equivalent (Figure 5G). Treatment of mice with either streptozotocin and hydrocortisone appeared to increase the relative immunoreactivity of NT in exocrine and endocrine pancreatic tissue (Figure 5C). These data reveal that both xenin and NT are produced by islets and suggest functional effects in response to streptozotocin and hydrocortisone treatments.

**Effects of NT and xenin on rodent BRIN BD11 and human 1.1B4 cell proliferation**

GLP-1 enhanced ($P<0.01$) proliferative frequency in BRIN BD11 and 1.1B4 β-cells (Figure 6A,B). Similarly, NT and xenin significantly ($P<0.05$) increased proliferation in human 1.1B4 β-cells (Figure 6A,B). Xenin also increased ($P<0.05$) proliferation frequency in BRIN BD11 β-cells, however NT failed to enhance proliferation frequency in these cells (Figure 6A). Representative images of Ki67 stained BRIN BD11 and 1.1B4 cells under each culture condition are shown in Figure 6C. Such data indicate that xenin and NT act to increase β-cell proliferation.

**Protective effects of NT and xenin on streptozotocin-induced DNA damage in rodent BRIN BD11 and human 1.1B4 cells**

Streptozotocin (5 mM) significantly ($P<0.001$) decreased both BRIN BD11 and 1.1B4 β-cell viability, which was fully countered by co-incubation with $10^{-6}$ M GLP-1 (Figure 7A,B). NT and xenin also had positive effects on BRIN BD11 and 1.1B4 β-cell viability following streptozotocin insult, with NT fully reversing the detrimental effect of streptozotocin in BRIN BD11 cells and xenin having a similar beneficial effect in 1.1B4 cells (Figure 7A,B). However, cell viability of BRIN BD11 cells exposed to streptozotocin and xenin was still
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reduced \((P<0.05)\) compared to control culture (Figure 7A), with a similar observation apparent in 1.1B4 cells treated with streptozotocin and NT (Figure 7B). Streptozotocin also increased \((P<0.001)\) increased % tail DNA and olive tail moment in rodent and human β-cell lines, which was fully or partially reversed by co-incubation with GLP-1 (Figure 7C-F). In agreement, NT fully reversed the negative effect of streptozotocin on BRIN BD11 cell % tail DNA (Figure 7C). Co-incubation with xenin had a similar beneficial \((P<0.05)\) effect on streptozotocin-induced DNA damage, but % tail DNA was still elevated \((P<0.001)\) when compared to control cultures (Figure 7C). The negative effect of 5 mM streptozotocin on olive tail moment in BRIN BD11 and 1.1B4 β-cells was partially countered \((P<0.05\) to \(P<0.001)\) by NT and xenin, but levels were still elevated compared to respective control cells (Figure 7E,F). Representative comet images from each culture condition in BRIN BD11 and 1.1B4 cells are shown in Figure 7G. Combined with data on Ki-67 staining, these observations suggest that xenin and NT increase β-cell mass.

Discussion

The demonstration of NT and NTSRs in the endocrine pancreas, as shown here and previously (Béraud-Dufour et al., 2010; Fernstrom et al., 1981; Trandaburu et al., 2003), suggests an important role for the neurotensinergic system in the regulation of islet function. In this regard, NTSR expression levels within the islets of NIH Swiss mice in the current study corresponds well with previous reports (Coppola et al., 2008; Béraud-Dufour et al., 2009). Despite the presence of NTSRs on all cell types examined, differences in the relative expression of subtypes between primary mouse islets (predominantly NTSR2) and cultured rodent or human β-cells (predominantly NTSR1 and NTSR3, respectively) were evident, highlighting a potential limitation of studying NT related effects in these cell lines. Nevertheless, overall observations in both rodent and human β-cell lines were faithfully replicated in isolated mouse islets, confirming applicability of the chosen experimental systems, and possible threshold of receptor expression levels required to mediate biological responses. Such data may also suggest significant overlap in the functional pathways triggered by NTSR1-3 in β-cells. Moreover, there is a strong possibility that some of the biological actions of xenin are completely distinct from modulation of NTSR related pathways (Mazella et al., 2012). In agreement with this, the ability of xenin to augment GSIS was only partially reversed by the NT receptor antagonist, SR142948A. In addition,
levocabastine the NTSR2 agonist in mice but antagonist in humans (Vita et al., 1998), fully blocked the modulatory effects of NT on insulin secretion in BRIN BD11 cells, but only had a moderate impact on xenin-mediated actions. Further investigations, such as β-cell specific NT receptor knockdown studies, would be required to fully delineate xenin receptor interactions at the level of the β-cell. Whilst, NT also stimulated insulin secretion at low glucose concentrations, GSIS was not potentiated from rodent and human β-cell lines, as well as isolated mouse islets, in full accord with previous reports (Dolais-Kitabgi et al., 1979; Béraud-Dufour et al., 2010).

Effects of NT on the regulation of insulin secretion have been investigated at the receptor level and shown to be associated to formation of a NTSR2/NTSR3 complex (Mazella et al., 2012). We therefore investigated cellular mechanisms of action of both peptides on the modulation of insulin secretion. NT did not alter BRIN BD11 β-cell membrane potential and [Ca^{2+}]_i, suggesting more distal effects on insulin secretion. Interestingly, NT was previously demonstrated to activate Ca^{2+} flux in INS-1E β-cells (Béraud-Dufour et al., 2009), although the inhibitory effect of NT on GSIS was absent in this cell line. Whilst xenin also had no effect on [Ca^{2+}]_i in BRIN BD11 cells, it did induce a slight, but measurable, membrane depolarisation, suggesting possible involvement of K_{ATP} channel-dependent pathways. Small effects on membrane potential without concomitant increases in [Ca^{2+}]_i indicate the potential of alternative signal transduction pathways in the insulinotropic actions of xenin. A similar, but less obvious, effect of xenin on membrane potential was previously observed in BRIN BD11 cells at 16.7 mM glucose (Taylor et al., 2010). In addition, modulation of cAMP levels does not appear to be involved in xenin-induced insulin secretion (Taylor et al., 2010). Thus, further detailed studies are required to uncover the cellular and molecular mechanisms leading to NT and xenin mediated effects on β-cell secretory function, and to determine if xenin-induced changes on β-cell membrane potential are physiologically relevant. Indeed, in contrast to the current observations, others have reported limited direct effects of xenin on insulin secretion, with β-cell neuronal stimulation by xenin believed to be more important (Wice et al., 2010). This difference may require further investigation although, in agreement with our in vitro and ex vivo investigations, xenin significantly augmented GSIS and improved glucose tolerance following an intraperitoneal glucose challenge in mice (Taylor et al., 2010; Martin et al., 2012; Gault et al., 2015; Parthsarathy et al., 2016). NT had no effect on GSIS in mice, and while this contrasts with our in vitro findings, it is likely related to concentrations of NT exposed directly to islet cells.
Although there is some evidence for a role of NT and xenin in glucose homeostasis (Dolais-Kitabgi et al., 1979; Taylor et al., 2010), the possible impact of these peptides in diabetes is not fully understood. Previous studies suggest that increased islet nerve innervation, and subsequent action of peptidergic neurotransmitters, is a distinguishing feature of diabetes (Ahren et al., 2006). In agreement, NT levels appear to be increased in human diabetes and obesity (Melander et al., 2012; Li et al., 2016), with the action of xenin shown to be altered in type 2 diabetes (Wice et al., 2012; Chowdury et al., 2016). We therefore assessed islet expression and localisation of NT and xenin in both streptozotocin- and hydrocortisone-induced diabetic mice. mRNA for both COPA, a ubiquitously expressed gene that gives rise to xenin (Chow and Quek, 1997), and NT were present in islets and we were able to evidence xenin expression in pancreatic α- and β-cells. Co-localisation studies in streptozotocin mice revealed that glucagon-containing α-cells exhibited significantly increased co-expression of xenin, but β-cells had decreased co-localisation with xenin. Considering that α-cell area was increased in these mice, the data would suggest equivalent elevations in islet-specific xenin concentrations, although it should be noted that overall pancreatic xenin levels were not significantly altered by streptozotocin treatment. Indeed, xenin was also found to be co-localised with insulin, and as would expected, streptozotocin mice had dramatically decreased β-cell area. However, circulating xenin levels were markedly elevated in these mice. This might suggest a possible role for xenin in the maintenance of islet architecture following β-cell loss, as recent observations suggest that α-cells play a role in the stimulation of β-cell regeneration (Li et al. 2017). Interestingly, although hydrocortisone treatment increased both β-cell number and co-expression of xenin with insulin, circulating xenin levels were unaltered in these mice. Thus, it is possible that secretion of pancreatic α- and β-cell, as well as gut derived xenin is differentially regulated.

It is interesting to note that GIP, known to be co-located and secreted with xenin from duodenal cells (Anlauf et al., 2000), and act in conjunction with xenin (Taylor et al., 2010), is also expressed in pancreatic α-cells (Fujita et al., 2010). However, GIP levels appear to be unaltered following streptozotocin insult in mice (Vasu et al., 2014), thus xenin may have an important GIP-independent role in cellular adaptations to insulin deficiency. Interestingly, glucose and arginine were both stimuli for islet release of xenin, emphasising a potential paracrine or autocrine role for xenin in modulating islet function. Moreover, xenin immunoreactivity in both α- and β-cells, with pancreatic levels similar to intestinal xenin concentrations as measured by ELISA, would further support this concept. Endocrine and
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Exocrine expression of NT was increased by both streptozotocin and hydrocortisone treatment in nerves innervating the pancreas, implying key adaptive functions for NT in situations of both insulin deficiency and resistance (Fernstrom et al., 1981; Sheppard et al., 1985). To further examine the role of NT and xenin in diabetes, we assessed the proliferative and anti-apoptotic actions of both peptides in cultured rodent and human β-cells.

Xenin significantly augmented rodent and human β-cell proliferation, whereas NT had positive effects only on human 1.1B4 cells. Notably, 1.1B4 β-cells represent a fusion of human islets with human pancreatic cancer PANC-1 cells (McCluskey et al., 2011), and NT receptor activation has been shown to augment PANC-1 proliferation (Wang et al., 2011). Therefore, further studies with primary human islets are required to delineate the importance of receptors derived from islet, as opposed to PANC-1 cells, for xenin and NT mediated benefits on human β-cell proliferation. Nonetheless, NT has long been recognised as a potent cellular growth factor (Wood et al., 1988), with trophic effects related to activation of NTSR3/sortlin (Devader et al., 2013). The superior proliferative actions of xenin compared to NT in the current study are therefore intriguing, and merit further investigation. Moreover, the elevated plasma levels of xenin observed in streptozotocin-induced diabetic mice could suggest an important role for this peptide in situations of severe β-cell destruction. The anti-apoptotic role of NT in various cell types has already been reviewed in some detail (Devader et al., 2013). In agreement, we demonstrated that NT protected both BRIN BD11 and 1.1B4 β-cells from streptozotocin-induced DNA damage, to a similar or superior degree, as GLP-1 (Thiriet et al., 2005). Previous research has shown that NT protects β-cells against apoptosis through activation of a NTSR2/NTSR3 heteromer, stimulating PI-3 kinase activity which in turn phosphorylates Akt (Coppola et al., 2009), and it would be interesting to assess levels of this enzyme in future studies. Notably, xenin was marginally less efficacious than NT in terms of protecting cultured β-cells from apoptosis. Thus, xenin is thought to be an antagonist to NTSR2, and activate NTSR3 with only low affinity (Sterl et al., 2016), which could justify its reduced effectiveness. Xenin did fully protect human 1.1B4 β-cells from cytotoxic effects of streptozotocin, although NTSR3 expression was particularly abundant in 1.1B4 cells, which may help explain this finding.

In conclusion, the current observations provide further evidence that both NT and xenin play a role in regulating the endocrine pancreas. We also showed that NT and xenin are endogenously produced within the pancreas, and the impact of this still requires further detailed examination. Potential benefits of NT and xenin on β-cell growth and survival...
suggest that modulation of NTSR signalling could have potential benefits for the treatment of diabetes.

**Materials and methods**

**NTSR, NT and gene expression**

Real-time PCR was used to determine expression of NTSRs, mRNA was extracted from rodent BRIN BD11 and human 1.1B4 β-cells, as well as freshly isolated islets from NIH Swiss mouse (12-14 week-old, Harlan, UK), using an RNeasy Mini Kit following manufacturer’s instructions (Qiagen, UK), as described previously (Khan et al., 2017). All RT-PCR primer sequences are provided in Table 1. Briefly, mRNA (3 µg) was converted to cDNA using SuperScript II Reverse Transcriptase kit (Invitrogen, Paisley, UK). Amplification conditions were set at 95°C for initial and final denaturation, 58°C for primer annealing and 72°C for extension for 40 cycles, followed by a melting curve analysis, with temperature range set at 60°C to 90°C. Expression of NT and α-coat protein (COPA), the gene responsible for synthesis of xenin, were also determined in mouse islets. Data were analysed using ΔΔCt method and normalised to Actb/ACTB expression.

**In vitro studies**

Evaluation of the actions of NT and xenin on β-cells were assessed in vitro using both rodent and human clonal β-cell lines as well as isolated mouse pancreatic islets. Briefly, islets were isolated from mice C57BL/6 mice by standard collagenase digestion, as described previously from our laboratory (Khan et al., 2017). Fresh islets were preincubated for 30 min at 37°C in Krebs–Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM HEPES, 0.1% bovine serum albumin and 1.1 mM glucose, prior to conducting studies detailed below. General culture conditions and characteristics of β-cell lines have been reported previously (McClenaghan et al., 1996; Hamid et al., 2002; McCluskey et al., 2011).

**Secretion of insulin and xenin**

BRIN BD11 and 1.1B4 cells were seeded (150 000/well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37°C. Following 40 min pre-incubation (1.1 mmol/l glucose; 37°C), cells were incubated (20 min; 37°C) in the presence of 5.6 or
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16.7 mmol/l glucose, as appropriate, with test peptides. After 20 min incubation, buffer was removed from each well and aliquots stored at -20°C prior to determination of insulin by an in-house radioimmunoassay (Flatt and Bailey, 1982). To assess the impact of NTSRs on insulin secretion, BRIN BD11 cells were incubated at 16.7 mM glucose with NT or xenin (10⁻⁶ M) alone or in combination the NTSR antagonist SR142984A (10⁻⁶ M; Tocris Bioscience) or the murine NTSR2 agonist levocabastine (10⁻⁶ M; Sigma), and insulin secretion determined as described above. In addition, to determine potential mechanisms of action, membrane potential and intracellular Ca²⁺ were also measured following treatment with test peptides (10⁻⁶ M) in BRIN BD11 cells at 5.6 and 16.7 mM glucose using a Flexstation scanning fluorometer (FLIPR Calcium 5 assay kit, Molecular Devices, Sunnyvale, USA) as described previously (Miguel et al., 2004). Furthermore, insulin secretion from NIH Swiss mouse pancreatic islets in response to 2.8 mM glucose, 16.7 mM glucose alone and in the presence of NT and xenin (10⁻¹₀ – 10⁻⁶ M) was determined as above, but with a 60 min test incubation period. In addition, xenin secretion from groups of 50 isolated NIH Swiss mouse islets (n=4; 60 min) in response to glucose (20 mM) and arginine (30 mM) was also measured. Following removal of the test solutions in islet studies, 200 µl of acid–ethanol solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) H₂O) was added to islets for overnight extraction of cellular protein. All samples were stored at -20°C for measurement of insulin or xenin concentrations, as described in the Biochemical analyses section.

In vitro β-cell proliferation and cellular stress

To assess the effects of NT and xenin on rodent BRIN BD11 and human 1.1B4 β-cell proliferation, cells were seeded at a density of 150 000 cells per well and cultured overnight (18 hs) in the presence of NT or xenin (10⁻⁶ M), and compared to positive control GLP-1 (10⁻⁶ M). Cells were washed with PBS and fixed using 4% paraformaldehyde. After antigen retrieval with citrate buffer at 95°C for 20 min, tissue was blocked using 2% BSA for 45 minutes. The slides were then incubated with rabbit anti-Ki-67 primary antibody, and subsequently with Alexa Fluor® 594 secondary antibody. Nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole). Slides were viewed using fluorescent microscope (Olympus System Microscope, model BX51; Southend-on-Sea, UK) and photographed by DP70 camera adapter system. Proliferation frequency was determined in a blinded fashion and expressed as % of total cells analysed. Approximately 150 cells per replicate were analysed. For analysis of the ability of NT and xenin to protect against streptozotocin-induced
DNA damage, BRIN BD11 and 1.1B4 cells were seeded as above. Cells were then exposed to streptozotocin (5 mM) in the presence or absence of NT and xenin (10^{-6} M) for 2 hours, with GLP-1 (10^{-6} M) as positive control. Cell viability was determined using the standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In a separate series, cells were harvested and a comet assay was performed as described previously (Khan et al., 2016). Resulting gels were stained using DAPI (100 μg/ml) and slides were viewed in a blinded manner under an appropriate filter using an Olympus fluorescent microscope. Comet score software (Version 1.5) was used for the analysis of % tail DNA (100 cells per gel) and olive tail moment.

**Animals**

All animal studies were carried out using adult male C57BL/6 or NIH Swiss mice (12-14 weeks of age, Harlan Ltd, UK), housed individually in air conditioned room at 22±2°C with 12 hours light and dark cycle and *ad libitum* access to drinking water and standard rodent diet (10% fat, 30% protein and 60% carbohydrate; Trouw Nutrition, Northwich, UK). All experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986.

**Acute *in vivo* glucose homeostatic and insulin secretory effects**

Plasma glucose and insulin responses were evaluated after intraperitoneal (i.p.) injection of glucose alone (18 mmol/kg body weight) or in combination with test peptides (25 nmol/kg body weight) in overnight (18 h) fasted NIH Swiss mice. This dose was chosen based on our previous experience with gluco-modulatory hormones (Khan et al., 2016, 2017).

**Islet histology in insulin-deficient and insulin-resistant diabetic mice**

Multiple low dose streptozotocin (50 mg/kg body weight, i.p.) was used to induce insulin-deficient diabetes in C57BL/6 mice, whereas insulin-resistant diabetes was provoked by daily hydrocortisone injections (70 mg/kg body weight, i.p.), as described previously (Khan et al., 2017). After 10 days, terminal plasma samples were collected for determination of circulating xenin. Pancreatic tissues excised, divided longitudinally, and processed for determination of xenin content following acid-ethanol hormone extraction, or fixed in 4% PFA for 48 h at 4°C. Fixed tissues was subsequently dehydrated using a series of increasing strength ethanol solutions and processed for embedding in paraffin wax (Vasu et al., 2014; Moffett et al.,
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Tissue blocks were sectioned (8 μm) using a Shandon Finesse 325 microtome (Thermo Scientific, Hemel Hempstead, UK) and picked for staining at intervals of 10 sections. After deparaffinising, sections were rehydrated using a series of decreasing strength ethanol solutions. Antigen retrieval was carried out using a citrate buffer (pH 6.0) at 94°C for 20 min, sections were then blocked using 2% BSA and incubated overnight at 4°C with appropriate primary antibody (Table 2). The specificity of the xenin antibody was examined by blocking experiments using the native peptide. Thus, pre-incubation with xenin (10⁻³ M) dramatically decreased the signal from the xenin antibody (Phoenix Pharmaceuticals, H-046-74), but was unable to completely block it. This likely relates to the ubiquitous nature of COPA and possible splice variants that could give rise to similar, but distinct peptide isoforms. The slides were then incubated with appropriate secondary antibodies (Alexa Fluor® 594 for red and Alexa Fluor® 488 for green; Table 2) and stained with nuclear DAPI staining. Slides were mounted with anti-fade mounting medium, viewed using a fluorescent microscope (Olympus System Microscope, model BX51; Southend-on-Sea, UK) and photographed using a DP70 camera adapter system (Vasu et al., 2014; Moffett et al., 2014). All staining procedures and image analysis were carried out in a blinded manner. Approximately 100 islets were analysed per group. Islet parameters were determined using the ‘closed polygon’ tool in Olympus CellF® analysis software. For co-localisation studies, xenin expression in α- or β-cells was determined by counting cells with xenin and glucagon or insulin expression and expressing as % of total α-or β-cells, as appropriate.

Biochemical analyses

Blood samples were collected from the cut tip on the tail vein of conscious mice at the time points indicated in Figure 4. Blood glucose was measured directly using a hand-held Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin analysis, blood samples were collected into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany), immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 x g and stored at -20 °C, prior to determination of insulin by radioimmunoassay (Flatt and Bailey, 1982). Xenin concentrations were determined using a xenin fluorescent EIA kit (Xenin-25, Human, Rat, Mouse, FEK-046-74, Phoenix Europe GmbH, Germany) following the manufacturer’s instructions.
Statistical analysis

Statistical analyses were performed using GraphPad PRISM software (Version 5.0). Values are expressed as mean±S.E.M. Comparative analyses between groups were carried out using a One-way ANOVA with Bonferroni post hoc test or student’s unpaired t-test, as appropriate. The difference between groups was considered significant if $P<0.05$.

Acknowledgements

This study was supported by an EFSD/Boehringer Ingelheim grant, donation from the SAAD Trading and Contracting Company and award of an Ulster University Vice Chancellor’s research scholarship to DK.

Conflict of interest statement

The authors have no conflict of interest to declare.
References


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Tables and figures

Table 1  Gene symbol, common name and sequences of mouse, rat and human PCR primers employed.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Official gene symbol</th>
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Table 2  Target, host and source of primary and secondary antibodies employed for immunofluorescent studies.

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**Figure 1** Expression of NTSRs by rodent and human β-cells as well as NT and COPA by isolated mouse islets.

NTSR mRNA expression in (A) rodent BRIN BD11 β-cells, (B) isolated C57BL/6 mouse islets and (C) human 1.1B4 β-cells. (D) NT and COPA expression in freshly isolated NIH Swiss mouse islets. mRNA expression was normalised relative to β-actin expression and also compared against expression of the classic islet-cell GPCR, GIPR. Values are mean ± SEM (n = 5). **P<0.01, ***P<0.001 compared to (A-C) GIPR or (D) insulin expression. All mRNA expression was normalized to Actb/ACTB expression.
Figure 2  Effects of NT and xenin on insulin release from rodent BRIN BD11, human 1.1B4 β-cell lines and isolated mouse islets and secretion of xenin from isolated mouse islets. (A,B) BRIN BD11, (C) 1.1B4 cells or (D) isolated mouse islets were incubated (A-C 20 min; D 60 min) with either 5.6 or 16.7 mM glucose, as appropriate, and the effects of test peptides (10^{-12} – 10^{-6} M) determined. Effects of the (E) NT receptor antagonist, SR142948A (10^{-6} M) and (G) murine NT receptor-2 agonist, levocabastine (10^{-6} M), on NT and xenin (10^{-8} – 10^{-6} M) modulation of insulin release from BRIN BD11 cells at 16.7 mM glucose. (F) Effects of glucose (20 mM) and arginine (30 mM) on xenin secretion from mouse islets in 60 min incubations. Total islet xenin content was (n=6) 12.9 ± 1.7 pg/50 islets. Values are mean ± SEM (n=8 in A-C,E,G and n=4 in D,F). *P<0.05, **P<0.01, ***P<0.001 compared to (A-E,G) 5.6 mM glucose control or (F) 5 mM glucose control. ΔP<0.05, ΔΔP<0.01, ΔΔΔP<0.001 compared to 16.7 mM glucose control. ♂P<0.05, ♂♂♂P<0.001 compared to NT alone. ψψP<0.01 compared to xenin alone.
Figure 3  Effects of NT and xenin on membrane potential and $[\text{Ca}^{2+}]_i$ in a rodent BRIN BD11 cell line.

(A,C) Cells were incubated with 5.6 mM glucose in the presence of NT or xenin ($10^{-6}$ M) and membrane potential or $[\text{Ca}^{2+}]_i$, assessed over a 5 minute period, with KCl (15 mM) and alanine (10 mM) as positive controls, respectively. (B,D) area under curve data is also shown. Values are mean ± SEM (n=6). *$P<0.05$, **$P<0.001$ compared to 5.6 mM glucose. $\Delta\Delta\Delta P<0.001$ compared to respective positive control.
Figure 4  Acute effects of NT and xenin on glucose tolerance and insulin response to glucose in overnight fasted mice.

(A) Blood glucose and (C) plasma insulin levels were assessed immediately before and after intraperitoneal administration of NT or xenin (25 nmol/kg bw) together with glucose (18 mmol/kg bw). Respective (B) blood glucose and (D) plasma insulin area under curve data is also shown. Values are mean ± SEM (n=6 mice). *P<0.05, **P<0.01, ***P<0.001 compared to glucose alone control.
Figure 5  Effects of streptozotocin and hydrocortisone treatment of mice on islet NT and xenin expression, localisation and circulating xenin levels.

C57BL/6 mice received daily injections of streptozotocin (50 mg/kg bw) or hydrocortisone (70 mg/kg bw) for 5 or 10 days respectively before examination of pancreatic histology on day 10. (A-C) Representative islet images from control, streptozotocin and hydrocortisone treated mice showing (A) xenin (red) staining with glucagon (green), (B) insulin (green) staining with xenin (red) and (C) insulin (red) staining with NT (green). Arrows indicate (A) xenin and glucagon co-localisation, (B,C) xenin or NT, as appropriate, and insulin positively stained cells. Nuclei are demonstrated using DAPI staining (blue). (D,F) Quantification of co-localisation of xenin with (D) glucagon and (F) insulin. (E,G) Measurement of (E) circulating plasma and (G) pancreatic xenin concentrations in control, streptozotocin and hydrocortisone treated mice. (G) Control mouse intestines included for reference. Values are mean ± SEM (n=6 mice). *P<0.05 compared to control mice.
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Figure 6 Effects of NT and xenin on rodent BRIN BD11 and human 1.1B4 β-cell line proliferation.

(A,B) Proliferation frequency in (A) BRIN BD11 and (B) 1.1B4 cells cultured with GLP-1, NT and xenin (all at 10^{-6} M) for 18 h. (C) Representative images showing Ki-67 (red) and DAPI (blue) staining in the presence (18 h) of GLP-1, NT and xenin. Values are mean ± SEM (n=4). *P<0.05, **P<0.01 compared to control cultures.

Figure 7 Effects of NT and xenin on protection of rodent BRIN BD11 and human 1.1B4 β-cell lines from streptozotocin induced cellular stress.

(A,B) Cell viability, (C,D) % tail DNA and (E,F) olive tail moment were assessed in response to 2 h exposure to 5 mM streptozotocin with and without co-incubation with GLP-1, NT and
xenin (all at $10^{-6}$ M) in (A,C,E) BRIN BD11 and (B,D,F) 1.1B4 β-cells. (G) Representative images showing comets of control, streptozotocin alone and in combination with NT and xenin ($10^{-6}$ M) in both cell types. Arrows indicate cells with comet tails. Values are mean ± SEM (n=4). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to control cultures. $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared to STZ (5 mM) alone.