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Lack of cholinergic innervation in gastric mucosa does not affect gastrin secretion or basal acid output in neurturin receptor GFRα2 deficient mice

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Key points

- Nervous control over gastric function is mediated via intrinsic neurons in the gastric myenteric ganglia. The majority of these neurons are cholinergic and are innervated by preganglionic efferents from the vagus nerve. Intact vagal innervation is crucial for gastric acid secretion and mucosal maintenance since vagotomy is known to abolish both basal and stimulated acid secretion and unilateral vagotomy causes gastric mucosal atrophy.
- Neurturin, a neurotrophic factor signalling via GDNF-family receptor α2 (GFRα2) is an important factor for parasympathetic innervation of many target tissues, but its role in gastric innervation is unknown.
- GFRα2-deficient (KO) mice lack virtually all cholinergic nerve fibres and associated glial cells in the gastric mucosa, yet have normal gastric morphology, gastrin secretion, and basal and maximal histamine-stimulated acid secretion.
- Blocking of myenteric ganglia with hexamethonium severely decreased basal acid secretion in wild-type mice but had no effect on the GFRα2-KO animals. Carbachol-stimulated acid secretion was higher in GFRα2-KO mice. Blocking of muscarinic receptors with atropine inhibited basal acid secretion in both genotypes suggesting that constitutive activity of muscarinic receptors may facilitate basal acid secretion.

Abstract Efferent signals from the vagus nerve are thought to mediate both basal and meal-induced gastric acid secretion, and provide trophic support of the mucosa. However, the underlying mechanisms are incompletely understood. Neurturin, signalling via glial cell line-derived neurotrophic factor (GDNF)-family receptor α2 (GFRα2), is essential for parasympathetic innervation of many target tissues but its role in gastric innervation is unknown. Here we show that most nerve fibres in wild-type mouse gastric mucosa, including all positive for gastrin-releasing peptide, are cholinergic. GFRα2-deficient (KO) mice lacked virtually all cholinergic nerve fibres and associated glial cells in the gastric (oxyntic and pyloric) mucosa but not in the smooth muscle, consistent with the selective expression of neurturin mRNA in the gastric mucosa. 2-Deoxyglucose and hexamethonium failed to affect acid secretion in the GFRα2-KO mice indicating the lack of functional innervation in gastric mucosa. Interestingly, basal and maximal histamine-induced acid secretion did not differ between wild-type and GFRα2-KO mice. Moreover, circulating gastrin levels in both fasted and fed animals, thickness of gastric mucosa, and density of parietal and different endocrine cells were similar. Carbachol-stimulated acid secretion was higher in GFRα2-KO mice, while atropine reduced basal secretion similarly in both genotypes. We conclude that cholinergic innervation of gastric mucosa depends on

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neurturin-GFRα2 signalling but is dispensable for gastrin secretion and for basal and maximal acid output. Basal acid secretion in the KO mice appears to be, at least partly, facilitated by constitutive activity of muscarinic receptors.

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Abbreviations 2-DG, 2-deoxyglucose; ECL, enterochromaffin-like; GDNF, glial cell line-derived neurotrophic factor; GFRα2, GDNF family receptor alpha-2; GRP, gastrin-releasing peptide; HDC, histidine decarboxylase; KO, knockout; PACAP, pituitary adenylate cyclase-activating polypeptide; PGP9.5, protein gene product 9.5; SP, substance P; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; VIP, vasoactive intestinal peptide.

Introduction

The two most important determinants of gastric acid secretion from the parietal cells are central input via the vagus and meal-stimulated gastrin release from the antrum. The stimulation of gastric acid secretion via the gastrin–enterochromaffin-like (ECL) cell–histamine pathway and its inhibition by somatostatin (from D-cells) is quite well established but the mechanisms underlying neuronal control of gastric acid secretion are complex and incompletely understood (Black & Shankley, 1987; Debas & Carvajal, 1994; Chen *et al.* 2005; Schubert & Peura, 2008; Ericsson *et al.* 2010*b*).

The vagus nerve controls gastric secretion via the intrinsic neurons in the gastric myenteric ganglia that provide the efferent innervation of the gastric mucosa. Most of these intramural neurons are cholinergic in all mammalian species studied (Pfannkuche *et al.* 1998; Nakajima *et al.* 2000; Pimont *et al.* 2003) and many of them coexpress neuropeptides including vaso-active intestinal peptide (VIP) and gastrin-releasing peptide (GRP) (Ekblad *et al.* 2000). In addition, gastric myenteric ganglia in both rodents and humans contain non-cholinergic neurons, including nitrergic inhibitory interneurons (Nakajima *et al.* 2000; Berthoud *et al.* 2001; Pimont *et al.* 2003). Studies in rats suggest that virtually all gastric myenteric neurons may be innervated by preganglionic vagal nerve fibres (Holst *et al.* 1997; Berthoud *et al.* 2001). Acetylcholine released from the postganglionic cholinergic nerve fibres in the gastric mucosa can increase gastric acid secretion directly by stimulating M3 muscarinic receptors on parietal cells and by inhibiting somatostatin release from D-cells. In addition, the neuropeptides released from the intrinsic nerves can regulate gastric acid secretion via the endocrine cells, and vice versa, somatostatin can regulate the activity of the intrinsic neurons in gastric mucosa (Schubert & Peura, 2008). The vagus and M3 receptors are required for basal acid secretion in different *in vivo* animal models and in humans (Schirmer, 1989; Debas & Carvajal, 1994; Aihara *et al.* 2003) and are also thought to provide trophic support to gastric mucosa in rats (Håkanson *et al.* 1984).

Plasticity and redundancy of gastric acid regulatory mechanisms have been reported in knockout mouse models (Chen *et al.* 2004; Zhao *et al.* 2008). For example, in gastrin–cholecystokinin double-mutant mice, basal and food-stimulated gastric acid secretion is normalized apparently by the cholinergic vagal pathway. In these mice, carbachol paradoxically reduces gastric acid secretion, possibly via an increased secretion of somatostatin in the oxyntic mucosa (Chen *et al.* 2004). Importantly, it is still unknown why vagotomy (or atropine) blocks both basal and gastrin- or histamine-stimulated acid response, as is the mechanism by which the vagus controls the mucosal cells (Chen *et al.* 2005).

Neurturin, signalling via the GDNF-family receptor α2 (GFRα2), is essential for the target innervation of many parasympathetic and a small subset of enteric neurons (Rossi *et al.* 2003, 2005). However, the role of neurturin-GFRα2 signalling in the stomach is unknown. Here, we show that GFRα2-deficient (KO) mice have a specific loss of the efferent cholinergic innervation in the gastric mucosa. This allowed us to investigate whether basal and stimulated gastric acid secretion, gastrin secretion and trophic support of the gastric mucosa requires the intrinsic cholinergic innervation.

Methods

Ethical approval

All animal experiments were carried out in accordance with the Guidelines laid down with the European Communities Council Directive (86/609/EEC) and were approved by the County Administrative Board of Southern Finland.

Animals

Two- to ten-month-old, age-matched GFRα2-KO and wild-type mice of both sexes in a congenic C57BL/6JOlaHsd background were used. All mice used for histology, PCR and gastrin measurements were littermates from heterozygous matings. Most mice used

to study acid output were the F1 offspring of several pairs of wild-type and GFRα2-KO parents. Mice were genotyped as described earlier (Rossi *et al.* 1999) and kept in standard, specific pathogen-free conditions under a constant dark/light cycle. The average body weight of GFRα2-KO animals used for gastric acid measurements was ∼20% (range 17–25%) less than that of their age-matched wild-type controls. However, there was no correlation between the body weight and the gastric pH, acid content or secretion.

Immunohistochemistry and microscopy

Mice were anaesthetized with an overdose of pentobarbital (200 mg/kg, I.P.; Orion, Espoo, Finland) and transcardially perfused first with cold PBS, pH 7.4, followed by 4% paraformaldehyde or 4% carbodiimide (for histamine-immunostaining) (Panula *et al.* 1988) in PBS. The stomachs were removed and postfixed at +4°C for 2–3 h or overnight (depending on antibody used), cryoprotected in 30% sucrose and mounted in Tissue Tek (Sakura Finetek, Torrance, CA, USA). Stomachs were cut into 10–20 μm sagittal sections and mounted on Super Frost Ultraplus microscopic slides (Thermo Fisher Scientific, Rockford, IL, USA) and stained using standard immunofluorescence techniques.

Primary polyclonal antibodies were against bombesin/GRP (rabbit, a kind gift from Dr Panula; Panula *et al.* 1982), galanin (rabbit, Millipore), GFRα2 (goat; R&D Systems Europe, Abingdon, UK), ghrelin (goat; Santa Cruz Biotechnology, Santa Cruz, CA, USA), H^+,K^+-ATPase β-subunit (rabbit, Thermo Fisher), histamine (rabbit; Panula *et al.* 1988), protein gene product 9.5 (PGP9.5; rabbit; Millipore, Billerica, MA, USA), S100β (rabbit; Swant, Bellizona, Switzerland), substance P (SP) (rabbit, Millipore), tyrosine hydroxylase (TH) (rabbit; Millipore), vesicular acetylcholine transporter (VAChT) (rabbit; Phoenix Pharmaceuticals, Burlingame, CA, USA), vasoactive intestinal peptide (VIP) (rabbit; Progen Biotechnik, Heidelberg, Germany). Secondary antibodies were from Jackson Immuno-Research Europe (Suffolk, UK). Confocal microscopy images were taken using a ×25 water immersion objective, and maximum intensity projections were generated using Zeiss LSM5 software (Carl Zeiss, Jena, Germany).

The density of nerve fibres and cells was quantified by a researcher blind to the genotype from microscopic images taken with ×20 magnification from nine sections through the stomach of each animal using systematic random sampling. The density of S100β-, TH-, VAChT- and VIP-positive fibres in gastric oxyntic mucosa was estimated by counting fibres crossing a standardized grid. The volume density of VAChT- and VIP-positive fibres in the inner, circular muscle layer of the oxyntic mucosa was estimated by counting the number of cut nerve profiles inside a circular probe randomly positioned on the muscle layer. The density of parietal cells and other cell types in the gastric mucosa was expressed as the number of clearly positive cell profiles per gland and length of mucosal surface, respectively.

Neurturin and GDNF mRNA *in situ* hybridization on cryosections from mouse stomach was performed as described (Rossi *et al.* 1999). No specific labelling above background was seen in adjacent sections hybridized with a sense probe.

Gastric juice pH

The animals were fasted overnight and killed by cervical dislocation. The stomachs were immediately removed and the gastric juice pH was measured with a flat-top pH probe (Blue Line 27 pH, Schott, Germany).

Acute fistula method

Briefly, the mice were fasted overnight with free access to water and anaesthetized with urethane (1.75 g kg^{-1}, I.P.; Sigma-Aldrich, St Louis, MO, USA) and kept on a heating pad while their body temperatures were monitored with a rectal probe. An incision was made in the abdomen to expose the duodenum and stomach. A flexible cannula was inserted into the stomach through a small incision made in the duodenum and secured in place around the pylorus. The cannula was attached to a small syringe that allowed the stomach to be flushed.

The gastric acid content was determined from the combined first (approximately five) washes with 0.3 ml of warm (39°C) saline until pH was >6.0. Basal secretion was then collected for 2 × 30 min by rinsing with 3 × 0.3 ml of saline. After the basal period, the mice were given 2-deoxyglucose (2-DG, 400 mg kg^{-1}, I.P.), histamine (10 mg kg^{-1}, I.P.) or carbachol (30 μg kg^{-1}, S.C.). These doses were chosen based on previous studies and our pilot experiments. For example, the histamine dose of 10 mg kg^{-1} was previously shown to lead to maximal acid stimulation (Tanaka *et al.* 2002). All drugs were from Sigma-Aldrich. Drug-stimulated acid secretions were collected up to 60 min after which the stomach was removed and the animals were killed by cervical dislocation. Gastric acid content in the samples was determined as above and expressed as micromoles of H^+ per hour.

Gastric acid output without anaesthesia

The mice were fasted overnight with free access to water. Drugs (or saline) were injected subcutaneously 40 min (histamine 10 mg kg^{-1}; pentagastrin 0.5 mg kg^{-1}) or 70 min (hexamethonium 20 mg kg^{-1}; atropine 3 mg kg^{-1})

before the mice were killed by cervical dislocation. The gastric acid content was determined by manual titration to pH 7.0 against 10 mM NaOH and expressed as μmol H^+ (g body weight)$^{-1}$).

Plasma gastrin levels

Blood samples (~100 μl) were collected in the morning from the saphenous vein of both fed and overnight (~14 h)-fasted mice (3- to 5-months-old, both sexes). Terminal blood samples (~0.5 ml) were collected through a cardiac puncture. The blood was centrifuged (13,000 *g*, 4°C) for 2 min and the separated plasma was stored at –80°C until analysis. Plasma gastrin levels were determined by using a commercially available RIA kit (MD302, Euro-Diagnostica, Malmö, Sweden) according to the manufacturer's instructions.

Real-time PCR

To assess mRNA expression of histidine decarboxylase (HDC) and different muscarinic receptor subtypes in the stomach, freely fed animals were killed and stomachs were dissected, washed and immediately frozen in liquid nitrogen and stored at –80°C. The tissues were lysed with QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and the mRNA was isolated according to the product protocol. Two micrograms of mRNA was used to generate first-strand cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers. SYBR Green-based real-time PCR was performed in triplicate with a Bio-Rad CFX96 system (Bio-Rad Laboratories) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control. GAPDH expression in the stomach did not differ between the genotypes. Analysis of data was done using the comparative Ct method. The primers were (5′-3′): HDC-fw: CTTTCTCATCCCGGCTACTATCCA, HDC-rev: ACCGCGTTGTCTTCCTCCTGT, M1-fw: TTCTGATCCC TGCTGTGTGG GAAT, M1-rev: TTGGCTCCTG ACTTCCTGCC TAAA, M2-fw: TACTTCTTGT TCAGCCTGGC CTGT, M2-rev: AGGCCAGTAG CCAATCACAG TGTA, M3-fw: TTGGTCCAAT GCCAATTCAG CAGG, M3-rev: AGACCCAGGC AGACCAATTT CTGA, M4-fw: ATGTGTGACT GCCATCGAGA TCGT, M4-rev: GATGAAGGCC AGCAGAATGG CAAA, M5-fw: TTCCCAGTGT CCAAAGACCC TTCA, M5-rev: ACAGAAGGTG GAAACCAGGA CCAT.

Statistical analysis

Values are presented as mean ± SEM. Data between two groups was compared using the two-tailed Student's *t* test, assuming unequal variance. Differences between multiple groups were determined by one-way or two-way ANOVA, followed by a Student–Newman–Keuls multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

Profound loss of gastric mucosal cholinergic innervation and associated glial cells in GFRα2-KO mice

Intrinsic cholinergic nerve fibres comprise the bulk of gastric mucosal innervation in rats (Ekblad *et al.* 2000). We used neuronal and glial markers to compare innervation of the gastric wall in adult wild-type and GFRα2-KO mice. Numerous VAChT- and VIP-positive nerve fibres were seen in the basal part of wild-type mouse gastric (oxyntic and pyloric) mucosa (Fig. 1*A* and *C* and not shown). Double staining showed that most, if not all, VAChT-positive nerve fibres in wild-type mouse gastric mucosa were bombesin/GRP-positive (Supplemental Fig. 1*A*–*C*), and most of them also coexpressed VIP (Supplemental Fig. 1*D*–*F*). Importantly, the VAChT-, GRP- and VIP-positive innervation of the mucosa was profoundly reduced throughout the glandular part of the stomach in GFRα2-KO mice (Fig. 1*B* and *D* and Supplemental Fig. 2*A* and *B*). Quantification revealed that the density of VAChT- and GRP-positive nerve fibres of the oxyntic mucosa was reduced by more than 90% in GFRα2-KO compared with wild-type mice (Fig. 1*E*, $P = 0.004$ and Supplemental Fig. 2*C*). Also the density of VIP-positive nerve fibres was reduced by ~80% (Fig. 1*F*, $P = 0.02$). A qualitatively similar loss of VAChT- and VIP-positive fibres was also seen in the pyloric mucosa (data not shown). In contrast, the density of VAChT- and VIP-positive nerve fibres in the gastric smooth muscle layer (Fig. 1*C* and *F*) was not different between the genotypes.

Interestingly, the number of glial cells was also reduced in the gastric mucosa but not in the gastric smooth muscle or in the myenteric ganglia of GFRα2-KO mice (Fig. 2*A* and *B*). Quantification revealed that the density of S100β-positive glial cells (not shown) and their processes in the gastric mucosa was reduced by ~80% in GFRα2-KO as compared with wild-type mice (WT: 1.8 ± 0.15, $n = 4$ *vs.* KO: 0.39 ± 0.06, $n = 3$, arbitrary units, $P = 0.002$).

In contrast to the dense intrinsic cholinergic innervation, PGP9.5-positive and VAChT-negative, putative non-cholinergic nerve fibres in wild-type mouse gastric mucosa were relatively sparse and appeared unaffected in the GFRα2-KO mice (Fig. 2*C* and *D*). Few sympathetic TH-positive nerve fibres were seen in the mouse gastric mucosa (Supplemental Fig. 1*K*) and their density was similar between the genotypes (WT:

0.50 ± 0.01, $n = 4$ *vs.* KO: 0.46 ± 0.03, $n = 3$, arbitrary units, $P = 0.3$). Finally, a few galanin-positive nerve fibres were present similarly in both genotypes in the gastric submucosa and muscle layers (but not in the mucosa) (Supplemental Fig. 2*D* and *E*).

Localization of GFRα2 and neurturin in mouse gastric wall

Consistent with our previous results that myenteric cholinergic neurons and associated glial cells express GFRα2 (Rossi *et al.* 2003), GFRα2 immunoreactivity was colocalized with VAChT-positive nerve fibres (Supplemental Fig. 1*G–I*) and with S100β-positive enteric glial cells in the gastric mucosa and muscle layers (Fig. 2*A*). The few sympathetic (TH+) nerve fibres in the stomach mucosa were GFRα2 negative (Supplemental Fig. 1*J–L*), agreeing with the lack of GFRα2 expression in noradrenergic sympathetic neurons and nerve fibres in other tissues (Rossi *et al.* 2005).

A previous study reported that the preferred GFRα2 ligand neurturin is expressed in adult mouse gastric mucosa (Golden *et al.* 1999). Extending this observation, we found that neurturin mRNA was expressed selectively in the basal part of the gastric mucosa (in the oxyntic and pyloric parts but not in the gastric smooth muscle layers) both in 2-week-old (Supplemental Fig. 3) and adult mice (Fig. 2*E* and *F*). No expression of GDNF mRNA above the background was found in the gastric mucosa in adjacent sections (Supplemental Fig. 3). Thus, neurturin–GFRα2 receptor signalling is needed for the development and/or maintenance of the intrinsic cholinergic innervation and associated glia in the mouse gastric mucosa.

Normal morphology of gastric mucosa in GFRα2-KO mice

The overall morphology and the thickness of gastric (oxyntic and pyloric) mucosa in GFRα2-KO mice appeared normal in haematoxylin and eosin (H&E)-stained sections (Fig. 3*A* and not shown). Immunostaining of cryostat sections revealed a similar distribution and density of H^+,K^+-ATPase-positive parietal (Fig. 3*B*) and histamine-positive ECL (Fig. 3*C*) cells in the gastric oxyntic mucosa between the genotypes. Histidine decarboxylase (HDC) mRNA expression levels in the stomach wall were comparable (WT: 0.8 ± 0.2, $n = 5$ *vs.* KO: 1.0 ± 0.1, $n = 4$, $P = 0.1$). Also the distribution and density of somatostatin- (Fig. 3*D*) and ghrelin- (Fig. 3*E*) positive cells in the gastric mucosa did not differ between the genotypes.

Gastric pH, basal acid secretion and gastrin levels are not altered in GFRα2-KO mice

Surprisingly, despite the virtually complete lack of cholinergic innervation of the gastric mucosa, GFRα2-KO

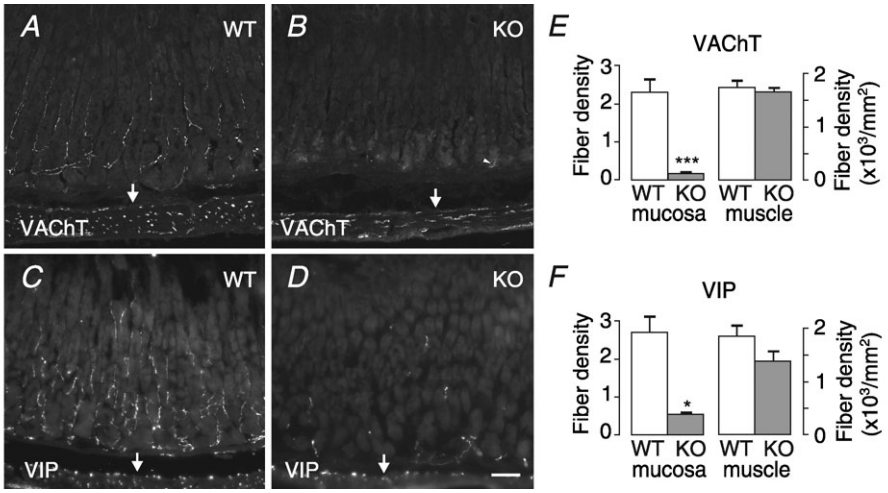

Figure 1. Loss of cholinergic innervation in GFRα2-KO mouse gastric mucosa
Representative sections from wild-type (WT) and GFRα2-KO (KO) mouse gastric mucosa (oxyntic part) immunostained for VAChT (*A* and *B*) and VIP (*C* and *D*). *A*, numerous VAChT-positive cholinergic fibres are seen in wild-type mouse gastric mucosa and in the underlying circular smooth muscle layer. *B*, density of VAChT-positive nerve fibres is profoundly reduced in the GFRα2-KO mouse gastric mucosa but not in the muscle layer (arrows). Arrowhead marks unspecific staining of gland lumen. *C* and *D*, density of VIP-positive nerve fibres is profoundly reduced in the GFRα2-KO mouse gastric mucosa but not in the underlying muscle layer (arrows). The density of VAChT- (*E*) and VIP- (*F*) positive fibres in the mucosa (in arbitrary units) and muscle was estimated from wild-type ($n = 4$–5) and GFRα2-KO ($n = 3$–4) mice using systematic random sampling. $^*P = 0.02$; $^{***}P = 0.004$, using a *t* test. Scale bar: 50 μm.

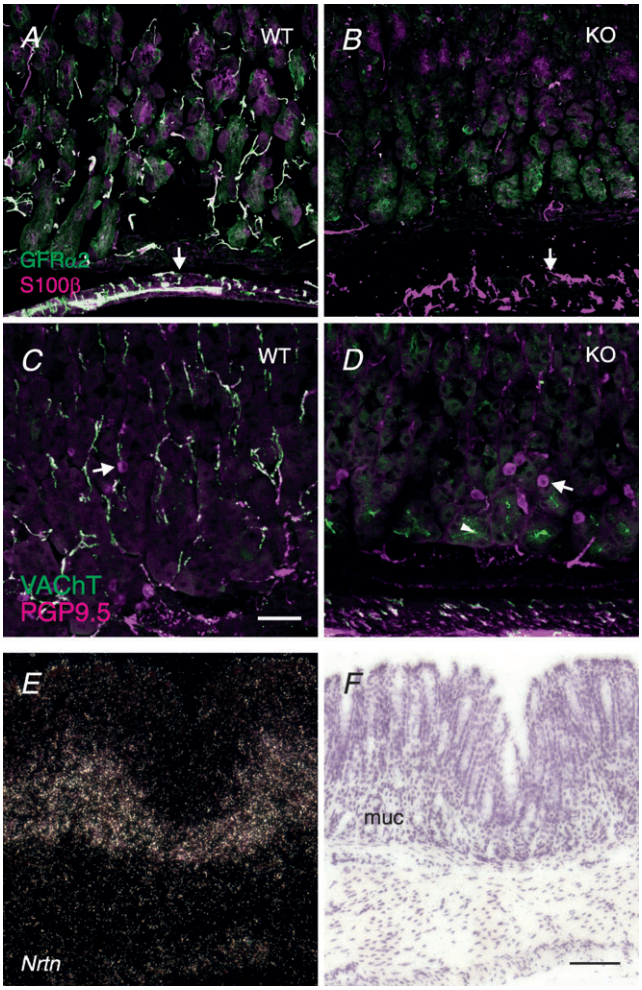

Figure 2. Lack of enteric glia in GFRα2-KO gastric mucosa and expression of neurturin mRNA in postnatal mouse stomach
A and *B*, double-staining with GFRα2 (green) and the glial marker S100β (magenta). *A*, in wild-type mouse stomach, S100β-positive glial cells and their processes coexpress GFRα2 both in the oxyntic mucosa and in the muscle layer (arrows). *B*, density of S100β-positive glial processes is greatly reduced in the GFRα2-KO mouse gastric mucosa but not in muscle layer (arrows). *C* and *D*, double-staining with cholinergic marker VAChT (green) and the pan-neuronal marker PGP9.5 (magenta). *C*, PGP9.5-positive nerve fibres in wild-type mouse gastric mucosa are concentrated in the basal half of the mucosa and most of them are cholinergic (white). Arrows point at PGP9.5-positive and VAChT-negative (magenta) putative endocrine cells in the mucosa. *D*, in GFRα2-KO mice, the PGP9.5-positive nerve fibres are virtually absent in the gastric mucosa, but the PGP9.5-positive endocrine cells remain. Note many PGP9.5-positive nerve fibres in the submucosa and muscle layer. Arrowhead marks faint unspecific staining of gastric gland lumen by the VAChT antibody. *E*, *in situ* hybridization of a stomach section from adult mice shows that neurturin (Nrtn) mRNA is selectively expressed in the basal half of the gastric mucosa but not in the muscle layers. *F*, corresponding bright-field image of the dark-field image (*E*). muc, mucosa. Scale bar: 50 μm.

mice had a normal gastric pH after the overnight fast (Fig. 4*A*). Consistent with this finding, gastric acid content (Fig. 4*B*) and basal acid secretion under urethane anaesthesia (Fig. 4*C*) did not differ between GFRα2-KO and wild-type control mice. Moreover, plasma gastrin levels were similar between the genotypes both in fasted and in fed animals (Fig. 4*D*).

Maximal histamine-induced acid secretion is normal in GFRα2-KO mice

Administration of histamine at 10 mg kg^{-1} (I.P.) is known to induce a maximal acid output response in mice (Tanaka *et al.* 2002). Importantly, this dose of histamine produced a similar acid secretory response in both wild-type and GFRα2-KO mice (Fig. 5*A*), indicating that the gastric acid secretory capacity of GFRα2-KO mice was not impaired.

Reduced vagally stimulated but increased carbachol-stimulated acid secretion in GFRα2-KO mice

Central hypoglycaemia induced by 2-DG stimulates gastric acid secretion via the vagus nerve (Becker *et al.* 1988). As expected, 2-DG induced a robust (~8-fold) acid secretion in wild-type but not in the GFRα2-KO mice (Fig. 5*B*). Muscarinic receptor stimulation by carbachol (30 μg kg^{-1}, S.C.) induced a 2-fold higher ($P < 0.01$) gastric acid secretion in GFRα2-KO mice than in wild-type controls (Fig. 5*C*). This result was supported by repetition with an independent cohort of mice using 20 μg kg^{-1} (I.P.) of carbachol (data not shown). Muscarinic receptor (M1 to M5) mRNA levels in the stomach (measured by real-time PCR) were similar between the genotypes (Supplemental Table 1).

Maximal gastric acid output without anaesthesia is similar between the genotypes

Urethane anaesthesia is known to inhibit basal and stimulated acid secretion by stimulating somatostatin release (Yang *et al.* 1990). To exclude the possibility that the anaesthesia (or the surgery) would affect acid secretion differently between the genotypes, we measured basal and secretagogue-stimulated acid output in intact, non-anaesthetized animals. Consistent with the results from the acute fistula model, basal acid secretion was similar in wild-type and GFRα2-KO mice, and stimulation by a maximal dose of histamine (10 mg kg^{-1}, S.C.) produced a similar (about 3-fold) increase in gastric acid output in both genotypes (Fig. 6). Pentagastrin (0.5 mg kg^{-1}, S.C.) increased acid output (by about 2-fold) in wild-type mice but not in GFRα2-KO mice (Supplemental Fig. 4).

Atropine but not hexamethonium reduces basal acid secretion in GFRα2-KO mice

The ganglion blocker hexamethonium is known to reduce basal acid secretion similarly to surgical vagotomy (Kay & Smith, 1951; Bunce & Parsons, 1977). Hexamethonium (20 mg kg^{-1}, s.c.) reduced basal acid output in wild-type mice by ∼70% compared with saline treatment ($P < 0.001$). In contrast, hexamethonium treatment did not affect basal acid output in GFRα2-KO mice (Fig. 6). Interestingly, the muscarinic receptor antagonist atropine (3 mg kg^{-1}, s.c.) reduced basal acid output similarly both in wild-type and in GFRα2-KO mice (Fig. 6).

Discussion

The main findings of this study are as follows. (1) GFRα2-KO mice lack virtually all efferent cholinergic innervation of the gastric oxyntic and pyloric mucosa resulting in a severely diminished acid secretory response

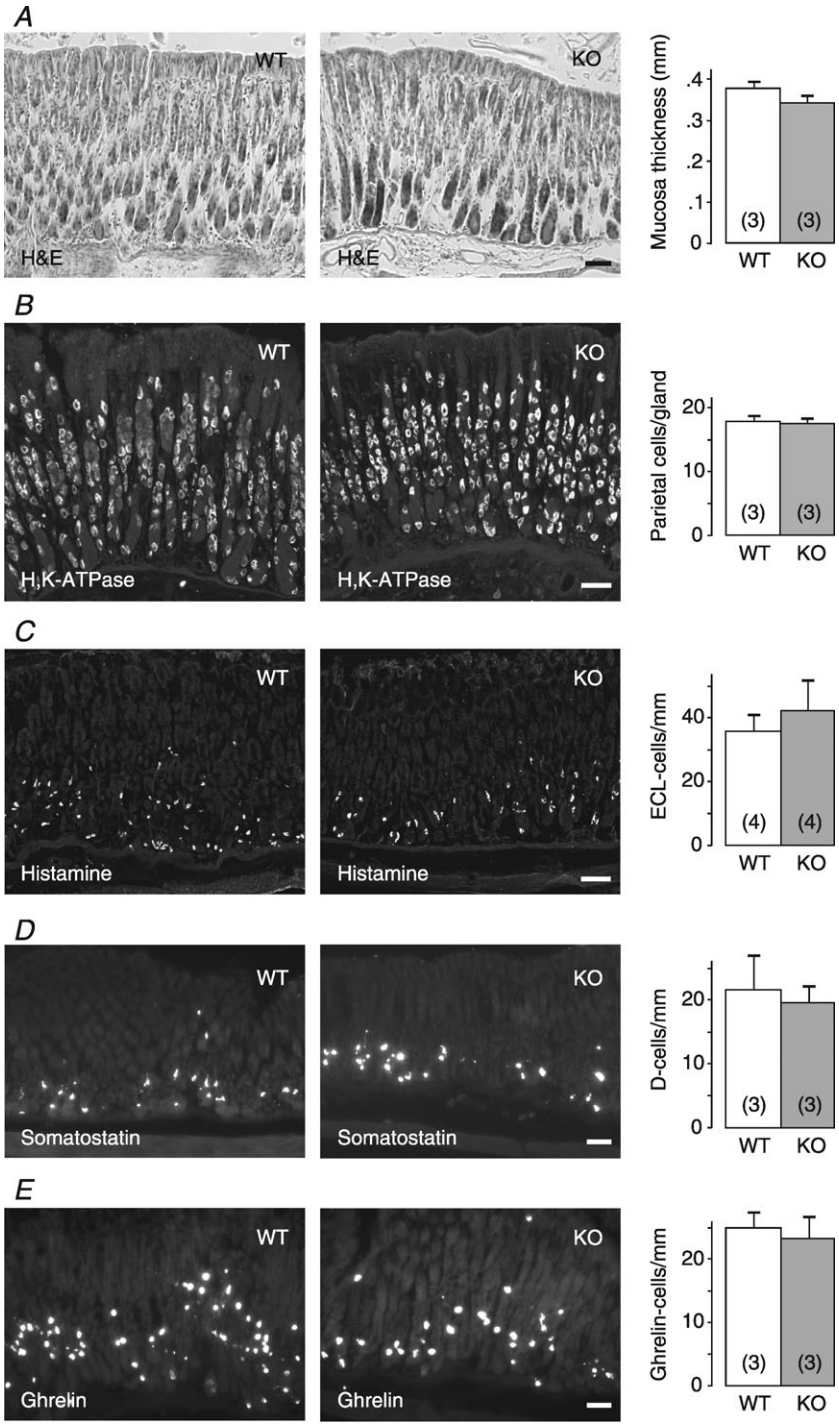

Figure 3. Normal morphology of the gastric mucosa in GFRα2-KO mice
A, representative images of H&E-stained sections from wild-type (WT) and GFRα2-KO (KO) gastric mucosa (oxyntic part). Thickness of the mucosa is similar between the genotypes ($P = 0.2$). *B–E*, different cell types in the gastric mucosa were stained and quantified using antibodies for H^+,K^+-ATPase (*B*), histamine (*C*), somatostatin (*D*) and ghrelin (*E*): The distribution and density of the different cell types in the oxyntic mucosa is similar between the genotypes ($P = 0.7$–0.8). Number of animals per group is shown in parentheses. Scale bar: 50 μm.

to vagal stimulation by 2-deoxyglucose and no inhibition by hexamethonium. (2) Despite the loss of functional innervation gastric gland development, circulating gastrin levels, as well as basal and maximal histamine-stimulated gastric acid output were not impaired. (3) In comparison to wild-type mice, GFRα2-KO mice display a similar response to atropine but an increased sensitivity to carbachol suggesting that constitutive activity of muscarinic receptors facilitates basal acid secretion in the mice.

Cholinergic innervation of the gastric mucosa requires neurturin–GFRα2 signalling

Physiological stimuli from both inside and outside the stomach converge on the effector neurons in the gastric myenteric plexus that are the primary regulators of acid secretion (Schubert & Peura, 2008). Most of the effector neurons are cholinergic (60–65% of all myenteric neurons in rat) which provide the bulk of the gastric mucosal innervation but also project to the gastric muscle (Nakajima *et al.* 2000; Schicho *et al.* 2003). Subsets of the gastric cholinergic neurons coexpress neuropeptides including SP, VIP, GRP and pituitary adenylate cyclase-activating polypeptide (PACAP; Ekblad *et al.* 2000). The neuropeptides may mark putative functional specialization of gastric myenteric neurons: intrinsic neurons that coexpress GRP project predominantly to the mucosa, whereas intrinsic neurons that express SP project mainly to the muscle layer (Berthoud, 1996; Ekblad *et al.* 2000). We show here that the cholinergic innervation of gastric mucosa is profoundly (>90%) reduced in

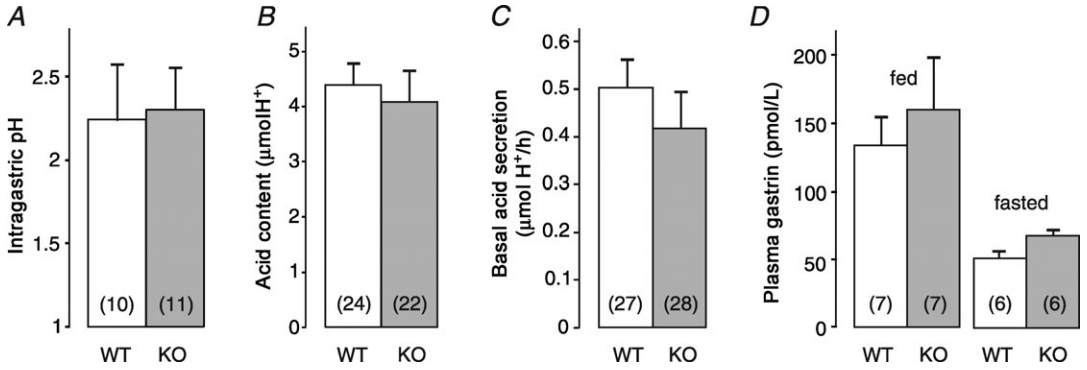

Figure 4. Similar gastric pH, acid content, basal acid secretion and gastrin levels in fasted wild-type and GFRα2-KO mice
A, gastric pH of drug-naive animals ($P = 0.8$). *B*, acid content in the stomach ($P = 0.7$). *C*, basal gastric acid secretion measured using the gastric fistula method under urethane anaesthesia ($P = 0.4$). *D*, plasma gastrin levels ($P = 0.6$). Number of animals per group is shown in parentheses.

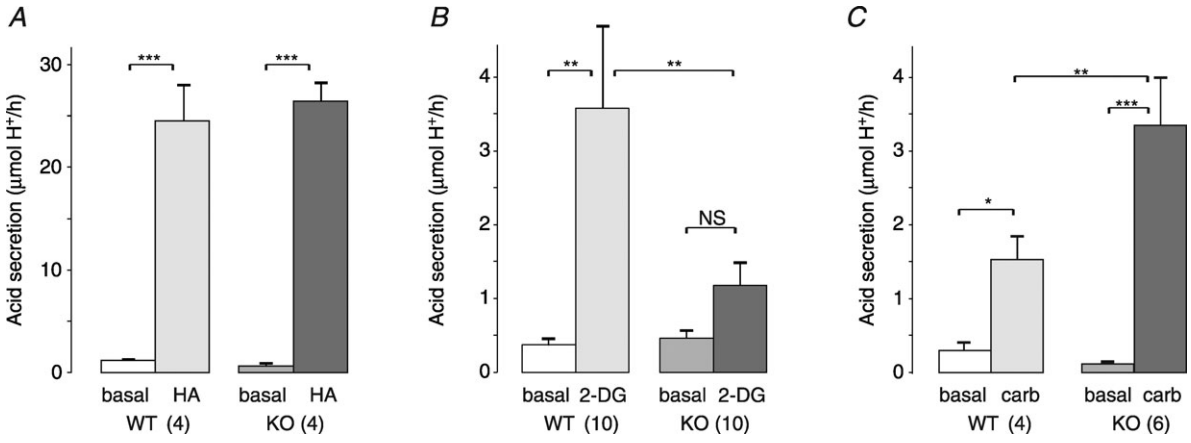

Figure 5. Drug-stimulated gastric acid secretion in fasted wild-type and GFRα2-KO mice
Acid secretion was measured under urethane anaesthesia using the gastric fistula method. Basal acid secretion was measured for 2 × 30 min before the stimulation. *A*, maximal acid output induced by histamine (HA, 10 mg kg^{-1}, I.P.) was similar between the genotypes. *B*, 2-deoxyglucose (2-DG, 400 μg kg^{-1}, I.P.) induced gastric acid secretion in wild-type but not in GFRα2-KO mice. *C*, carbachol (carb, 30 μg kg^{-1}, S.C.) induced more acid secretion in GFRα2-KO mice than in the wild-type controls. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. Number of animals per group is shown in parentheses.

adult GFRα2-KO mice compared with wild-type animals, whereas cholinergic innervation of gastric muscle layers is not affected. We propose that the selective expression of neurturin mRNA in the gastric mucosa but not in the myenteric layer, explains the selective loss of cholinergic innervation only in the gastric mucosa in GFRα2-KO mice.

Gastric mucosal morphogenesis and maturation of secretory function takes place during the first postnatal weeks in rodents (for a review see Keeley & Samuelson, 2010). The selective expression of neurturin in the 2-week-old mouse gastric mucosa suggests that the development of gastric mucosal intrinsic innervation requires neurturin–GFRα2 signalling during first postnatal weeks. Consistent with this finding, our results indicate that the innervation deficit in gastric mucosa is present already in 2-week-old GFRα2-KO mice (Supplemental Fig. 5). Furthermore, the continuing expression of neurturin mRNA in the gastric mucosa (and its receptors in the myenteric neurons) in adult mice suggests that the maintenance of gastric mucosal innervation may also depend on neurturin–GFRα2 signalling. Although the exact identity of neurturin-expressing cells in the gastric mucosa is not known, the pattern of expression in the basal part of the mucosa suggests localization of neurturin mRNA in the endo/paracrine cells.

Non-cholinergic intramural neurons in the stomach include inhibitory nitrergic interneurons (20–25% of all intramural neurons in rat) many of which also express VIP and mainly innervate the gastric muscle and myenteric plexus (Pfannkuche *et al.* 1998; Nakajima *et al.* 2000; Berthoud *et al.* 2001; Schicho *et al.* 2003). The normal density of VIP- and PGP9.5-positive innervation of the gastric muscle layers in the GFRα2-KO mice suggests that the non-cholinergic myenteric neurons do not require GFRα2 signalling. Interestingly, enteric glial cells were also largely missing (~80% reduction) in the gastric mucosa of the GFRα2-KO mice. In some parasympathetic target organs, such as salivary and lacrimal glands and pancreas, which also lack virtually all cholinergic innervation in GFRα2-KO mice (Rossi *et al.* 2003), the glial (Schwann) cells are not affected (Supplemental Fig. 6). Since the sympathetic and sensory innervation of these tissues is more abundant than in the gastric mucosa, we propose that the unaffected non-cholinergic innervation in these tissues may provide enough trophic support for the accompanying glial cells. Our results indicate that GFRα2 signalling is selectively needed for the development and/or maintenance of intrinsic cholinergic neurons and accompanying glial cells in the gastric mucosa. Since the cholinergic nerve fibres comprise the bulk of mucosal innervation, most of the intrinsic neurons that innervate the gastric mucosa require GFRα2 signalling for target innervation.

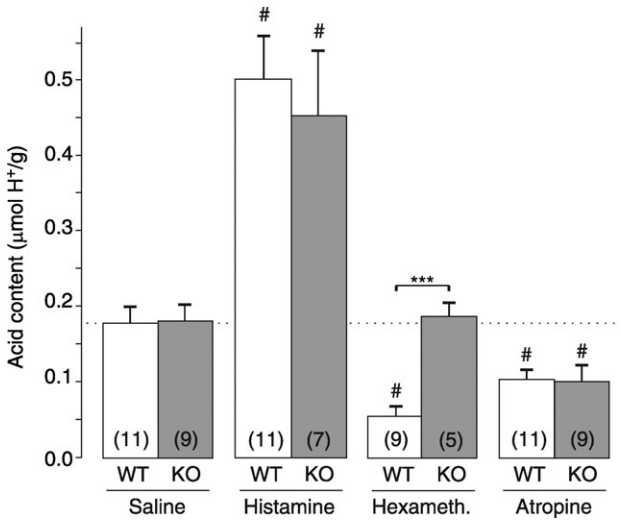

Figure 6. Effect of histamine, hexamethonium and atropine on gastric acid output in fasted wild-type and GFRα2-KO mice without anaesthesia
Histamine (10 mg kg^{-1}), hexamethonium (20 mg kg^{-1}), atropine (3 mg kg^{-1}) or saline were administered subcutaneously 40–70 min before the animals were killed and gastric acid content analysed. ***$P < 0.001$ between genotypes; #$P < 0.001$ compared to saline. Number of animals per group is shown in parentheses.

Intrinsic cholinergic innervation is not required for long term trophic support of gastric mucosa in mice

Gastrin is a major stimulant of gastric mucosa proliferation. In addition, the vagus nerve is thought to exert trophic support on the gastric mucosa since unilateral vagotomy in rats leads to atrophy of the gastric mucosa and reduces the density of ECL cells without altering gastrin levels (Håkanson *et al.* 1984). Since the gastric mucosa is not directly innervated by the efferent vagus, it is thought that the vagal control of ECL cells is mediated via the intramural neurons (Chen *et al.* 1999). One candidate is the neuropeptide PACAP that can regulate ECL cell proliferation (Lauffer *et al.* 1999) and gastric mucosal growth (Lu *et al.* 2011). Since PACAP is coexpressed in the VIP- and GRP-positive cholinergic nerve fibres in wild-type mouse gastric mucosa (Sundler *et al.* 1992), most if not all PACAP-containing innervation is expected to be missing in GFRα2-KO mouse gastric mucosa. However, since the thickness of gastric mucosa and the density of ECL cells were similar between GFRα2-KO mice and their wild-type littermates, trophic support of gastric mucosa does not seem to require cholinergic innervation in mice. Consistent with this, the importance of vagus on ECL cell proliferation has been questioned in other species including humans (Wangberg *et al.* 1996; Peghini *et al.* 2002). Yet, since the trophic effect of gastrin seems to depend on the M3 receptor (Aihara *et al.* 2003) and that the cholinergic innervation and muscarinic signalling have trophic effects

on other epithelia (Rossi *et al.* 1999; Knox *et al.* 2010), further studies using the GFRα2-KO mice seem warranted.

Cholinergic innervation of the gastric mucosa is dispensable for gastrin secretion

Gastrin secretion from pyloric G-cells is stimulated by the luminal food as reflected by 2- to 3-fold higher serum gastrin levels in fed mice (Feng *et al.* 2010). In addition to the food-related luminal stimuli, gastrin secretion is thought to be regulated by messengers from other endocrine/paracrine cells and local neurons (Schubert & Makhlouf, 1992). However, the role of gastric mucosal innervation on basal and food-stimulated gastrin secretion has been controversial. Although GRP/bombesin is a potent stimulator of gastrin secretion (Schubert & Makhlouf, 1992; Ericsson *et al.* 2010*a*), studies using GRP receptor antagonists in humans indicate that GRP is dispensable for normal basal and food-induced gastrin secretion (Hildebrand *et al.* 2001). A recent antrum microdialysis study in rats concluded that food-stimulated gastrin release depends on a complex interplay between local neurons and the vagus (Ericsson *et al.* 2010*b*). Food-induced gastrin release was reduced by local infusion of neuronal blocker TTX but was paradoxically increased by unilateral vagotomy. This suggested that gastrin release depends on 'local' neurons and is predominantly inhibited by the vagus nerve. It should be noted that TTX and vagotomy also block the afferent (sensory) innervation of the gastric mucosa. In contrast, the GFRα2-KO mice lack only the efferent cholinergic innervation but have an apparently normal afferent innervation of the gastric mucosa. Since both fed and fasted circulating gastrin levels in the GFRα2-KO mice were similar to those in wild-type littermates, the efferent cholinergic nerve fibres in the gastric mucosa and their neurotransmitters (including GRP/bombesin) may not be necessary (and thus may not be physiologically important) in the control of normal gastrin secretion. Nevertheless, further studies on the role of local neurons in gastrin release seem warranted, e.g., whether anacidity-induced hypergastrinaemia that is proposed to be mediated by GRP/bombesin or by sensory neurons (Nojima *et al.* 2000) is affected in the GFRα2-KO mice.

Lack of efferent cholinergic innervation in the gastric mucosa does not affect basal and maximal histamine-stimulated acid secretion in GFRα2-KO mice: putative compensation by constitutive muscarinic receptor activity

Tonic activity of the efferent vagus is thought to be necessary for basal gastric acid secretion since vagotomy reduces basal gastric acid secretion by 70–90% in experimental animals as well as in humans (Håkanson *et al.* 1982; Debas & Carvajal, 1994). Basal acid secretion is also reduced by atropine and in M3 muscarinic receptor knockout mice (Aihara *et al.* 2003), consistent with the idea that acetylcholine, released from the intrinsic cholinergic mucosal nerve fibres following vagus nerve activity, stimulates basal gastric acid secretion via M3 receptors on parietal cells. In contrast, we found that basal gastric acid secretion was not reduced in the GFRα2-KO mice that lack virtually all cholinergic innervation of the gastric mucosa indicating that the basal acid secretion may not require efferent cholinergic innervation (and release of acetylcholine) in the gastric mucosa.

Cholinergic stimulation of gastric acid secretion is mediated by M3 and M5 muscarinic receptors in mice (Aihara *et al.* 2005), whereas activation of M2 or M4 receptors is thought to inhibit gastric acid secretion (Schubert & Peura, 2008). The increased carbachol-response in the GFRα2-KO mice suggests net sensitization of muscarinic receptor signalling in the gastric mucosa, although the underlying mechanism remains unclear. The possibility that residual acetylcholine release from the muscle layers (combined with the muscarinic receptor sensitization) would explain the maintained basal secretion can be excluded since hexamethonium treatment did not reduce basal acid secretion in the GFRα2-KO mice. This result also suggests that the KO mice have a complete loss of functional efferent innervation of the gastric mucosa.

Most if not all G protein-coupled receptors, including muscarinic receptors, are constitutively active, i.e., they can signal in the absence of agonist, although the physiological significance of constitutive receptor activity is less clear (Casarosa *et al.* 2010). Thus, muscarinic receptor blockers such as atropine are in fact negative antagonists (= inverse agonists) that inhibit both agonist-stimulated and constitutive, agonist-independent activity of the receptors (Burstein *et al.* 1997). Our finding that atropine reduced the basal acid output in the GFRα2-KO mice (to an extent similar to that in wild-type mice) suggests that constitutively active muscarinic receptors on parietal and/or D-cells may facilitate basal acid secretion *in vivo*. Confirmation of this hypothesis will require data from isolated gastric glands or cells. Interestingly, the lack of cholinergic innervation in GFRα2-KO mice is also associated with increased responsiveness to muscarinic agonists in urogenital organs (Nangle & Keast, 2006). Thus, we suggest that the increased carbachol responses in GFRα2-KO mice may reflect a compensatory upregulation of constitutive muscarinic receptor activity. However, we cannot exclude the possibility that there is also an increased sensitivity to histamine, which would indicate a more general sensitization of the system. In other words, this

phenotype may reflect a functional adaptation to the life-long loss of a cholinergic/peptidergic input to ECL, D- and parietal cells. Assuming that GFRα2 signalling is also required for the maintenance of mucosal cholinergic innervation, a inducible GFRα2-knockout might allow the time course of the adaptation to be addressed.

Our results indicate that *in vivo* parietal cells can secrete normal amounts of gastric acid without the efferent cholinergic innervation in the mucosa during the basal state (Fig. 7). This conclusion is supported by previous studies using the isolated mouse stomach preparation (Black & Shankley, 1985; Schubert *et al.* 1987), as well as antrum microdialysis in conscious rats (Ericsson *et al.* 2010*b*) in which neuronal blocker TTX had no (or only a small) effect on basal gastric acid secretion. Understanding why basal acid secretion is reduced by blocking the vagal input but apparently not by blocking the myenteric neurons will require a more detailed knowledge of the gastric myenteric network function. Since gastric myenteric neurons that are encircled by a dense ring of vagal preganglionic varicosities are presumably more easily exited than others (Schemann & Grundy, 1992; Holst *et al.* 1997) tonic vagus activity may stimulate basal acid secretion via a selective subpopulation of gastric myenteric neurons. Future studies to directly monitor and manipulate the activity of the intramural nerve plexus *in vivo*, e.g., by optogenetic tools, may allow the underlying network mechanisms to be dissected.

Vagotomy is also known to rapidly and permanently inhibit the maximal histamine-stimulated gastric acid secretion (Vallgren *et al.* 1983) but the underlying mechanism has remained unclear (Chen *et al.* 2005). Our observation that the maximal histamine-induced acid secretion was not affected in GFRα2-KO mice suggests that the parietal cells can maintain a normal acid secretory capacity to histamine without the intrinsic cholinergic innervation of the gastric mucosa. This is consistent with the previous studies in isolated mouse stomach

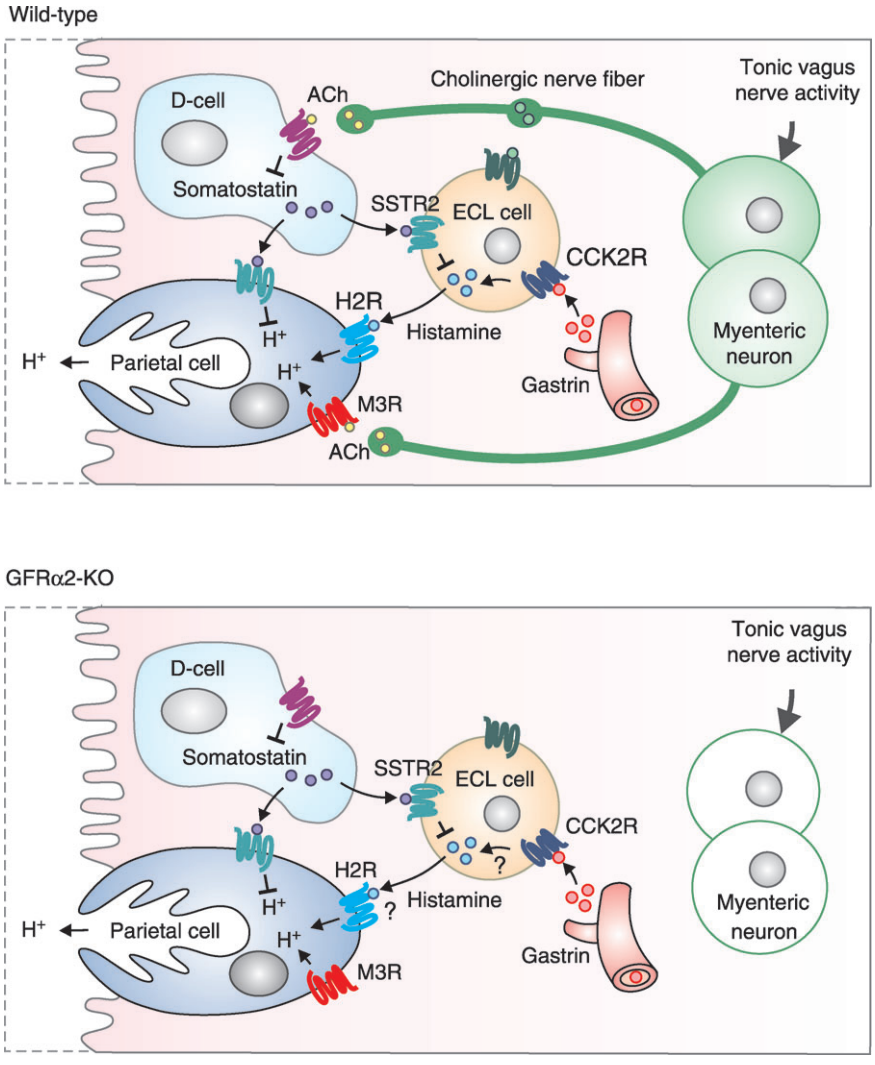

Figure 7. Efferent innervation of gastric mucosa is dispensable for basal acid secretion in GFRα2-KO mice
Upper panel, a model of basal gastric acid secretion in wild-type mice. Circulating gastrin (from antral G-cells, not shown) is thought to stimulate basal acid secretion largely by releasing histamine from ECL cells. Somatostatin from D-cells appears to exert tonic inhibition on acid secretion primarily by inhibiting histamine from ECL cells. Acetylcholine released from the intrinsic neurons in response to tonic vagus activity is thought to promote acid secretion via M3 receptors (M3R) on parietal cells but also by inhibiting tonic somatostatin release from D-cells. Lower panel, lack of cholinergic innervation in the gastric mucosa of GFRα2-KO mice does not impair basal acid secretion presumably partly because of compensatory upregulation of muscarinic receptor constitutive activity. The gastrin–histamine pathway may also be altered in the KO mice (as indicated by question marks; see Discussion for details). The role of neuropeptides (VIP, PACAP and GRP that are coexpressed in the mucosal cholinergic nerve fibres) in basal acid secretion is not clear. CCK2R, gastrin receptor; H2R, histamine H2 receptor, M3R, muscarinic M3 receptor; SSTR2, somatostatin receptor.

preparations in which TTX treatment did not affect the maximal histamine-stimulated acid response (Angus & Black, 1982). We cannot exclude possible changes in histamine sensitivity or in the histamine–muscarinic signalling interaction in the GFRα2-KO mice at this stage. Further studies are also warranted to address the relative contribution of constitutive activity of different muscarinic (and possibly other) receptors to gastric functions.

A previous *in vivo* microdialysis study in rats suggested that the vagus nerve controls ECL cell sensitivity to gastrin (Norlen *et al.* 2005). This may explain why gastrin stimulated less acid output in the GFRα2-KO mice compared with wild-type controls. Confirmation of this hypothesis will require additional dose–response studies for gastrin-induced histamine mobilization and acid output.

In conclusion, the present work demonstrates that the efferent cholinergic innervation of the gastric mucosa requires neurturin–GFRα2 signalling but is not necessary for normal gastrin secretion, for basal or maximal acid secretion, or for trophic support of the gastric mucosa by the vagus. Our results suggest that basal acid output from the parietal cells in the GFRα2-KO mice can be maintained without innervation possibly in part by constitutively active muscarinic receptors (Fig. 7). Other possible compensatory mechanisms, such as sensitization to histamine, remain to be studied. In contrast, meal-induced acid secretion depends on the efferent cholinergic innervation consistent with the lack of gastric acid secretory responses to vagal stimulation in the GFRα2-KO mice. The GFRα2-KO mouse offers a unique model of a selective loss of the efferent cholinergic innervation in the gastric mucosa for future studies on the role of intrinsic neurons in gastric mucosal functions.

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Author contributions

The experiments were performed in the lab of M.S.A. J.K., J.R. and K.-H.H. acquired and analysed the data, M.S.A. designed and supervised the study. All authors contributed to the interpretation of data and writing the manuscript with intellectual consent. All authors approved the final version of the manuscript.

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