Title: A novel role for the extracellular matrix glycoprotein-Tenascin-X in gastric function

Running title: The role of Tenascin-X in murine gastric function

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Rubina Aktar¹, Madusha Peiris¹, Asma Fikree¹, Simon Eaton², Stamati Kritas³, Stephen J. Kentish³, Eduardo J. A. Araujo¹, Cristiano Bacarin⁴, Amanda J. Page³, Nicol C. Voermans⁵, Qasim Aziz¹*, and L. Ashley Blackshaw¹*

Author affiliation: ¹Blizard Institute, Queen Mary University of London, London, U.K.²Institute of Child Health, University College London, London, U.K. ³South Australian Health and Medical Research Institute, University of Adelaide, Australia. ⁴Department of Histology, Centre for Biological Sciences, State University of Londrina, Brazil. ⁵Department of Neurology, Radboud University Medical Centre, Nijmegen, Netherlands. *Joint senior authors

Corresponding Author: Dr Rubina Aktar, Wingate Institute, 26 Ashfield Street, London E1 2AJ. r.aktar@qmul.ac.uk, Tel: +442078322646, Fax: 02073752103.

Key points summary

- Tenascin X (TNX) functions in the extracellular matrix of skin and joints to maintain correct intercellular connections and tissue architecture.

- TNX is associated exclusively with vagal-afferent endings and some myenteric neurones in mouse and human stomach respectively.

- TNX-deficient mice have accelerated gastric emptying and hypersensitivity of gastric vagal mechanoreceptors that can be normalised by an inhibitor of vagal-afferent sensitivity.

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- Cultured nodose ganglion neurones showed no changes in response to capsaicin, cholecystokinin and potassium chloride in TNX-deficient mice.

- TNX-deficient patients have upper gastric dysfunction consistent with those in mouse-model. Our translational studies suggest abnormal gastric sensory function may explain upper gut symptoms present in TNX deficient patients, thus making it important to study gastric physiology.

- TNX deficiency should be evaluated routinely in patients with connective tissue abnormalities which will enable better understanding of its role and allow targeted treatment. For example, inhibitors of vagal afferents-Baclofen could be beneficial in patients. These hypotheses need confirmation through targeted clinical-trials.

**ABSTRACT**

Tenascin X (TNX) is a glycoprotein that regulates tissue structure through anti-adhesive interactions with collagen in the extracellular matrix. TNX deficiency causes a phenotype similar to hypermobility Ehlers Danlos Syndrome (hEDS) involving joint hypermobility, skin hyperelasticity, pain and gastrointestinal (GI) dysfunction. Previously, we have shown TNX is required for neural control of the bowel by a specific subtype of mainly cholinergic enteric neurones and regulates sprouting and sensitivity of nociceptive sensory endings in mouse colon. These findings correlate with symptoms shown by TNX-deficient patients and mice. We set out to identify if TNX is similarly present in neural structures found in mouse and human gastric tissue. We then determined whether TNX has a functional role, specifically in gastric motor and sensory function and nodose ganglia neurones. We report TNX was present in calretinin-immunoreactive extrinsic nerve endings in mouse and human stomach. TNX deficient mice had accelerated gastric emptying and markedly increased vagal afferent responses to gastric distension that could be rescued with GABA$_B$ receptor agonist. There were no changes in nodose ganglia excitability in TNX deficient mice suggesting vagal afferent responses are likely due to altered peripheral mechanosensitivity. In TNXB-deficient patients significantly greater symptoms of reflux, indigestion, and abdominal pain was reported. Here we report the first role for TNX in gastric function and further studies are required in TNX deficient patients to determine if symptoms can be relieved using GABA$_B$ agonists.
INTRODUCTION

TNX is an extracellular matrix glycoprotein belonging to a family of tenascins and other molecules that govern tissue structure (Valcourt et al., 2015). Its absence leads to heritable connective tissue disorders with a phenotype similar to hypermobility Ehlers Danlos Syndrome (hEDS) (Zweers et al., 2004) although TNX deficient patients are now classified as a separate subgroup (Malfait et al., 2017). We recently showed that TNX is expressed not only in connective tissue, but also in colonic intrinsic neurones, and that it is required for normal neural control of colonic motility (Aktar et al., 2018). Our data suggested a direct role in cholinergic control, but we also found that absence of TNX leads to hypertrophy and hypersensitivity of nociceptive sensory nerve endings in the colon which do not themselves express TNX, therefore suggesting an indirect role in pain. These observations in knockout mice correlated closely with GI symptoms in genetically confirmed TNX-deficient patients with hEDS, including disrupted motility and bowel habit, and visceral pain (Aktar et al., 2018). There remains, however, a large gap in our understanding, since patients with hEDS suffer from symptoms that are localised in both upper and lower GI tract. The most common upper GI symptoms include bloating, reflux, disordered gastric emptying of a meal, nausea and indigestion (Castori et al., 2010; Mathias et al., 2012; Fikree et al., 2014). We set out to investigate firstly if there was a systematic link between TNX gene deficiency and symptoms in humans, and secondly what is the underlying mechanism. In particular, vagal afferent fibres innervating the upper gastrointestinal tract play a critical role both in initiation of symptoms and reflexes controlling several functions (Page & Blackshaw, 2009). Vagal afferents are comprised of two main types: firstly mucosal endings that respond to touch and to chemical stimuli; secondly muscular endings that respond optimally to mechanical stretch or tension (Page et al., 2002). These gastric mechanoreceptors have been anatomically identified as intraganglionic laminar endings (IGLE). Another population of intramuscular arrays (IMA) may also play a role in mechanosensation (Zagorodnyuk et al., 2001). They project to the brain stem whereupon they provide input to central sensory pathways and motor programmes (Browning & Travagli, 2010).

We first investigated the correlation between TNX gene deficiency and symptom severity in a hEDS patient cohort. We then determined TNX expression in calretinin-immunoreactive structures analogous with vagal afferent endings in both humans and mice. Electrophysiological recordings were...
used to show if TNX knockout mice have altered sensitivity of vagal afferents to gastric distension. The association of changes in vagal afferent function with overall gastrointestinal function was explored by measuring gastric emptying in vivo. We also investigated a means of normalising afferent function by activation of inhibitory GABA$_B$ receptors on gastric vagal afferents. What emerges is an inhibitory role for TNX in a specific population of upper GI mechanosensory neurones, which may underlie fundamental aspects of sensory signal transduction at afferent endings. Our findings further indicate these endings may be therapeutic targets for a range of symptoms in patients with TNX deficiency and may have a role in other patients such as those with hEDS.

**MATERIALS AND METHODS**

**Ethical Approval**

Full-thickness gastric tissue (non-pathological) was obtained from cancer patients (>10cm away from tumors) undergoing gastrectomy using approved Human Research Ethics from Bart’s and London NHS Trust with informed consent (NREC 09/H0704/2). Unfortunately we could not obtain tissue from TNX deficient patients, however, TNX deficient patients gave written informed consent prior to completing questionnaires.

All studies were completed according to the animal ethics policy and the study is in compliance with guidelines (Grundy, 2015). All mice used in these studies were killed by asphyxiation using carbon dioxide in accordance with the UK Home Office (Schedule 1, Animals Act 1986) for all experimental procedures. All human questionnaire studies were not registered in a database in accordance with the Helsinki Declaration.

**Mouse Tissue**

Mice used in this study originated from parent mice with a C57BL/6N background and donated by Professor Manuel Koch (University of Cologne). The generation of these mice have been previously described (Mao et al., 2002), (Aktar et al., 2018). Knockout and wild-type mice were phenotypically healthy, grew similarly and were of similar weight at the time of the study (WT: 19.9±0.8g and KO: 20.8±1.1g). All mice were reared and transported under conditions specified in the UK’s Animal Welfare Act 2006. Mice aged between 10-12 weeks were used and were killed by a rising concentration of CO$_2$ asphyxiation (Schedule 1, Animals Act 1986, U.K. Home Office).
**Immunohistochemistry**

Immunolabelling with rabbit polyclonal TNX (1:200, Santa Cruz, sc-25717), calretinin (1:500, Swant, CG1 and 6B3) and calcitonin gene-related peptide CGRP (1:400-Abcam-ab36001 and Thermo Fisher- ABS026-05-02) was assessed in human and mouse specific stomach regions. Additionally, choline acetyl transferase ChAT (1:400-Abcam-ab18736) was used in brainstem sections.

**Immunohistochemistry process**

Tissue was fixed in 4% paraformaldehyde, 10µm sections and wholemounts were prepared, then blocked in universal blocking serum (Dako) and incubated overnight at 4°C with primary antibodies. Slides/whole mount tissue was then incubated for 1hr at room temperature with AlexaFluor conjugated secondary antibody as appropriate. Slides/whole mounts were then mounted using a coverslip and Vectashield Hardset Mountant conjugated with DAPI (H-1500) and left to dry before viewing under the microscope at 40x.

**Vagal Afferent Recording Studies**

Methodology used here was modified from Page (Page et al., 2002), as whole stomach was used. Once a distension-sensitive fibre was identified, 1ml of Krebs solution was infused via a cannula into the mouse stomach for 1min. After 1min the cannula was removed and the stomach was allowed to drain naturally. The empty stomach was then allowed to rest for 5min before repeating this process twice more. Separate TNX-KO mice were used for baclofen studies, whereby post two distension recordings baclofen (100µM) was infused into the organ bath as well as injection directly into the bath. After 10mins of baclofen infusion, two further distensions were made with a 5min rest in between to measure the effects of baclofen on afferent firing. Gastric compliance was measured using a pressure transducer which was attached to the cannula used to infuse the stomach with with standard volumes of Krebs solution. Single units were discriminated using wavemark analysis via Spike2 software (Cambridge Electronic Design Ltd.).

**Gastric Emptying Studies**

Mice were fasted overnight, placed in separate chambers, then consumed an egg yolk meal containing 1µl/1g C\textsuperscript{13} octanoic acid (99% enrichment, Cambridge Isotope Laboratories, Andover, MA, USA). Once fully consumed, breath samples were collected at intervals up to 150min and analysed.

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for $^{12}\text{CO}_2/^{13}\text{CO}_2$ using the isotope-ratio mass spectrometer (Thermo Finnigan, Germany). Excretion data was analysed using non-linear regression analysis for curve fitting to obtain gastric emptying half-life (T½) and time taken for solid digestion into the duodenum (T-lag) (Ghoos et al., 1993).

**Primary cell culture and calcium imaging of nodose ganglia**

Primary cell cultures from the nodose ganglia of 12 week old WT and TNX-KO mice were prepared. Both nodose ganglia were removed under a stereomicroscope and placed immediately in cold F12 complete nutrient medium. The ganglia were dissociated by enzymatic digestion in two steps. First by collagenase II and dispase 3mg/ml in HBSS with agitation at 5min intervals over 30mins and then by collagenase II alone for 15mins. After incubation the ganglia were rinsed in cold HBSS and F12 and further dissociated by trituration using a fine pipette. Cells were then pelleted, rinsed in HBSS and resuspended in Neurobasal A medium. The cells were plated on poly-D-lysine coated 24 well plates and placed in a 5% CO$_2$ incubator at 37 °C for 2h to allow cell adherence. After 2h, warmed complete NBA medium (supplemented with B-27, penicillin/streptomycin and glutamax) was added to each well and incubated for 48hrs before experiments. After 2 days of incubation cell cultures were loaded with Fluo-4-AM as described in protocol detects calcium flux and was loaded 1hr before rinsing the cells with Krebs and reloadding with Krebs. Changes in intracellular Ca$^{2+}$ concentration [Ca$^{2+}$] are reflected in Fluo-4-AM fluorescence intensity and recorded at 525/50 nm. Baseline activity of identified neurones was recorded for 120s and then stimulated with either capsaicin (100 nM), CCK (10nM) or KCl (50mM) for 120s. Images were acquired every 5s with a confocal Zeiss LSM 880 with Airyscan using Zen software. Changes in fluorescence intensity were measured by drawing a region of interest around neurones, then measuring change in fluorescence intensity. The total number of neurones that responded to stimuli were then averaged over a single time point. Peak intensity within the first 10s was measured in cell cultures from both mouse groups. Identified neurones that changed in intensity due to the stimuli was described as a responder, while a non-responder was described as neurones that showed no changes in fluorescence intensity. The total number of neurones that responded to capsaicin, cholecystokinin (CCK) and KCl was individually noted. We sampled nodose ganglia neurones randomly to see if TNX deficient neurones respond differently as a population; more subtle changes may be revealed using patch clamp recordings from neurone retrogradely labelled from the stomach.
Patient genotyping

11 patients (3 males and 8 females) identified from Radboud University Medical Centre took part and were genotyped for TNXB (Schalkwijk et al., 2001). Mutation analysis was performed using next-generation sequencing (NGS) testing TNXB DNA mutation, as previously described (Demirdas et al., 2016). Additionally, serum samples were analysed for TNX glycoprotein using ELISA with rabbit anti-TNX.

GI symptom questionnaire

Patients with TNX deficiency completed a Flemish version of the validated Gastrointestinal Symptom Rating Scale (GSRS) (Svedlund et al., 1988). Questionnaire responses were translated and scored for 5 different domains – reflux, abdominal pain, constipation, indigestion, and diarrhoea. Scores ranged from 1 (no symptoms) to 7 (unbearable symptoms). GSRS scores for abdominal pain and upper GI symptoms for reflux and indigestion were analysed in TNX deficient patients compared to a reference Swedish population of similar age and sex (Dimenas et al., 1996).

Experimental design and Statistical analysis

Immunohistochemistry: Qualitative images were obtained for both human and mouse stomach. Controls with no primary antibody were performed with each run of immunohistochemistry to confirm the specificity of the primary antibody, which resulted in no fluorescence above background. Western blot with this antibody in humans revealed a band at the predicted weight of 268kD. Moreover TNX was absent in all TNX-KO gut tissue. Images were obtained using Metamorph software on Olympus MM Leica (sections) or Zen software on Zeiss LSM 710 or Zeiss LSM 880 (confocal imaging-wholemounts) at 40x magnification.

Vagal afferent recordings: A total of 12 WT and 12 KO mice (6 females and 6 males in both groups) were used to measure spontaneous and post distension afferent activity in the stomach. Separate baclofen experiments in N=4 WT vs. N=4 KO mice (2 females and 2 males in both groups) were also performed. Data were statistically analysed with an unpaired t test and individual data were plotted for all data sets with a p-value of <0.05 was deemed significant.

Gastric emptying: A total of N=9 WT vs. N=15 KO mice (5 females and 4 males in WT and 7 females and 8 males in KO) were used. Statistical analysis was performed with an unpaired t test to compare
between mouse groups and individual data were plotted with a \( p \)-value of \(<0.05\) was deemed significant.

**Calcium Imaging:** A total of \( N=5\) WT vs. \( N=5\) KO mice (3 females and 2 males in both groups) were used. In total 25 WT and 17 KO individual nodose neurones were stimulated with KCL and capsaicin respectively while 18 WT and 34 KO individual nodose neurones were stimulated with CCK. Statistical analysis was performed using an unpaired \( t \)-test. Individual data were plotted and a \( p \)-value of \(<0.05\) was deemed significant.

**Patient questionnaires:** 11 patients (3 males and 8 females) with TNX deficiency were used and compared to a reference Swedish population \( N=2162\). The reason for the small sample size in TNX deficient group was due to a small number of patients known to have TNX deficiency since it is uncommonly measured. Questionnaire data are shown as mean values with standard deviation. Statistical analysis was performed using an unpaired students \( t \)-test for each symptom, \( p<0.05\).

All data are expressed as individual data points and variability within the data was represented using 95% confidence intervals of the mean. Statistical analysis was performed using GraphPad Prism (V.7.02, GraphPad Software, Inc).

**RESULTS**

**TNX localisation in Mouse and Human stomach**

Calretinin-IR is a well-established marker for vagal afferents in rodent upper gut, and reliably labels intraganglionic laminar endings (IGLE) and intramuscular arrays (IMA) (Berthoud et al., 1995; Fox et al., 2000; Phillips & Powley, 2000; Powley et al., 2013). In the mouse stomach, fibres positive for TNX+calretinin were seen in circular muscle (Fig 1B). Fibres in stomach showed partially overlapping IR for calretinin and TNX and corresponded anatomically and neurochemically to IMA (Fig 1B) and IGLE (Berthoud & Neuhuber, 2000). TNX and calretinin-IR colocalisation was also observed surrounding myenteric ganglia in IGLEs (Fig 1A), which have been previously described as tension-sensitive vagal afferents (Berthoud & Powley, 1992; Phillips & Powley, 2000; Zagorodnyuk et al., 2001). Correspondingly, TNX-IR was found in a subpopulation of cell bodies of vagal afferent neurones in the nodose ganglia (Fig 1C) and more centrally in punctate endings within the nucleus.
tractus solitarius, some in close proximity to dendrites of TNX-negative ChAT-positive neurones (presumably vagal preganglionic motoneurones) in the adjacent dorsal motor nucleus of the vagus (Fig 1D). Peripherally, qualitative analysis shows some TNX-IR cell bodies were present in gastric myenteric ganglia of human fundus (Fig 1E) but not in mouse (Fig 1A). CGRP did not co-label with TNX in human fundus (Fig 1F). Similar to the mouse circular muscle, TNX-IR IMA were also found in human smooth muscle (Fig 1G).

**Gastric Vagal Afferent Sensitivity in Mice**

The pattern of TNX localisation in calretinin-IR endings in the stomach suggested a role in vagal afferent (IGLE and IMA) function, therefore we investigated if there was an alteration in mechanosensory and electrophysiological properties of gastric vagal afferents that lack TNX. Single afferent fibre recordings of gastric tension receptors that are mechanosensitive to contraction and distension exhibiting slow adaptation to innocuous wall tension (Page et al., 2002) were analysed. Spontaneous firing was significantly increased by 66% in TNX-KO compared to WT (0.23± 0.06 WT vs 0.69± 0.08 KO, p=0.0003) (Fig 2C). Similarly, responses to distension were significantly increased in TNX-KO (2.37± 0.67 WT vs 9.67± 1.2 KO, p<0.0001) (Fig 2D). Distension-induced changes in intragastric pressure showed no difference in area under curve (1598.9± 168.2 WT vs 2055.6± 177.9 KO, p=0.7326) (Fig 2E). Therefore, gastric compliance is unaltered suggesting increased afferent sensitivity in TNX-KO is a property of the afferent neurones, not their environment.

**Rescue of afferent hypersensitivity in TNX-KO mice**

Addition of the GABA$_B$ receptor agonist- baclofen caused a significant reduction in spontaneous firing (0.69± 0.08 KO vs 0.13± 0.029 KO+baclofen, p<0.0001), Fig 2C) and afferent firing during distension in TNX-KO (9.67± 1.2 KO vs 1.55± 0.253 KO+baclofen, p<0.0001), Fig 2D). Afferent firing post baclofen was reduced in TNX-KO mice similar to levels observed in WT mice (Fig 2C).

**Gastric Emptying**

Since vagal afferents showed mechanical hypersensitivity in the absence of TNX, we asked if this translated to reflex control of gastric motility by observing gastric emptying in TNX-KO. In WT, a gradual increase in labelled CO$_2$ ultimately peaked at 90 mins, while TNX-KO mice had a rapid increase which peaked at 60 mins (Fig 3A). Starting at 100 mins, CO$_2$ gradually declined in WT mice...
unlike TNX-KO that had a rapid decline at 75mins, demonstrating faster gastric emptying (Fig 3A). The T½ (158min± 21.8 WT vs 103min±12.9 KO, p=0.0277) (Fig 3B) and T-Lag (24min± 2.7 WT vs 38min± 5.7 KO, p=0.0194) (Fig 3C) were significantly reduced in TNX-KO suggesting that propulsive motility of the stomach is enhanced.

**Excitability of cultured nodose ganglia**

Vagal afferent hypersensitivity could be due to altered generation of action potentials by mechanical stimuli at the endings or by altered excitability of the neurone as a whole. To determine which is most likely, nodose ganglia neurones were isolated from their target and studied in culture. They grew similarly (Fig 4A) in both WT and KO mice indicating a lack of influence of TNX on cytoarchitecture and viability. Responsiveness was assessed by peak calcium responses within the first 10s post-KCl, CCK and capsaicin, which showed no differences between WT or TNX-KO neurones (Fig 4B). Additionally there were no differences in the number of responders versus non-responders to KCl, CCK and capsaicin between groups (Fig 4C). Thus the changes we observed are most likely due to mechanical hypersensitivity.

**Gastrointestinal Symptoms in TNX deficient patients**

TNX deficient patients showed significantly increased severity of GI symptoms overall (p<0.0001) compared to healthy controls (Fig 5). Specifically, symptoms of gastroesophageal reflux, abdominal pain and indigestion were all significantly increased.

**DISCUSSION**

Having recently discovered a novel role for TNX in enteric neurones of the lower GI tract, it followed that there was a likely role also in the upper gut, particularly in view of the fact that patients with TNX deficiency have upper as well as lower GI symptoms. We found a strong correlation between TNX gene deficiency and upper GI symptom severity in a patient cohort. Correspondingly, TNX was normally present in calretinin-immunoreactive structures, analogous with vagal afferent endings in both humans and mice. Electrophysiological recordings showed that TNX knockout mice have greatly enhanced sensitivity of vagal afferents to gastric distension. This is associated with accelerated...
gastric emptying in vivo, and matches the upper GI symptoms reported by patients. We also show that increased afferent function can be reversed by activation of inhibitory GABA\(_B\) receptors on gastric vagal afferents. Thus TNX plays an inhibitory role in a specific population of upper GI mechanosensory neurones, and may underlie aspects of sensory signal transduction at afferent endings. Our findings further indicate these endings may be therapeutic targets for a range of symptoms in patients with TNX deficiency and may have a role in other patients such as those with hEDS.

TNX-IR was found in a small population of nodose ganglion neurones and in sparse central vagal terminals in the NTS. Therefore we corroborated our finding in hindgut that TNX is expressed by GI neurones, but with the exception that expression in upper gut is in extrinsic, rather than intrinsic neurones. This indicates a very different role for TNX in the upper GI tract. TNX-IR was seen often in endings that colocalised with calretinin. It is established from other work that these are vagal afferent terminals with cell bodies in the nodose ganglia (Berthoud et al., 1995; Fox et al., 2000; Phillips & Powley, 2000; Powley et al., 2013), which fits with the lack of TNX-IR intrinsic cell bodies. Correspondingly, Based on this finding of TNX expression in extrinsic neurones, it was important to determine if TNX influences vagal afferent function, which we investigated with a gastric vagal afferent preparation adapted from those we have used before (Page et al., 2002) to reveal differences between genotypes. We observed greater mechanosensitivity and baseline discharge of individual low-threshold distension-sensitive afferents, known as tension receptors (Page et al., 2002). This was in the absence of changes in gastric compliance, suggesting it was a property of the nerve endings only. This notion was further supported by studies of nodose ganglion cell bodies, which showed no difference in responsiveness after application of KCl, CCK or capsaicin. This leads us to believe that TNX plays a specialised role in the anchoring of nerve terminals to their targets, perhaps by allowing flexibility between the two. Thus, without TNX the afferent ending becomes less dynamic in its attachment and reaches full mechanical distortion with less movement relative to the surrounding tissue. We speculate on this in Fig 6, in which the spacing of mechanosensory terminals and smooth muscle elements is critical for normal afferent signalling. It will require further studies with electron microscopy to see if this anchoring role is tenable. In our previous study of high threshold nociceptive colonic afferents in the splanchnic nerves (Aktar et al., 2018), we also found increased

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mechanosensitivity in the TNX-KO, but TNX labelling was not associated with the endings themselves and was in fact mutually exclusive with their marker – CGRP. In that case there was approximately a 50% greater mechanosensitivity in the knockout, whereas here we found a 400% greater mechanosensitivity, which is perhaps not surprising given the direct association of TNX with vagal afferents compared with the distinct location of TNX relative to splanchnic afferents. This is by far the biggest difference in vagal function we have seen in several comparisons of knockouts of sensory genes with wild types, suggesting the role of TNX is critical. The indirect role of TNX in splanchnic afferents is supported by the fact that they appeared to sprout into the otherwise TNX-negative mucosa (Aktar et al., 2018) (suggesting a barrier function), whereas their direct role in vagal afferents is supported by the observation that their anatomical relation to other tissues was not noticeably altered in TNX-KO, but their function was markedly different.

The main expected consequence in vivo of increased gastric vagal afferent sensitivity, upon reaching the central nervous system (CNS) would be increased perception of non-nociceptive mechanical forces in the stomach, but mouse models are not available for this. Therefore we investigated another major consequence of vagal afferent activation, which is the control of gastric emptying (Browning et al., 2014). This is mediated via a vago-vagal reflex in the brain stem, where indeed we saw localisation of TNX presumably at the central endings of vagal afferents. It is known that distension of the proximal stomach promotes motor activity of the distal stomach via this reflex, which serves to augment the antral pump, which in turn facilitates emptying of solids from the stomach through the pylorus into the duodenum (Grundy et al., 1989), (Andrews et al., 1980). There was a faster rate of gastric emptying of a solid meal in TNX-KO that was comparable to the greater afferent sensitivity, which supports a role for TNX in this pathway in vivo. Although studies of gastric emptying specifically in TNX-deficient patients are lacking, some studies of emptying in ungenotyped hEDS patients show increased solid emptying (Menys et al., 2017), suggesting the same phenomenon. Through comparison of GSRS questionnaire data, we were able to determine if TNX-deficient patients reported upper GI symptoms differently to controls, and we found that in the cases of perception of indigestion and reflux, scores were approximately double in the TNX-deficient cohort compared to controls. This is in addition to lower GI symptoms we already reported in this cohort.
The data presented here would suggest that all upper GI symptoms in TNX-deficient patients (and possibly therefore those reported in a large number of hEDS patients) may have a common origin in the hypersensitivity of vagal afferents. In that case it would make these endings a potential therapeutic target. This has been suggested before, when the triggering of transient lower oesophageal sphincter relaxations by vagal afferents through a similar pathway was exploited as a therapeutic target for gastro-oesophageal reflux disease (Tonini et al., 2004). Specifically, the expression of GABA\(_B\) receptors by vagal afferents was exploited as a means of reducing their excitability, and thus the strength of their signal to the central motor programme giving rise to sphincter opening that allows acid reflux (Zhang et al., 2002). This was done, and almost succeeded in clinical trials, despite the lack of any evidence for hypersensitivity of vagal afferents in gastrooesophageal reflux disease (GORD). Here though, we have a demonstrable hypersensitivity that is specific to mechanical stimuli, that we know is susceptible to inhibition via GABA\(_B\) receptors. Therefore we sought to rescue the hypersensitivity of vagal afferents using the prototypic GABA\(_B\) receptor agonist baclofen. This was highly effective, with profound inhibition of responses to distension as well as spontaneous activity. Unfortunately baclofen has considerable effects on the CNS (Dario et al., 2007), so it was not possible to determine if these effects seen in the organ bath were translatable to in vivo experiments on gastric emptying. For that a more peripherally restricted compound would be required.

Generally speaking, mouse and human data were comparable in this study in terms of differences between TNX-deficient and control cohorts. However, not all parameters could be assessed, such as gastric emptying in TNX-deficient patients (for ethical reasons related to their primary referral), and upper GI symptoms in mice (because of lack of outcome markers). In the case of TNX localisation, we were able to compare directly the two species, and we did find discrepancy. Whereas the mouse lacked completely any somatic labelling in gastric myenteric neurones for TNX, human specimens invariably showed gastric myenteric neurones were TNX-IR, albeit at a much lower abundance than those in the lower GI tract. This may suggest the neuronal subtypes may differ between species, and indeed it has been noted before that very few or no AH/Type II myenteric neurones are found in the upper GI tract of some species, but not others (Mazzuoli & Schemann, 2012). Of importance is the observation that larger mammals are more likely to possess these neurones, which means that TNX-
IR may be serving as a marker for this population, along with calretinin (although not exclusively). This would fit with the far higher prevalence of TNX+calretinin-IR enteric neurones in the colon, where AH/Type II neurones are common in all species. It was not possible to observe fine anatomical detail of neurones labelled in this study, and therefore their Dogiel type, but the colocalisation of TNX with calretinin is a further indicator that this is their phenotype. Intrinsic AH/Type II neurones more closely resemble extrinsic sensory neurones than other functional subtypes in the ENS, so it may be that TNX associates exclusively with a non-nociceptive sensory phenotype, since of course vagal afferents are also sensory neurones.

Perineuronal nets (PNN) are a feature of central synapses, and are formed by the interaction of another tenascin, tenascin-R, with various ECM components (Sorg et al., 2016). PNN are involved in structural connections between neurones and in synaptic plasticity (Kwok et al., 2011). We have no indications from our data as to whether or not TNX fulfils such a role in the gut or in the dorsal vagal complex, since labelling of other ECM components is required to do this definitively. However, it is intriguing how IGLE form a basket-like network around myenteric ganglia, which may serve a structural role in the delineation of discrete ganglia in the ENS. The location and orientation of IGLE and IMA would simultaneously assist in their mechanosensory function by detecting force impinging between and within smooth muscle layers.

Could differences in afferent endings lacking TNX reflect a role for TNX in correct spacing of afferent terminals relative to host tissue? By comparison, the ECM molecule agrin is thought to be important in forming neuromuscular junctions and maintaining synapses between cholinergic preganglionic axons and sympathetic neurons (Gingras et al., 2002). Mice lacking a part of the agrin molecule show marked reduction in acetylcholine receptor which indicates this molecule is crucial in normal synapse function in the CNS (Gingras et al., 2002), (Gautam et al., 1996 4716). Moreover, agrin increases nicotinic transmission at the synapse by modulating the space between the gap-junction-mediated electrical coupling (Martin et al., 2005). Similar to agrins, TNX may have a role in modulating the vagal afferent microenvironment by regulating the intercellular spaces between the vagal afferent terminals and smooth muscle or interstitial cells of Cajal. Therefore, it is likely that TNX plays a similar role to other ECM proteins in modulating neural connectivity. Perhaps TNX is important in maintaining the
shape of vagal afferent endings, therefore the absence of TNX may transduce mechanical forces less dynamically. The morphology of vagal afferent endings in the KO model was not altered therefore this needs to be further explored at the ultrastructural level.

In TNX deficiency, in addition to extracellular abnormalities, ion channels that detect mechanical sensitivity may be altered, thus affecting the movement of sodium influx initiating the action potential. This change could then result in increased afferent firing observed in the KO. Interestingly, TNR is shown to modulate the activity of sodium channels that are involved in action potential generation (Weber et al., 1999), in particular cells containing β1 and β2 subunits (Xiao et al., 1999). TNR deficient mice show no change in the distribution of sodium channels, but action potentials recorded from optic nerves showed a significant decrease in conduction velocity (Weber et al., 1999). Since TNX and TNR are within the same family, the functional importance of voltage gated sodium channels may similarly be affected by their removal.

In conclusion, we propose that TNX serves at least two important roles in gastrointestinal neural function, in addition to its established role in the structure and rigidity of somatic tissues. These are: a positive influence on cholinergic neurotransmission in intrinsic enteric neurones in the intestine, and a negative influence on mechanical coupling of vagal afferent endings in the stomach. These seemingly opposite effects may both ensue from changes in ultrastructure.
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Additional information

Competing interest – The authors declare no competing financial interests

Author Contributions:

All authors had access to the study data and reviewed and approved the final manuscript. Professor Aziz and Professor Blackshaw are joint senior authors.

1. Study concept and design- LAB, QA, RA

2. Acquisition of data- RA, MP, EJA, CB, NCV

3. Analysis and interpretation of data- RA, LAB, MP, SE

4. Drafting of the manuscript- RA

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FIGURE LEGENDS

Figure 1: TNX is expressed in neural structures in the stomach. Representative Immunohistochemical images taken from whole mount mouse fundus labelled with TNX and calretinin in IGLE surrounding the MP but not in cell bodies (A). TNX-IR was commonly found in calretinin-IR fibres in the smooth muscle layer of the stomach (B-Merge). The cell bodies of vagal afferents in the nodose ganglia also showed positive TNX and calretinin co-labelling in sections (C-Merge). TNX positive endings found in the mouse NTS (nucleus tractus solitarius). Unlike mouse, human fundus sections showed TNX- and calretinin-IR cell bodies in the MP (E-Merge) that were distinct from CGRP fibres (F-Merge). TNX also co-labelled calretinin-positive endings in the circular smooth muscle (G-Merge). All images from mouse and human stomach are whole mount confocal z images except for nodose ganglia and circular muscle image taken with an epi-fluorescent microscope. DMN denotes dorsal motor nucleus. Scale bar in panels A, B and C=25μm, C, D, E= 30μm
Figure 2: Vagal afferent mechanosensitivity in WT and TNX-KO mice. Responses to distension from single vagal afferent units innervating the stomach in WT vs. KO. Nerve discharge rate (histogram) was increased in KO in both spontaneous and distended (1mL of fluid in stomach) conditions (A). B shows the corresponding raw trace to the nerve discharge rate. Spontaneous (C, $p=0.0003$) and distension ($p<0.0001$) induced afferent firing (D) was significantly increased in TNX-KO compared to WT, (n=14 WT afferent units vs. n=18 KO afferent units). Addition of Baclofen (100µM) significantly reduced both spontaneous ($p<0.0001$) and distension ($p<0.0001$) induced firing back to similar levels observed in WT mice (C, D, n=23 KO afferent units). Baclofen also reduced WT spontaneous ($p<0.0001$) and distension ($p=0.028$) induced firing (C, D, n=22 WT afferent units). There was no change in gastric compliance to 1ml Krebs over 1 min in WT vs TNX-KO mice (N=12 WT vs. N=12 KO). (E). Statistical analysis was performed using an unpaired t-test.
Figure 3: Gastric emptying in WT and TNX-KO mice. Mean results showing $^{13}$CO$_2$ excretion curve in WT vs. KO. KO curve (grey) shows an increase in gastric emptying rate since the curve has shifted to the left (A). The half-life ($T_{1/2}$) was significantly reduced in the KO (103±12.9 min) $p=0.0277$ (B). The time taken for solid food to be broken down ($T_{Lag}$) was significantly reduced in the KO: 24min ±2.7 $p=0.0194$. (N=9 WT vs. N=15 KO). Statistical analysis was performed using an unpaired t-test.
Figure 4: Capsaicin and CCK effects in cultured nodose neurones

Differential interference contrast (DIC) image of neurones in culture, at baseline and stimulated neurone (A). The average peak intensity within the first 10s shows no change in response to KCl, capsaicin or CCK (B). Numbers of responsive and non-responsive neurones show no change in WT and KO with KCl, capsaicin or CCK stimulation (C). n=25 WT vs. n=17 KO KCL/capsaicin experiments and n=18 WT vs. n=34 KO CCK experiments.
Figure 5: Gastrointestinal symptoms in TNX deficient patients and healthy controls. The severity of gastrointestinal symptoms assessed using the gastrointestinal symptom rating scale (GSRS) scores (means and 95% CI) in patients with TNX deficiency (N=11) and healthy controls (N=2162). Abdominal pain, reflux and indigestion was significantly higher in TNX deficient patients compared to Swedish controls (****, p< 0.0001). Statistical analysis was performed using a two-way ANOVA (Sidak multiple comparison test).
Figure 6: Hypothesized anti-adhesive mechanisms by which TNX supports sensory function. In the stomach, vagal afferent endings are maintained at an optimal attitude to the host tissue by TNX, whether that be ganglia or muscle, or possibly interstitial cells (not shown). Without TNX, the ECM is improperly supported so excessive strain, both resting, and particularly active, is exerted on the mechanosensitive elements of the afferent ending.
Biography: Dr Rubina Aktar

I completed my PhD in 2016 and currently working as a Post-Doctoral Research Fellow at the Wingate Institute of Neurogastroenterology. My research interests encompass the normal and disease mediated function of enteric neurons. My PhD topic centred on the role of extracellular matrix molecule-Tenascin-x and how it affects gut function. Specifically, I am studying the cellular/molecular mechanisms underlying connective tissue defects in gut function. The motivation to study this area is based on an increase prevalence of gastrointestinal symptoms in patients with connective tissue abnormality like the hypermobile Ehlers-Danlos group. Gut symptoms in this cohort of patients are parallel to symptoms in patients with irritable bowel syndrome and functional gut disorders. Furthermore, I have been involved in and have a keen interest in understanding visceral pain mechanisms, the role of microbiota in gut function and how nutrients and food affect appetite.