Role of the vagus in the reduced pancreatic exocrine function in copper-deficient rats

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Babic T, Bhagat R, Wan S, Browning KN, Snyder M, Fortna SR, Travagli RA. Role of the vagus in the reduced pancreatic exocrine function in copper-deficient rats. Am J Physiol Gastrointest Liver Physiol 304: G437–G448, 2013. First published December 28, 2012; doi:10.1152/ajpgi.00402.2012.—Copper plays an essential role in the function and development of the central nervous system and exocrine pancreas. Dietary copper limitation is known to result in noninflammatory atrophy of pancreatic acinar tissue. Our recent studies have suggested that vagal motoneurons regulate pancreatic exocrine secretion (PES) by activating selective subpopulations of neurons within vagovagal reflexive neurocircuits. We used a combination of in vivo, in vitro, and immunohistochemistry techniques in a rat model of copper deficiency to investigate the effects of a copper-deficient diet on the neural pathways controlling PES. Duodenal infusions of Ensure or casein, as well as microinjections of sulfated CCK-8, into the dorsal vagal complex resulted in an attenuated stimulation of PES in copper-deficient animals compared with controls. Immunohistochemistry of brain stem slices revealed that copper deficiency reduced the number of tyrosine hydroxylase-immunoreactive, but not neuronal nitric oxide synthase- or choline acetyltransferase-immunoreactive, neurons in the dorsal motor nucleus of the vagus (DMV). Moreover, a copper-deficient diet reduced the number of large (>11 neurons), but not small, intrapancreatic ganglia. Electrophysiological recordings showed that DMV neurons from copper-deficient rats are less responsive to CCK-8 or pancreatic polypeptide than are DMV neurons from control rats. Our results demonstrate that copper deficiency decreases efferent vagal outflow to the exocrine pancreas. These data indicate that the combined selective loss of acinar pancreatic tissue and the decreased excitability of efferent vagal neurons induce a deficit in the vagal modulation of PES.

pancreas; vagus; vagovagal reflexes

IT HAS BEEN KNOWN FOR SEVERAL decades that copper plays an important role in the development and function of the central nervous system (9, 11, 14). Among the many consequences of copper deficiency is a decrease in myelin and norepinephrine concentrations in the rodent brain (11, 30, 37, 38), and recent evidence shows that copper deficiency also reduces field-evoked colonic contractility (18).

In humans, the essential role of copper in proper brain development is best illustrated in children, where copper deficiency results in the typical, severe neuronal degeneration observed in Menkes disease (42). Copper deficiency is also emerging as a consequence of gastrectomy and gastric bypass surgery (13, 19), perhaps as a result of bypassing the duodenum and proximal jejunum, where copper is usually reabsorbed (49).

Dietary copper also plays a crucial role in the proper development of the exocrine pancreas. A unique finding in copper-deficient rats is the extensive, selective, noninflammatory degeneration of pancreatic acinar tissue. It appears, however, that acute pancreatitis is not associated with the acinar cell loss. In addition, pancreatic duct cells remain unaffected by copper deficiency, although the islets of Langerhans display some level of hyperplasia (15, 16, 41, 43, 54).

The pancreas, like the upper gastrointestinal (GI) tract, is under the modulatory control of the vagus nerve and vagovagal reflexes. While several features of vagovagal reflexes innervating the upper GI tract are shared by different organs, we have also demonstrated that efferent pathways innervating different targets have unique neurochemical and electrophysiological properties. In particular, we and others have demonstrated that separate vagal pathways regulate exocrine and endocrine pancreatic secretions (1, 3, 4, 6, 10, 31–35, 40, 52, 53). In relatively recent studies, we used electrophysiological approaches to demonstrate that pancreas-projecting neurons in the dorsal motor nucleus of the vagus (DMV) can be subdivided into separate populations on the basis of their responses to pancreatic polypeptide (PP), sulfated CCK-8 (CCK-8s), and glucagon-like peptide 1 (GLP-1) (2, 51–53). In a more recent study, we demonstrated with in vitro and functional experiments that DMV neurons that regulate insulin release respond to GLP-1 and group III metabotropic glutamate receptor agonists, whereas neurons that regulate pancreatic exocrine secretions (PES) respond selectively to PP and CCK-8s (2). Given the peripheral impairment of acinar pancreatic tissue in copper deficiency, we designed a set of in vivo and in vitro experiments to test the effects of a copper-deficient diet on vagovagal reflexes controlling PES and on the efferent vagal motoneurons.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats of either sex were used in all experiments, which were approved by the Penn State University Institutional Animal Care and Use Committee. Pregnant dams (n = 26) were fed a copper-deficient diet (catalog no. TD-80388, Harlan-Teklad) starting at gestational day 13. Control dams (n = 36) were fed standard rat chow. Experiments were performed on rats at 31–66 days of age; most were carried out at ~40–45 days of age, since the mortality rate of rats fed the copper-deficient diet increased markedly beginning at 6 wk of age, and few animals survived beyond 8 wk postpartum.

Pancreatic duct cannulation and duodenal infusion of nutrients. Animals were fasted overnight (with water provided ad libitum) and anesthetized with thiobutabarbital (Inactin, 100–135 mg/kg ip), and a midline laparotomy was performed. The common bile-pancreatic duct was cannulated with polyethylene (PE-10) tubing to allow collection of PES at 10-min intervals. A cannula (PE-190 tubing) was inserted through a small (2- to 3-mm) incision into the duodenum ~1 cm distal to the pylorus to allow infusion of a 10% solution of casein (pH 7.4) or Ensure at a rate of 4 ml/h for 75 min. The abdominal laparotomy was closed with 6-0 suture, and the rat was placed on a heated pad to maintain body temperature at 37 ± 1°C.

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The total volume of PES was measured before protein content was assessed in 5-μl samples using the BCA protein assay kit (Pierce, Rockford, IL) and expressed as micrograms of protein per 10 min. Sample collection began after ~1 h of stabilization. Baseline and stimulated protein secretion was measured over a 30-min control period before and 90 min after drug treatment, respectively.

**Microinjection in the dorsal vagal complex.** After cannulation of the pancreatic duct, rats were placed in a stereotaxic frame, and the lower medulla was exposed. A glass micropipette (30- to 40-μm tip diameter) was directed into the dorsal vagal complex (DVC) under microscopic guidance (from calamus scriptorius: +0.2–0.5 mm rostrocaudal, 0.1–0.3 mm mediolateral, and –0.5 mm dorsoventral) for drug delivery. Vehicle (PBS) or CCK-8s (450 pmol) was applied in a 60-nl volume by pressure ejection over a 1-min period. Fluorescent microspheres (Fluoresbrite Carboxy NYO, Polysciences, Warrington, PA) were included in the injectate for post hoc verification of the injection site.

At the end of the experiment, animals were perfused transcardially with physiological saline followed by 4% paraformaldehyde in 0.1 M PBS. The brain stem was removed and placed in postfixative solution containing 20% sucrose overnight. Coronal sections (50 μm) through the DVC were cut on a microtome and analyzed with a Nikon E400 microscope equipped with fluorescence filters to reconstruct the location of the microinjection.

**Immunohistochemistry.** In another set of rats, 50-μm-thick brain stem sections from control (n = 8) and copper-deficient (n = 12) rats were processed for nitric oxide (NO) synthase (NOS), tyrosine hydroxylase (TH), or choline acetyltransferase (ChAT) immunoreactivity (IR). After three 10-min rinses in Tris-PBS (TPBS) containing 0.3% Triton X-100 and 0.05% thimerosal, sections were incubated in a solution of 30% methanol-1% H2O2 to eliminate endogenous peroxidase activity. After three additional 10-min rinses in TPBS, sections were blocked in 10% normal horse serum in TPBS for ≥30 min. Sections were incubated in the primary mouse monoclonal anti-neuronal NOS (1:1,000 dilution; catalog no. N2280, Sigma, St. Louis, MO), mouse anti-TH (1:1,000 dilution; catalog no. 22941, Immunostar, Hudson, WI), or goat anti-ChAT (1:5,000 dilution; catalog no. AB144P, Millipore, Billerica, MA) antibody for 3 days at 4°C. Sections were then rinsed three times in TPBS and incubated with biotinylated antiserum (for detection of immunoreactive peroxidase) for 60 min at room temperature. Sections were then rinsed and placed in biotin-SNAP-conjugated donkey anti-mouse for TH and NOS or donkey anti-goat (for ChAT staining) (1:500 dilution; Jackson ImmunoResearch) overnight. After three rinses in TPBS, tissue was incubated in ExtrAvidin-horseradish peroxidase (HRP; catalog no. E2886, Sigma) for 4–6 h. The HRP reaction was visualized with nickel-cobalt-enhanced diaminobenzidine or Vector SG using glucose oxidase. Sections were rinsed, mounted on gelatin-coated microscope slides, and dehydrated, and coverslips were applied.

Sections were examined under bright-field microscopy and photographed with a SPOT-RT color camera (Diagnostic Instruments, Sterling Heights, MI), and the number of TH-, NOS-, and ChAT-IR neurons in the DVC was counted.

**Labeling of intrapancreatic ganglia.** Intrapancreatic ganglia were labeled by injection of Fluoro-Gold (500 μg·ml−1·rat−1·ip). After 3 days, the animals were perfused transcardially with heparinized saline, and the pancreas was removed. The pancreatic tissue was fixed in 4% paraformaldehyde overnight, rinsed, flattened, and embedded in gelatin. Sections (100 μm thick) of the pancreas were cut on a vibratome and mounted on gelatin-coated slides, and coverslips were applied with Fluoromount-G (Southern Biotech, New Orleans, LA). Sections were examined under a fluorescence microscope equipped with UV filters and photographed with a SPOT-RT camera to allow reconstruction of the location and number of intrapancreatic ganglia. The number of Fluoro-Gold-containing neurons in each intrapancreatic ganglion was counted. A different group of pancreata from age-matched control and copper-deficient rats (~45 days old, n = 3 for both) was treated as described above, embedded in paraffin, cut into 10-μm-thick sections, and reacted for hematoxylin-eosin stain.

**Retrograde tracing and tissue preparation.** The fluorescent tracer 1,1’-dioleyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) was applied to the pancreas following the method described previously (5, 52). Briefly, 12- to 14-day-old rats were anesthetized with isoflurane (2.5% with air, 600 ml/min), and a deep level of anesthesia was maintained throughout the surgical procedure. The abdominal area was cleaned with alcohol prior to laparotomy. The pancreas was isolated gently from the surrounding viscera and placed on sterile gauze moistened with saline. DiI crystals were placed on the surface of the body of the pancreas and embedded in place using a fast-hardening epoxy resin. The epoxy resin was allowed to dry for 3–5 min, the entire surgical area was cleaned with sterile saline, the wound was sutured using 5-0 thread, and the animal was allowed to recover for ≥10–15 days before experimentation.

The method used to prepare the tissue slice for electrophysiological study is described elsewhere (7, 46). Briefly, the rats were anesthetized deeply with isoflurane (3%) and killed by bilateral pneumothorax, and the brain stem was removed and placed immediately in oxygenated chilled Krebs solution. Four to six coronal slices (300 μm thick) containing the DVC were cut and incubated in Krebs solution at 25–30°C for 90 min. A single slice was placed in a custom-made perfusion chamber (500-μl volume) on the stage of a Nikon E600FN microscope, held in place by a nylon mesh, and perfused with oxygenated, warmed (32 ± 1°C) Krebs solution.

**Identification of labeled DMV neurons and electrophysiological recording.** Electrophysiological recordings were made only from the bright fluorescently labeled pancreas-projecting (DiI-filled) neurons identified using tetramethylrhodamine isothiocyanate epifluorescence filters. Once the identity of DiI-filled neurons was confirmed, electrophysiological recordings were made under bright-field illumination using differential interference contrast (Nomarski) optics.

Whole cell patch-clamp recordings were made using borosilicate patch pipettes that had a tip resistance of 2–5 MΩ and were filled with potassium gluconate or KCl intracellular solution. N-(2-aminoethyl)-biotinamide HCl (Neurobiotin, 2.5 mg/ml) was included in the recording pipette to stain the neuron for later morphological analysis. The recordings were made using an Axopatch 200B or 1D amplifier (Axon Instruments, Union City, CA), and only recordings with a series resistance <20 MΩ were accepted. In all current-clamp experiments, the neurons were clamped to −80 mV (for recording of firing-rate changes) or at −65 mV (for recording of membrane depolarization/hyperpolarization). In voltage-clamp experiments, the neurons were held at −60 mV. An intracellular solution of potassium gluconate and 50 μM bicusculine in Krebs solution was used to isolate glutamate excitatory postsynaptic currents (EPSCs); intracellular KCl in 1 mM kynurenic acid in Krebs solution was used to isolate GABAergic inhibitory postsynaptic currents (IPSCs).

To assess the effects of drugs, each neuron served as its own control; i.e., the results obtained after drug (PP or CCK-8s) administration were compared with those obtained before drug administration. One cell per slice was tested. To construct the concentration-response curve, at least three concentrations were tested on the same neuron, and each drug was superfused for a period sufficient for the response to reach its plateau. A ≥10-min interval was allowed between each successive drug application.

Drugs were applied by superfusion; once the response reached plateau, the drug was washed out, and 10–15 min later the membrane was restored to control by direct-current injection before the protocols were repeated.

**Morphological reconstructions.** At the end of the experimental protocol for electrophysiological recording, Neurobiotin was injected into the DMV neuron (1-s depolarizing current pulses every 2 s for 20 min), and the brain stem slice was fixed overnight in Zamboni’s fixative at 4°C. The fixative was cleared from the slice with multiple washes of PBS-Triton X-100 (see below), and Neurobiotin was visualized using a cobalt-nickel enhancement of the avidin D-HRP technique, as described previously (7, 29). Neuronucida software (Microbrightfield, Williston, VT) was used to make three-dimensional...
reconstructions of the individual Neurobiotin-labeled neurons, digitized at a final magnification of ×600. The morphological features assessed were soma area and diameter, form factor (a measure of circularity for which a value of 1 indicates a perfect circle and 0 indicates a line; form factor = 4 πa × 1p2, where a is soma area and p is the perimeter of the soma in the horizontal plane), number of segments (i.e., branching of dendrites), and extension in the x- and y-axes. Data analysis was performed as described previously (7, 29).

Solution composition. Krebs solution consisted of (in mM) 126 NaCl, 25 NaHCO3, 2.5 KCl, 2.4 CaCl2, 1.2 NaH2PO4, and 11 dextrose and was maintained at pH 7.4 by bubbling with 95% CO2.

Intracellular solution contained (in mM) 128 potassium glutamate, 10 KCl, 0.3 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP, and 0.25 GTP; its pH was adjusted to 7.35 with KOH.

KC intracellular solution consisted of (in mM) 140 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 10 EGTA, 2 ATP-Na, and 0.25 GTP-Na; its pH was adjusted to 7.35 with HCl.

Zamboni’s fixative contained 1.6% (wt/vol) paraformaldehyde, 19 mM KH2PO4, and 100 mM Na2HPO4 in a solution of 240 ml of saturated picric acid-1,600 ml of H2O; its pH was adjusted to 7.4 with HCl.

PBS contained (in mM) 115 NaCl and 75 Na2HPO4·7H2O (0.15% Triton X-100). Avidin D-HRP solution consisted of 0.05% dianimobenzidine in PBS containing 0.5% gelatin supplemented with 0.025% CoCl2 and 0.02% NiNH4SO4.

Drugs and chemicals. Neurobiotin and avidin D-HRP were purchased from Vector Laboratories (Burlingame, CA) and DiI from Molecular Probes (Eugene, OR); all other drugs and chemicals were purchased from Sigma Aldrich.

Statistical analysis. Values are means ± SE. Emax was calculated using GraphPad Prism 5.0 software. Each neuron served as its own control. Student’s t-tests were used to compare the results before and after application of the drug. The χ2 test was used to compare the number of neurons responsive to PP or CCK-8s administration. The level of significance was defined as P < 0.05.

RESULTS

Copper-deficient diet decreases the number of intrapancreatic ganglia. We constructed a structural map of the intrapancreatic innervation of the pancreas. Copper-deficient animals (n = 7) displayed a marked degeneration of the acinar tissue compared with control animals (n = 5; Fig. 1). The total number of intrapancreatic ganglia, as well as the total number of neurons, was significantly reduced in copper-deficient rats compared with control animals (P < 0.05). In contrast, the number of neurons per ganglion was unaffected by copper deficiency. A detailed examination of the morphology of intrapancreatic ganglia revealed that copper deficiency decreased the number of ganglia containing ≥11 neurons (P < 0.05), especially in the tail of the pancreas, whereas the number of ganglia containing <10 neurons was unaffected by the copper-deficient diet (Fig. 1). Data are summarized in Table 1.

These data suggest that copper deficiency specifically reduces the number of large intrapancreatic ganglia. We then carried out a series of experiments to test whether there is a correlation between the involution of the pancreatic acini and responses to stimulation of vagovagal reflexes.

Duodenal infusions of nutrients induce a lower stimulation of PES in copper-deficient animals. In control animals, the baseline volume of PES was 180 ± 17.8 μl/10 min (n = 25). The baseline volume of PES was significantly lower in copper-deficient than control animals (77 ± 7.6 μl/10 min, n = 30, P < 0.05). None of the pharmacological treatments changed the baseline PES volume significantly. Microinjections of PBS into the DVC had no effect on PES in control (203 ± 32.4 and 198 ± 30.2 μg/10 min before and after PBS, respectively, n = 5) or copper-deficient (112 ± 12.1 and 97 ± 3.6 μg/10 min before and after PBS, respectively, n = 3) animals.

In control animals, duodenal infusion of Ensure increased PES from 179 ± 26.3 to 835 ± 289.9 μg protein/10 min (617 ± 262.6% of control, n = 4, P < 0.05). Peak increase was observed 40 min from the start of Ensure infusion, and PES returned to baseline values ~60 min after the end of Ensure infusion. In copper-deficient animals, duodenal infusion of Ensure increased PES from 106 ± 20.8 to 225 ± 47.2 μg protein/10 min (229 ± 58% of control, n = 4, P < 0.05 vs. baseline and P < 0.05 vs. increase in control animals). The increase in PES induced by infusions of Ensure was lower in copper-deficient than control animals, suggesting that the copper-deficient diet attenuates the nutrient-induced increase in PES (Fig. 2).

In a separate group of animals, casein was infused into the duodenum to determine whether the copper-deficient diet affects specifically protein-induced increases in PES. As reported previously (50), in control animals, infusions of casein increased PES from 124 ± 9.5 to 890 ± 33.5 μg protein/10 min (741 ± 67% of control, n = 6, P < 0.05). The peak increase was observed 50 min following the start of casein infusion and returned to baseline 50 min after cessation of the infusion. In animals fed the copper-deficient diet, duodenal infusion of casein had no effect on PES (42 ± 3.6 and 43 ± 4.2 μg protein/10 min before and during casein infusion, respectively, n = 4, P > 0.05), although it increased PES in one of five animals tested (from 122 to 590 μg protein/10 min, 485% of control; Fig. 2). These data suggest that a copper-deficient diet decreases the ability of duodenal protein infusion to stimulate PES.

Microinjection of CCK-8s into the DVC induces a lower increase in PES in copper-deficient animals. The finding that duodenal nutrient infusions had a decreased effect on PES in copper-deficient animals suggests that copper deficiency may alter vagovagal reflexes regulating PES. Thus we microinjected CCK-8s into the DVC to determine alterations in the effenter limb of pancreatic vagovagal reflexes. In control animals, microinjections of 450 pmol of CCK-8s into the DVC increased PES from 163 ± 52.0 to 650 ± 202.0 μg protein/10 min (489 ± 149.9% of baseline, n = 5, P < 0.05), with peak secretion at 10 min following the injection. In copper-deficient animals, microinjections of CCK-8s elicited a significantly lower increase in PES, from 63 ± 14.4 to 117 ± 33.0 μg protein/10 min (218 ± 39.5% of baseline, n = 9, P < 0.05 vs. control increase; Fig. 2).

These data suggest that copper deficiency impairs the efferent vagal output to the pancreas.

Copper-deficient diet decreases TH-IR but does not affect NOS- or ChAT-IR in the DMV. Immunohistochemistry for TH, NOS, and ChAT was carried out to determine whether copper deficiency alters the neurochemical phenotype of a subpopulation of DVC neurons. As reported previously (20, 25, 27), TH-, NOS-, and ChAT-IR neurons were observed throughout the rostrocaudal extent of the DVC. In control animals, an average of 31 ± 2.8, 26 ± 3.4, and 14 ± 4.4 TH-IR neurons per section were observed in the caudal, intermediate, and rostral DMV, respectively (n = 12). In copper-deficient rats (n = 16), the number of TH-IR neurons was reduced significantly in the caudal and intermediate DMV (20 ± 3.0 and 13 ± 2.0 neurons per section, respectively, P < 0.05 for both), but...
Fig. 1. Copper deficiency induces acinar degeneration and loss of large intrapancreatic ganglia. A and E: photomicrographs of pancreata from age-matched 60-day-old control (A) and copper-deficient (E) animals. Note extensive degeneration of acinar tissue in the pancreas from the copper-deficient animal. Scale bars, 5 mm. B and F: schematic drawings of a pancreas from a control (B) and a copper-deficient (F) animal showing distribution of intrapancreatic ganglia. Copper deficiency caused a selective reduction in intrapancreatic ganglia containing \( \geq 11 \) neurons in the tail of the pancreas. Symbols represent intrapancreatic ganglia containing a defined number of neurons: \( \bigcirc \), 1–5 neurons/ganglion; \( \bullet \), 6–10 neurons/ganglion; \( \bigcirc \), 11–15 neurons/ganglion; \( \blacklozenge \), 15–20 neurons/ganglion; \( \star \), >20 neurons/ganglion.

C and G: low-magnification photomicrographs of hematoxylin-eosin-stained pancreata from age-matched 45-day-old control (C) and copper-deficient (G) animals. Note degeneration of acinar tissue in the pancreas from the copper-deficient animal. Scale bars, 5 mm. D and H: high-magnification photomicrographs from pancreata shown in C and G. Note degradation of acinar tissue in the pancreas from the copper-deficient animal (H) compared with intact tissue from the control animal (D). Scale bar, 0.5 mm.

not in the rostral DMV (10 ± 2.9 neurons per section; Fig. 3). In contrast, the number of TH-IR neurons in the nucleus tractus solitarius (NTS) was not different between control (\( n = 4 \)) and copper-deficient (\( n = 6 \)) animals in any area of the NTS (\( P > 0.05 \); Fig. 3).

Immunohistochemistry for NOS showed that, in control animals (\( n = 12 \)), the caudal, intermediate, and rostral DMV contained 19 ± 3.6, 8 ± 2.0, and 17 ± 4.0 NOS-IR neurons per section. In copper-deficient animals (\( n = 10 \)), the number of NOS-IR neurons was not different from that in controls at any rostrocaudal level of the DMV. Similarly, the number of NOS-IR neurons in the NTS was not different between control (\( n = 4 \)) and copper-deficient (\( n = 5 \)) animals (\( P > 0.05 \); Fig. 3).

In control animals (\( n = 4 \)), an average of 31 ± 5.9, 133 ± 13.8, and 67 ± 31.6 neurons per section were ChAT-IR in the caudal, intermediate, and rostral DMV, respectively. The cop-
per-deficient diet did not alter the number of ChAT-IR neurons in the DMV (n = 4; Fig. 3).

These findings demonstrate that copper deficiency reduces the number of TH-IR, but not NOS- or ChAT-IR, neurons in the DMV, indicating a possible alteration of catecholaminergic regulation of PES.

Copper-deficient diet alters some membrane properties of pancreas-projecting DMV neurons. Our in vivo data suggest that copper deficiency may affect the efferent vagal output to the exocrine pancreas. To determine whether these changes are accompanied by alterations in basic electrophysiological and morphological properties of DMV neurons, we compared the properties of identified pancreas-projecting DMV neurons in copper-deficient animals with those in control animals.

The basic electrophysiological characteristics of pancreas-projecting DMV neurons from control (n = 89) and copper-deficient (n = 49) rats are summarized in Table 2. We were able to reconstruct the morphological properties of 44 and 22 DMV neurons from control and copper-deficient rats, respectively. Copper-deficient animals displayed an increased action potential duration and decay time of afterhyperpolarization, as well as a decreased firing frequency, in response to direct current injection (P < 0.05; Table 2).

Values are means ± SE. *P < 0.05 vs. control.
These data suggest that copper deficiency decreases excitability of pancreas-projecting DMV neurons.

Copper-deficient diet alters postsynaptic responses of pancreas-projecting neurons to CCK-8s and PP. To determine whether copper deficiency alters the responses of pancreas-projecting DMV neurons to CCK-8s and PP, we tested the effects of these peptides on postsynaptic responses of DMV neurons. The postsynaptic responses to perfusion with CCK-8s were tested in 164 and 55 neurons from control and copper-deficient rats, respectively. As reported previously (52), in control rats, CCK-8s induced a postsynaptic response in 98 of 164 neurons (i.e., 60%).

Perfusion of the slice with 100 nM CCK-8s increased the firing rate in 25 neurons from 5 ± 2.1 to 94 ± 13.4 action potentials/min (P < 0.05). The CCK-8s-induced increase in the firing rate was concentration-dependent (EC50 = 10.1 ± 0.3 nM, Emax at 100 nM, n = 4 – 8 per concentration). The CCK-8s response did not show tachyphylaxis; the first application increased the firing rate by 52 ± 14.1 action potentials/min, and the second application increased the firing rate by 47 ± 16.6 action potentials/min (n = 10, P > 0.05 vs. the 1st response). Lorglumide blocked the CCK-8s-induced increase in the firing rate in two of six neurons and reduced it from 133.7 ± 27.9 to 23.6 ± 9.7 action potentials/min in the other four neurons (i.e., 21 ± 7.8% of the increase in the absence of lorglumide).

These findings indicate that, as shown previously (52), the CCK-8s-induced depolarization of pancreas-projecting DMV neurons is mediated by CCK-A receptors.
Table 2. Electrophysiological and morphological properties of pancreas-projecting DMV neurons in control and copper-deficient animals

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<td><strong>Electrophysiological</strong></td>
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<td>n</td>
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<td>Input resistance, MΩ</td>
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<td>Action potential duration, ms</td>
<td>2.8 ± 0.1</td>
<td>3.3 ± 0.1*</td>
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<td>Afterhyperpolarization amplitude, mV</td>
<td>18.1 ± 0.9</td>
<td>20.0 ± 0.9</td>
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<td>Action potentials at 30 pA, s⁻¹</td>
<td>10 ± 0.8</td>
<td>8.9 ± 0.9</td>
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<td>Action potentials at 270 pA, s⁻¹</td>
<td>34.1 ± 2.1</td>
<td>29.4 ± 2.3*</td>
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| **Morphological**        |               |                 |
| characteristics          |               |                 |
| n                       | 44            | 22              |
| Dendritic x-plane, μm    | 342 ± 47.6    | 391.2 ± 76.5    |
| Dendritic y-plane, μm    | 168.6 ± 17.21 | 186.2 ± 31.9    |
| Soma area, μm²           | 303 ± 15.6    | 253.3 ± 24.67   |
| Soma diameter, μm        | 26.5 ± 0.8    | 24.1 ± 1.2      |
| Form factor (0 = line, 1 = circle) | 0.7 ± 0.04 | 0.7 ± 0.04     |
| No. of segments          | 7.2 ± 0.5     | 8.7 ± 1.1       |
| Segment length, μm       | 185.9 ± 23.5  | 201.2 ± 22.1    |

Values are means ± SE. DMV, dorsal motor nucleus of the vagus. *P < 0.05 vs. control.

projecting DMV neurons in copper-deficient than control animals (P < 0.05 by χ² test). Perfusion of slices with CCK-8s increased a concentration-dependent depolarization similar to that obtained in control neurons (EC₅₀ = 29.9 ± 0.2 nM, E₉⁰ at 100 nM, n = 12–16 per concentration), with no signs of tachyphylaxis (6.2 ± 1.7 and 7 ± 1.3 mV during the 1st and 2nd CCK-8s applications, respectively, n = 8, P > 0.05); the response was antagonized by pretreatment with lorglumide (7.7 ± 2.4 and 2.8 ± 2.1 mV in CCK-8s and CCK-8s + lorglumide, respectively, n = 4, P < 0.05).

Perfusion of the slice with 100 nM CCK-8s increased the firing rate in 27 neurons from 4.6 ± 3.1 to 137 ± 17.5 action potentials/min. The CCK-8s-induced increase in the firing rate was concentration-dependent (EC₅₀ = 10.3 ± 0.1 nM, E₉⁰ at 100 nM, n = 12). The CCK-8s response did not show tachyphylaxis; the first application of CCK-8s increased the firing rate by 66 ± 11 action potentials/min, and the second application increased the firing rate by 73 ± 41 action potentials/min (n = 7, P > 0.05 vs. the 1st response).

These data demonstrate that copper deficiency reduces the percentage of pancreas-projecting DMV neurons that respond to CCK-8s but does not change the pharmacology of CCK-8s effects.

The postsynaptic responses of identified pancreas-projecting neurons to PP were tested in 13 neurons from control and 74 neurons from copper-deficient rats. In control rats, perfusion of slices with 100 nM PP induced a postsynaptic response in 12 of 13 neurons (92%). PP induced a −10 ± 1.9 mV hyperpolarization in 10 neurons and a 9 ± 0.12 mV depolarization in 2 neurons. In control rats, the concentration-response curve to PP-induced hyperpolarization had an EC₅₀ = 1.2 ± 0.3 nM and E₉⁰ at 100 nM (n = 4–6 per concentration; Fig. 4). Given the limited...
occurrence of PP-induced depolarization, the concentration-response curve was not reconstructed. Perfusion of slices with TTX significantly decreased the magnitude of PP-induced hyperpolarization in five of eight neurons from −13 ± 3.1 to −5 ± 3.0 mV (P < 0.05). In the other three neurons, TTX reversed the effect of PP from −9 ± 2.4 to +3 ± 0.6 mV.

In copper-deficient rats, 42 of 74 neurons (57%, P < 0.05 vs. control by χ² test) responded to PP, and the remaining 32 neurons were unaffected. The concentration-response curve to PP-induced hyperpolarization in copper-deficient animals was shifted to the right compared with control animals, with EC₅₀ = 30 ± 0.1 nM (P < 0.05 vs. control) and Eₘₐₓ at 100 nM in copper-deficient rats (n = 5–10 per concentration; Fig. 4).

In copper-deficient rats, TTX pretreatment reduced the PP-induced hyperpolarization from −6 ± 3 to −2 ± 0.8 mV (n = 3), while in one neuron TTX reversed the effect of PP from −15 to +2.0 mV.

Taken together, these data suggest that a copper-deficient diet decreases the sensitivity of pancreas-projecting neurons to PP via a decrease in the number of responsive neurons and a significant rightward shift in the EC₅₀ curve.

We previously showed that CCK-8s and PP affect the same population of pancreas-projecting DMV neurons (2, 6, 52). To determine whether copper deficiency affects neurons responding to both of these peptides, we tested the effect of CCK-8s and PP in 34 neurons from control and 55 neurons from copper-deficient animals. In control animals, 13 of 34 neurons responded to CCK-8s and PP, 8 of 34 responded only to CCK-8s, 7 responded only to PP, and 6 did not respond to either peptide. In copper-deficient animals, the proportion of cells responding to different combinations of peptides was significantly different from that in control animals (P < 0.05 by χ² test): 27 of 55 neurons responded to both peptides, 9 of 55 responded only to CCK-8s, 3 responded only to PP, and 16 did not respond to either peptide. Further analysis revealed that the percentage of CCK-8s-responsive neurons that also responded to PP was higher in copper-deficient (27 of 36) than control (13 of 21) animals (P < 0.05 by χ² test). Furthermore, more cells that were responsive to PP also responded to CCK-8s (13 of 20 in control and 27 of 30 in copper-deficient animals, P < 0.05 by χ² test). These findings show that although copper deficiency reduces the number of cells that respond to CCK-8s or PP, a higher percentage of cells responded to both peptides.

**Copper-deficient diet does not change presynaptic responses to CCK-8s and PP.** To determine whether copper deficiency changes the effects of CCK-8s and PP on synaptic inputs to pancreas-projecting DMV neurons, we assessed the effects of these peptides on glutamatergic and GABAAergic inputs.

In control animals, baseline frequency of spontaneous EPSC (sEPSC) was 4.7 ± 0.7 events/s and baseline amplitude was 30.1 ± 2.3 pA. Perfusion with 100 nM CCK-8s increased sEPSC frequency from 4.3 ± 1.1 to 8.7 ± 2.1 events/s (209 ± 55% of control) in 8 of 20 neurons but did not affect the amplitude (28 ± 7.8 and 29 ± 6.7 pA before and during CCK-8s, respectively; Fig. 5). Baseline sEPSC frequency was significantly lower in copper-deficient rats than in control animals (2.3 ± 0.3 events/s, P < 0.05), whereas sEPSC amplitude in copper-deficient rats was not different from that in control animals (26.4 ± 1.1 pA). Perfusion with CCK-8s increased sEPSC frequency from 1.9 ± 0.4 to 4.5 ± 0.7 events/s (352 ± 65%, P < 0.05) in 16 of 36 neurons. These data suggest that copper deficiency did not affect the number of cells in which CCK-8s induced an increase in sEPSC frequency (P < 0.05 by χ² test) or the magnitude of the increase in sEPSC frequency (P > 0.05; Fig. 5).

In control animals, baseline spontaneous IPSC (sIPSC) frequency was 2.3 ± 0.4 events/s and baseline amplitude was 55.6 ± 6.7 pA. Perfusion with 100 nM CCK-8s increased sIPSC frequency in 9 of 18 neurons from 2 ± 0.5 to 5.5 ± 3.2 events/s (P < 0.05; Fig. 5). In eight of the nine neurons, perfusion with CCK-8s did not affect the sIPSCs amplitude (42.4 ± 8.3 and 43.3 ± 8.2 pA before and after CCK-8s perfusion, respectively, P > 0.05). In copper-deficient animals, neither the baseline sIPSC frequency (2.3 ± 0.4 events/s) nor the sIPSC amplitude (52.1 ± 4.2 pA) was different from that in control animals (P > 0.05). Perfusion with CCK-8s increased sIPSC frequency from 1.03 ± 0.23 to 1.86 ± 0.5 events/s (187 ± 62% of control) in 12 of 34 neurons but did not affect the amplitude (50 ± 5.8 and 46 ± 4.7 pA in control and CCK-8s, respectively, P > 0.05).

These data indicate that copper deficiency does not affect the ability of CCK-8s to affect synaptic transmission onto pancreas-projecting neurons.

As shown previously (6), perfusion with 100 nM PP decreased sEPSC frequency in 3 of 11 neurons from 4.9 ± 1.2 to 2.2 ± 0.7 events/s and had no effect in the remaining 8 neurons. In copper-deficient animals, perfusion with 100 nM PP decreased sEPSC frequency from 5.2 ± 1.5 to 2 ± 0.58 events/s in 11 of 25 neurons. PP had no effect on sEPSC amplitude in control or copper-deficient animals (P > 0.05).

In control animals, perfusion with 100 nM PP decreased sIPSC frequency from 4.8 ± 1.8 to 2 ± 1.0 events/s in 4 of 9 neurons but did not affect their amplitude (80 ± 13.6 and 75 ± 12.2 pA before and during PP perfusion, respectively, P > 0.05). In copper-deficient animals, perfusion with 100 nM PP decreased sIPSC frequency from 3.6 ± 0.8 to 1.6 ± 0.4 events/s in 8 of 18 neurons (P < 0.05). PP also decreased the sIPSC amplitude from 61 ± 6.7 to 23 ± 1.9 pA in three responsive neurons (P < 0.05). Neither the number of cells in which PP decreased the frequency of sEPSCs or sIPSCs nor the magnitude of the increase was different between copper-deficient and control animals (P > 0.05; data not shown).

These data suggest that copper deficiency does not change synaptic responses of pancreas-projecting DMV neurons to PP.

**DISCUSSION**

In this study, we report that, in rats fed a copper-deficient diet, 1) the number of large intrapancreatic ganglia is reduced, 2) the increase in PES induced by intraduodenal infusion of nutrients is attenuated, 3) the increase in PES obtained upon CCK-8s microinjection into the DVC is attenuated, 4) the number of TH-IR, but not NOS-IR, neurons in the DMV is decreased, and 5) the responsiveness of vagal motoneurons to exogenously applied CCK-8s and PP is reduced but synaptic transmission to these neurons is not changed. These findings suggest that, in copper-deficient animals, there is a diminished exocrine pancreatic function that results from the combination of alterations in pancreatic acinar tissue, the decreased number of intrapancreatic ganglia, and the reduced responsiveness of pancreas-projecting vagal neural pathways. These impair-
ments, however, do not appear to affect the synaptic inputs onto vagal motoneurons.

PES is regulated by neural and hormonal mechanisms (2, 24, 44). Under normal conditions, ingestion of nutrients activates vagovagal reflexes, i.e., activates vagal afferent fibers, which synapse onto second-order neurons in the NTS. NTS neurons integrate this sensory information and transmit these signals, including the stimulation of the exocrine pancreas to produce enzymes required for digestion, to the DMV, which provides motor output to the upper GI tract (47). In addition, microinjection of the GABA<sub>A</sub> receptor antagonist bicuculline, CCK-8s, or group II metabotropic glutamate receptor agonists into the DMV increases, whereas microinjection of PP or somatostatin decreases, PES (26, 33, 35, 50), demonstrating that these peptides affect PES by a direct effect on DMV neurons. In this study, we show that copper deficiency alters acinar tissue, as well as the neural pathways regulating PES.

In this study, we used duodenal infusions of Ensure or casein to stimulate PES. Our data show that copper deficiency attenuates the nutrient-induced increase in PES. This finding suggests that copper deficiency results in decreased vagovagal reflexes via a decreased sensitivity of vagal afferents to in-

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**Fig. 5.** Copper-deficient diet does not alter presynaptic responses of pancreas-projecting DMV neurons to CCK-8s. A and B: representative traces showing CCK-8s-induced increase in spontaneous inhibitory postsynaptic current (sIPSC) frequency in a control (A) and a copper-deficient (B) animal. C: CCK-8s-induced increase in spontaneous excitatory postsynaptic current (sEPSC) frequency in control (n = 8 of 20) and copper-deficient (n = 16 of 36) animals. In both instances, perfusion with CCK-8s increased sEPSC frequency. D: CCK-8s-induced increase in sIPSC frequency in control (n = 9 of 18) and copper-deficient (n = 12 of 34) animals. In both instances, perfusion with CCK-8s increased sIPSC frequency. E and F: percentage of pancreas-projecting neurons in which CCK-8s induced an increase in sEPSC (E) and sIPSC (F) frequency. *P < 0.05.
trudodenal nutrients, a reduction of motor output to the exocrine pancreas, or a decreased response of postganglionic neurons innervating the pancreas. Together, our observations demonstrate that, in copper-deficient animals, 1) DVC micro-injections of CCK-8s result in an attenuated increase in PES and 2) there is a loss of large intrapancreatic ganglia compared with controls. Electrophysiological experiments on identified pancreas-projecting DMV neurons show that these neurons are less responsive to CCK-8s or PP. Therefore, our data support the suggestion that copper deficiency also decreases the neural motor output to the pancreas.

In addition, our electrophysiological data indicate that copper deficiency alters the membrane properties of identified pancreas-projecting neurons by prolonging the action potential afterhyperpolarization and making the neurons less responsive to direct current stimulation. The possibility that copper deficiency also affects postganglionic mechanisms is supported by the observation that copper deficiency decreases the number of large intrapancreatic ganglia, affecting in particular the tail portion of the pancreas, i.e., the pancreatic region with the lowest density of vagal innervation.

The finding that copper deficiency reduced the number of TH-IR neurons in the DMV and the intrapancreatic ganglia is consistent with previous reports, which have demonstrated that animal models of copper deficiency, as well as Menkes disease, in humans result in neurodegeneration, abnormalities in myelination, and synaptogenesis (12, 23, 37, 39, 42, 48, 59). To the best of our knowledge, our study is the first to demonstrate that a copper-deficient diet also results in impairment of parasympathetic preganglionic neurons regulating PES. Furthermore, the results of our study suggest that copper deficiency does not result in an overall loss of neurons but, rather, affects specific neuronal populations. This suggestion is supported by our data showing a decrease in the number of TH-IR, but not NOS-IR, neurons in the DMV, as well as a reduction in the number of large intrapancreatic ganglia in copper-deficient animals. A reduction in the number of TH-positive neurons was expected, since copper is a cofactor for dopamine β-hydroxylase (17). It has been shown previously that copper deficiency reduces catecholamine levels in a region-specific manner, with a >50% decrease in medullary regions (37). The presence of NO in vagal brain stem neurocircuits is well established, as is its role in the modulation of gastric motility (25, 36, 45, 58). However, no information about the functions of NO in central pancreas-controlling vagal neurocircuits has been provided, although our data seem to indicate that nitrergic neurons in the DMV do not play a relevant role in PES modulation.

In this study, we use an animal model of copper deficiency to demonstrate that selective loss of the exocrine pancreas without damage to the endocrine pancreas induces changes in vagal pathways regulating PES. In particular, we show that responsiveness of pancreas-projecting neurons to PP and CCK-8s, peptides that selectively regulate the exocrine pancreatic functions (2, 52, 53), is diminished in animals fed a copper-deficient diet. Vagal pathways regulating upper GI functions exhibit a great deal of plasticity and can be modulated by various hormones and neurotransmitters and changing physiological conditions (reviewed recently in Ref. 8). The present study extends these observations by demonstrating for the first time that a rearrangement of effenter pathways can be induced by the atrophy of the target organ, as indicated by the reduced responses to exogenously applied CCK-8s and PP. Although the reason for different susceptibility of distinct neuronal populations to copper deficiency has not been investigated, it could reflect differences in their embryological origin, function, or neurochemical composition. As mentioned earlier, we demonstrated previously that pancreas-projecting neurons in the DMV can be subdivided into separate populations on the basis of their responses to PP, CCK-8s, GLP-1, and metabotropic glutamate receptor agonists (2, 51–53). Similarly, neurons in the intrapancreatic ganglia display diverse properties and neurochemical phenotypes, as well as diverse ganglionic localization, possibly reflecting diverse postganglionic neural circuits controlling the different pancreatic functions (reviewed in Refs. 28, 44, and 55). Furthermore, pharmacological differences in intrapancreatic ganglia should also be considered; for example, norepinephrine inhibits excitatory synaptic transmissions via actions on α2-adrenergic receptors in ganglia located in the head of the pancreas, whereas norepinephrine facilitates excitatory transmission via actions on α1-adrenergic receptors in ganglia located in the tail of the pancreas (56, 57). Interestingly, we observed a reduced number of large pancreatic ganglia primarily in the tail of the pancreas, the region with a lower density of vagal innervation. Although this finding may suggest that vagal innervation may protect intrapancreatic ganglia from copper deficiency-induced degeneration or prevent an overstimulation due to α1-adrenergic receptor activation, this possibility has not been investigated. Alternatively, differences in vulnerability of some neurons to copper deficiency may reflect differences in their metabolic activity, since it has been suggested that copper deficiency leads to neurodegeneration via mitochondrial inhibition (21, 22).

In summary, this study has demonstrated that copper deficiency results in degeneration of vagal and intrapancreatic pathways that regulate PES. Our results show that, in addition to the loss of pancreatic acinar tissue, deficiency of exocrine pancreatic function in copper deficiency is also partly due to the impairment of pre- and postganglionic neurons innervating the pancreas. Loss of vagal innervation of the exocrine pancreas, combined with the decrease of acinar tissue, likely exacerbates the loss of pancreatic function induced by copper deficiency.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.B., K.N.B., and R.A.T. are responsible for conception and design of the research; T.B., R.B., S.W., K.N.B., M.S., S.R.F., and R.A.T. performed the experiments; T.B., R.B., S.W., K.N.B., M.S., and R.A.T. analyzed the data; T.B., K.N.B., M.S., and R.A.T. interpreted the results of the experiments; T.B., R.B., S.R.F., and R.A.T. prepared the figures; T.B., R.B., K.N.B., and R.A.T. drafted the manuscript; T.B., K.N.B., and R.A.T. edited and revised the manuscript; T.B., R.B., S.W., K.N.B., M.S., S.R.F., and R.A.T. approved the final version of the manuscript.
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