Published in final edited form as:

Neurogastroenterol Motil. 2011 February ; 23(2): e69–e79. doi:10.1111/j.1365-2982.2010.01616.x.

Experimental spinal cord injury in rats diminishes vagally-mediated gastric responses to cholecystokinin-8s

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Abstract

Background—We have shown recently that our model of experimental high-thoracic spinal cord injury (T3-SCI) mirrors the gastrointestinal clinical presentation of neurotrauma patients, whereby T3-SCI animals show diminished gastric emptying and dysmotility. In the present study we used cholecystokinin as a model peptide to test the hypothesis that the T3-SCI induced gastroparesis is due, in part, to an impaired vagally-mediated response to gastrointestinal peptides.

Methods—We measured the responses to sulfated cholecystokinin (CCK-8s) in control and T3-SCI (3 or 21 days after injury) rats utilizing: 1) *c-fos* expression in the nucleus tractus solitarius (NTS) following peripherally administered CCK-8s; 2) *in vivo* gastric tone and motility following unilateral microinjection of CCK-8s into the dorsal vagal complex (DVC); and, 3) whole cell recordings of glutamatergic synaptic inputs to NTS neurons.

Results—Our results show that: 1) medullary *c-fos* expression in response to peripheral CCK-8s was significantly lower in T3-SCI rats 3 days after the injury, but recovered to control values at 3 weeks post SCI. 2) Unilateral microinjection of CCK-8s in the DVC induced a profound gastric relaxation in control animals, but did not induce any response in T3-SCI rats at both 3 and 21 days after SCI. 3) Perfusion with CCK-8s increased glutamatergic currents in 55% of NTS neurons from control rats, but failed to induce any response in NTS neurons from T3-SCI rats.

Conclusions—Our data indicate alterations of vagal responses to CCK-8s in T3-SCI rats that may reflect a generalized impairment of gastric vagal neurocircuitry, leading to a reduction of gastric functions after SCI.

Keywords

vago-vagal reflexes; brainstem; gastrointestinal motility; gastrointestinal transit

The CNS integration of gastric sensory and motor activity occurs within brainstem vagal circuits of the dorsal vagal complex (DVC), which comprises the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMV) and the area postrema (AP)¹. The integrative activity that occurs within the DVC nuclei is the result of inputs originating from higher central nervous system (CNS) areas ²⁻⁴, from spinosolitary inputs ⁵⁻⁷ as well as from

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Conflicts of interest

The authors disclose no conflicts

neurohormonal signals from the periphery. All of these signals finely tune the coordinated emptying of nutrients from the stomach.

One of the most studied peptides controlling gastric and digestive functions is cholecystokinin (CCK), which is released from the proximal small intestine in response to fat or protein content of a meal⁸. CCK release has profound effects on gastrointestinal functions⁹⁻¹¹, its vagal mechanisms of action include a paracrine activation of vagal afferent fibers along the gut wall (reviewed in¹²) as well as direct actions upon nodose ganglion^{13,14}, and DVC neurons¹⁵⁻¹⁸.

The most visible consequence of spinal cord injury (SCI) is the loss of sensory and motor function, however, SCI has also severe gastrointestinal (GI) consequences. Dysphagia, delayed gastrointestinal transit, gastric dilatation, nausea and emesis are in fact observed immediately after SCI^{19,20} and may persist for years after the initial injury²¹⁻²⁶. Each of these co-morbidities diminishes the long-term quality of life and nutritional status in SCI patients²⁷.

We and others have shown recently that models of experimental high-thoracic spinal cord injury (T3-SCI) mirror the GI clinical presentation of neurotrauma patients, indeed we reported that T3-SCI animals show a diminished food intake and gastroparesis^{28,29}, while other groups have also shown inhibition of gastric emptying and duodenal transit of liquids³⁰⁻³³. We have also shown a significant reduction in gastric motility and a diminished esophageal-gastric relaxation reflex in anesthetized T3-SCI rats 3 days after injury²⁸. We further demonstrated that the post-SCI dysmotility lasts as long as 6 weeks after injury and is accompanied by a delay in gastric emptying of a ¹³C-tagged solid meal²⁹. Based upon anatomical⁵⁻⁷ and physiological³⁴ evidence of afferent input to the NTS from the spinal cord, we originally hypothesized that T3-SCI interrupted ascending spinosolitary input to brainstem vagal nuclei thereby altering the sensitivity of vagal reflex function²⁸.

In the present work we used CCK as a model peptide with the aim to test the hypothesis that the T3-SCI induced gastroparesis is due, in part, to a direct impairment of vagally-mediated responses to gastrointestinal peptides.

Materials and Methods

All experiments were conducted on male Wistar rats ($n = 65$; Harlan, Indianapolis, IN). Rats were ≥ 8 weeks of age upon entrance into the experiment and were double housed in a room maintained at 21-24°C on a 12:12-hours light-dark cycle and food and water *ad libitum*. After surgery, animals were housed singly and observed daily. All procedures were performed according to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

Spinal Cord Injury Surgery and Post-operative Care

Animals were anesthetized with a 5% solution of Isoflurane with oxygen (400-600ml·min⁻¹) in accordance with the Animal Care and Use Committee Guidelines. Spinal cord injury and post-injury care was performed as described previously²⁸. Afterward, body and chow weights were recorded daily for all animals. Mean energy intake (MEI) was calculated by dividing the daily average of kcal consumed·100g⁻¹ of body weight for each 2 day period of monitored feeding.

Behavioral analysis—To assess the extent of contusion injury, showing deficits and partial recovery of hind limb movement, we performed the locomotor testing according to

Basso, Beattie, Bresnahan (BBB) locomotor rating scale (³⁵), assigning values ranging from 0 (no hindlimb movement) to 21 (normal locomotion).

In vivo studies—Following an overnight fast (water ad libitum), animals were deeply anesthetized with thiobutabarbital (Inactin®; 100–150 mg·kg⁻¹ IP) and administered dexamethasone® (1 mg·kg⁻¹ sc) to prevent cerebral edema. The trachea was intubated and a jugular catheter was inserted for intravenous delivery of drugs. The stomach was isolated and placed upon a moisturized gauze pad, an encapsulated miniature strain gauge (RB Products, Minneapolis, MN) was aligned with the circular smooth muscle fibers and sutured to the gastric corpus before closing the abdominal incision. Animals were placed in a stereotaxic frame and maintained at 37±1 °C using a feedback-controlled warming pad. The fourth ventricle and the vagal trigone area were surgically exposed. After 1 hour recovery period, a glass micropipette (30–40µm tip diameter) was directed into the left dorsal vagal complex (DVC) with the aid of a micromanipulator at the following co-ordinates: 0.1–0.3mm lateral from midline, 0.1–0.3mm rostral to calamus scriptorius and 0.5–0.7mm dorsoventral to the surface. Sixty nanoliters of phosphate buffer solution (PBS, in mM: 147.6 NaCl, 83.3 NaH₂PO₄, 12.9 KH₂PO₄) or CCK-8s (450 pmol dissolved in PBS) were microinjected using a picospritzer (Toohey, Pressure System Ile, Fairfield NJ).

Baseline gastric motility and tone were monitored continuously on a polygraph (model 79, Grass, Quincy, MA) and the effects of each treatment were compared to the average of the 2–5 minutes preceding the microinjection. A subset of rats, 20–30 minutes after the first injection of CCK-8s, received intravenous bethanechol (50 µg·kg⁻¹ bolus followed by continuous intravenous infusion 20 µg·kg⁻¹·h⁻¹ for 20 min) to increase baseline gastric motility. A second microinjection of CCK-8s into the DVC occurred after the bethanechol effects stabilized, usually within 20–30 minutes.

Histological Processing—At the conclusion of the experiment, rats were transcardially perfused with a bolus of lidocaine and heparin, PBS and PBS with 4% paraformaldehyde. The brainstem and spinal cord at the lesion level were removed and refrigerated overnight in PBS containing 20% sucrose and 4% paraformaldehyde. Alternate coronal slices (40µm thick) were processed (see below), captured using a digital camera attached to a Nikon Labophot-2 Microscope and imported into Adobe Photoshop CS2 for analysis.

Luxol Fast Blue and Cresyl Violet Staining—Spinal cord sections were stained with luxol fast blue (LFB) to show myelinated fibers and brainstem sections were stained with cresyl violet to verify the microinjection site. Sections were cleared, coverslipped and analysed. The proximity of the T3 lesion center to the cervical enlargement precluded an appropriate determination of spinal cord cross-sectional area in tissue rostral to the injury. LFB-stained myelin in injured tissue was then expressed as a percent of the total cross-sectional area as would be predicted by the intact tissue.

Histological Processing for c-Fos Protein—Following an overnight fast (water ad libitum), animals received CCK-8s (2µg·kg⁻¹ IP) and returned to the home cage. After 90min, each rat was deeply anesthetized and immediately perfused. The brainstem and spinal cord were removed as described above. After overnight fixation, free floating sections were processed for c-Fos immunohistochemistry using a standard protocol ¹⁶. Sections were rinsed in PBS, mounted on glass slides, dried overnight, cleared and cover slipped.

Electrophysiology—The methods of preparing the brainstem slices and the identification of NTS neurons have been described previously ^{36,37}.

Whole cell recordings of spontaneous glutamatergic excitatory postsynaptic currents (EPSC) were conducted on NTS neurons located dorsal to the vagal motoneurons and medial to the tractus solitarius at levels spanning from the posterior tip of the area postrema to approximately 0.5mm rostral to its anterior portion.

Data were sampled at 10kHz and filtered at 2kHz, digitized via a Digidata 1322 interface, acquired with a PC utilizing pClamp9 software (Molecular Devices) and analyzed with Mini Analysis software (Jaeger Software, Leonia, NJ).

Drugs were applied to the bath *via* a series of manually operated valves. Cells were classified as responsive if perfusion with CCK-8s (100nM) modified the frequency of spontaneous excitatory postsynaptic currents (sEPSC) by a minimum of 50% from baseline (measured as the average frequency during 1min of recording in control conditions *vs.* 30s of recording centered on peak drug response)

Drugs and chemicals—c-Fos antibody was purchased from EMD Biosciences/Calbiochem (San Diego, CA); Biotinylated goat anti-rabbit IgG from Jackson ImmunoResearch (West grove, PA); Vectastain Elite ABC Kit from Vector Labs (Burlingame, CA); DAB substrate from Pierce/Thermoscientific (Rockford, IL). Inactin®, CCK-8s and all other salts were purchased from Sigma (St. Louis, MO).

Statistical Analysis—Results are expressed as means $\pm$ S.E.M. with significance defined as $P < 0.05$. Between group results from *in vivo* studies were compared by one-way ANOVA and tukey *post hoc* analysis or paired *t*-test as appropriate (e.g., locomotor score *vs.* c-Fos count, respectively). Results from *in vitro* studies were compared before and after drug administration, with each neuron serving as its own control (Student's paired *t*-test). Intergroup comparisons were conducted using the Student's grouped *t*-test or the χ^2 test.

Results

Histological and Behavioral Assessments of Control and T3-SCI Rats

Locomotion scores collected at 3 days post-injury were 21 ± 0.0 and 0.3 ± 0.2 for control (N=32) and T3-SCI (N=33) rats, respectively ($p < 0.05$). In those animals surviving until 21 days post-injury, locomotion score were 21 ± 0.0 and 8.5 ± 0.2 for controls (N=10) and T3-SCI rats (N=10) ($p < 0.05$). This indicates that control animals displayed normal locomotion, while T3-SCI rats exhibited no- or slight movement at one hind limb joint at 3 days post-injury, but 3 weeks after SCI rats were capable of sweeping hind limb movements with no weight support or coordination.

On the third day after surgery, the body weight was $100 \pm 1\%$ and $92 \pm 1\%$ of pre-surgery weight for control and T3-SCI animals, respectively ($p < 0.05$). At this same timepoint, the mean energy intake (MEI) was 29 ± 1 and 12 ± 1 kcal·day⁻¹·100g body weight⁻¹ for control and T3-SCI, respectively ($p < 0.05$). By the third week after injury, body weight of control animals increased to $124 \pm 6\%$ of the pre-surgery weight, while the body weight of T3-SCI animals was $108 \pm 3\%$ of the pre-surgery weight ($p < 0.05$) despite the fact that MEI was similar across groups ($p > 0.05$).

Three days after injury, the total cross-sectional area of the thoracic spinal cord was similar in control and T3-SCI rats, however, only $13 \pm 2\%$ of the controls' white matter remained in T3-SCI rats (Fig. 1; $p < 0.05$). Three weeks after injury, the total cross-sectional area of the thoracic spinal cord was $59 \pm 4\%$ of control and the remaining white matter was $5 \pm 2\%$ of control (Fig. 1; $p < 0.05$ *vs* both control and T3-SCI 3 days after injury).

Taken together, our histological, locomotor, feeding and body weight results verify the consistency and effectiveness of our surgical procedures for inclusion of all T3-SCI animals in data analysis.

IP Injection of CCK-8s increases *c-fos* expression in the NTS of control but not T3-SCI rats

Following an overnight fast, intraperitoneal administration of 2 μ g/kg IP CCK-8s 3days following surgery resulted in *c-fos*-positive neurons throughout the caudal, intermediate and rostral NTS. Specifically, control rats (N=5) had 272 $\pm$ 35 and T3-SCI rats (N=5) had 77 $\pm$ 30 *c-fos*-positive NTS neurons/section ($p<0.05$). By contrast, the number of *c-fos*-positive neurons in the AP was not significantly different between control and SCI rats (83 $\pm$ 9 vs. 95 $\pm$ 6 neurons/section, respectively, $p<0.05$; Fig. 2). Three weeks after injury, the number *c-fos*-positive neurons throughout the NTS or the AP in response to 2 μ g/kg CCK-8s was the same in both control and T3-SCI rats (data not shown; $p>0.05$).

These data indicate that 3days after surgery NTS neurons, and by consequence the vagal circuits regulating gastric reflex function, are insensitive to modulation by peripheral CCK-8s. Our data also suggest that three weeks after injury the response of either vagal afferent fibers and/or vagal medullary circuits have recovered their function.

Microinjection of CCK-8s in the DVC induced gastric relaxation in control but not in T3-SCI rats

Microinjection of PBS in the left DVC did not induce any significant change in gastric tone in either control (n=5) or T3-SCI rats (n=5; $p>0.05$).

Three days after surgery, microinjection of CCK-8s in the left DVC decreased gastric tone by 0.33 $\pm$ 0.01 and 0.09 $\pm$ 0.05g in control (N=6) and T3-SCI (N=7) rats, respectively ($p<0.05$). Following microinjection, gastric tone returned to baseline values within 9.7 $\pm$ 2.2 and 3.7 $\pm$ 1.9 min in control and T3-SCI rats, respectively ($p<0.05$; Fig. 3).

To test whether the reduced effects of CCK-8s in T3-SCI rats were due to the decreased baseline tone of T3-SCI rats, in a different group of rats we increased gastric tone and motility via i.v. bethanechol (50 μ g/kg bolus followed by iv infusion with 20 μ g/kg/hr for 20 min). Microinjection of CCK-8s prior to administration of bethanechol decreased gastric tone by 0.21 $\pm$ 0.02 and 0.11 $\pm$ 0.02g, in control and T3-SCI rats, respectively (N=5 for both groups; $p<0.05$). Intravenous bethanechol increased basal gastric tone in both control and T3-SCI rats (0.75 $\pm$ 0.09g vs. 0.63 $\pm$ 0.09g, respectively; $p>0.05$). Upon stabilization of gastric motility and tone, CCK-8s was microinjected a second time while still perfusing the rat with bethanechol. In control animals, CCK-8s induced a 0.34 $\pm$ 0.05g relaxation ($p<0.05$ vs the pre-bethanechol relaxation), however, in T3-SCI rats CCK-8s induced a 0.13 $\pm$ 0.06g relaxation which was significantly lower than bethanechol-treated control ($p<0.05$) but did not significantly differ from the CCK-8s response obtained pre-bethanechol (Fig. 4; $p>0.05$).

The results obtained in T3-SCI rats 21days after injury were similar to the data obtained in T3-SCI rats three days after surgery. Data are summarized in Table 1.

Our results indicate a long-lasting depression of vagal responsiveness to exogenous CCK-8s in T3-SCI animals. The similar responsiveness to systemic bethanechol, at all time points tested in both control and T3-SCI rats, suggests that the cholinergic postganglionic drive of gastric tone remains patent in both acute and chronic T3-SCI animals.

CCK-8s increased sEPSC frequency of cNTS neurons in control but not in T3-SCI rats

Brainstem slices obtained from 5 control and 7 T3-SCI rats were used for *in vitro* study. There were no significant changes in basic membrane properties (i.e. input resistance, amplitude or duration of the afterhyperpolarization or action potential duration) between cNTS neurons from control or T3-SCI rats (data not shown).

Perfusion with CCK-8s increased the baseline frequency of sEPSCs from 1.5 ± 0.6 to 4.2 ± 1.0 events s^{-1} in 12 out of 22 cNTS neurons from control rats (Fig. 5, $p < 0.05$) leaving the amplitude of the sEPSC unaffected ($107 \pm 5\%$ of baseline amplitude; $p > 0.05$). Conversely, perfusion with CCK-8s did not alter the frequency (1.34 ± 0.29 and 1.37 ± 0.28 events s^{-1} in Krebs' and in CCK-8s, respectively; $p > 0.05$) nor the amplitude (27.5 ± 1.20 and 27.3 ± 0.88 pA in Krebs' and in CCK-8s, respectively; $p > 0.05$) in 10 out of 11 cNTS neurons from T3-SCI rats three days after injury; and, increased the frequency from 0.9 ± 0.6 to 1.9 ± 0.9 events s^{-1} in four of the nine neurons tested seven days after injury.

The unaffected amplitude, but increased frequency response of the sEPSC in NTS neurons from control animals following perfusion of CCK-8s suggests a pre-synaptic site of action. To demonstrate the viability of the pre-synaptic terminals in T3-SCI rats, we perfused the CCK-8s unresponsive neurons with 4-aminopyridine (4-AP, 5mM). Following application of 4-AP, 100% of the previously unresponsive neurons responded and the sEPSC frequency increased by $3371 \pm 933\%$, indicating that the synaptic actions of CCK-8s were ablated in T3-SCI rats because of alterations in the vagal circuitry rather than a general absence of neurotransmitter release from nerve terminals.

Discussion

In the present study, we have shown that the T3-SCI induced gastroparesis may be due, in part, to an impaired vagally-mediated response to GI peptides. Our experimental data indicate that: 1) Administration of peripheral CCK-8s in T3-SCI rats three days after injury induced less NTS *c-fos* expression than in control rats, however, *c-fos* expression within the AP was similar in both groups; 2) Administration of peripheral CCK-8s in T3-SCI rats three weeks after injury induced similar *c-fos* expression in T3-SCI and control rats; 3) Microinjection of CCK-8s in the DVC induced a profound gastric relaxation in control but not in T3-SCI rats; 4) the reduced gastroinhibitory action of CCK-8s in the DVC persists at three weeks after injury; 5) Perfusion of CCK-8s increased glutamatergic synaptic inputs to NTS neurons of control but not T3-SCI rats, although a partial recovery was observed in neurons from T3-SCI rats one week after injury.

Spinal transection and contusion injury models are routinely employed to study SCI³⁸. The later models are often preferred in that spinal transection does not accurately model the most common pattern of human SCI, whereby the spinal cord is contused rather than transected. However, the severity of spinal contusion injury selected for these experiments is anatomically quite similar to complete spinal cord transection in that very few luxol fast blue-labeled fibers are observed traversing the perimeter of the T3 SCI lesion epicenter. Our previous studies utilizing both contusion SCI and transection reveal only modest differences in the magnitude and duration of post-SCI gastroparesis²⁹. Therefore, we conclude that the severity of our contusion model is neurophysiologically similar to spinal transection and clinically complete contusion injury.

Our current studies did not focus upon the effects of SCI and the response of hypothalamic nuclei to CCK-8s. At present, reduction in caloric intake after SCI serves as a functional outcome measure to demonstrate consistency of our model with previous studies²⁷. However, we acknowledge that the NTS is pivotal for integrating signals from both viscera

and higher CNS areas, and the effect of post-injury changes in the neural signaling between the hypothalamus and the brainstem may prove critical to our complete understanding of the derangements in caloric intake and digestive processes after SCI.

Our proposal that vagally-mediated responses to GI peptides are impaired after SCI is based upon the following evidence. It is well accepted that peripheral CCK-8s activates C-type vagal afferent fibers and increases *c-fos* immunoreactivity in NTS cells of neurally intact animals³⁹⁻⁴¹. Previous experimental studies have demonstrated that reducing NTS afferent input with vagotomy⁴² or capsaicin⁴³⁻⁴⁵ equally reduces, though not completely eliminates, *c-fos* expression which suggests both a peripheral and a direct action at the level of brainstem vagal circuits. In the present study, peripheral CCK-8s increased the number of *c-fos* expressing NTS cells in food-deprived control rats significantly more than in T3-SCI rats 3 days after injury. In contrast, the numbers of *c-fos* positive cells in the AP from these same 3d T3-SCI animals were not different from control animals. This data suggests that following acute T3-SCI, NTS neurocircuitry is insensitive to peripheral CCK-8s.

Recent evidence further supports the hypothesis that the gastrointestinal effects of CCK-8s include direct actions at the level of brainstem vagal circuits^{18,46-48} including vagal afferent fibers that synapse on NTS neurons as well as at the level of subpopulations of NTS and DMV neurons^{15-17,49-52}. We have previously demonstrated that central administration of CCK-8s to the DVC induces gastric relaxation. Specifically, the gastroinhibitory effect of 450 pmol CCK-8s microinjection into the DVC was eliminated by vagotomy and systemic pretreatment with the nitric oxide synthase inhibitor L-NAME¹⁸. These data demonstrated that the effects of central CCK-8s microinjection are vagally-mediated and include a postganglionic NANC pathway. In the present study, we confirm that central NTS neurocircuitry is insensitive to 450 pmol CCK-8s microinjections into the DVC following SCI. Our data clearly show that DVC microinjection of CCK-8s fails to elicit an appropriate gastric relaxation in 3d T3-SCI rats and that insensitivity to CCK-8s persists in animals that survive 3w post-injury. These data coincide with the period of gastric dysmotility we have reported previously for T3-SCI²⁹.

Vagally mediated gastroinhibition is accomplished by either a withdrawal of cholinergic tone or an increase in NANC activity, much of which is mediated by the release of nitric oxide (NO) (reviewed by Travagli, et al.¹). In the present study, the profound absence of gastric motility in the T3-SCI preparation could indicate a “floor-effect” in the presence of maximal nitrergic inhibition. As such, DVC microinjection of CCK-8s would fail to induce any further detectable gastric relaxation. Neuronal nitric oxide synthase (NOS) and VIP are suppressed following SCI³³, however, *in vitro* membrane hyperpolarizations recorded in antral smooth muscle preparations responded similarly to NO donors in control and SCI rats, suggesting that SCI did not impair the capacity to respond to NO-mediated inhibitory signals.

Intravenous administration of the nonselective cholinergic muscarinic agonist bethanecol maximally activates gastric tone and motility and provides a pharmacological “pre-load” upon which we can detect the CCK-8s mediated gastric relaxation. Our demonstration of similar elevation in gastric tone of both control and T3-SCI rats confirms that the muscarinic receptors on the gastric smooth muscle remain functional following T3-SCI. However, DVC microinjection of CCK-8s continued to fail to elicit an appropriate gastric relaxation in the presence of bethanecol. This suggests that therapeutic targeting of peripheral receptors may promote motility without effectively restoring the total GI reflex function of the SCI patient.

Further confirmation that NTS neurocircuitry is insensitive to CCK-8s after SCI is provided by our *in vitro* whole cell recordings from the NTS showing a presynaptic action of CCK-8s

in controls^{15,16} but not in T3-SCI rats. Administration of 4-aminopyridine (4-AP), which increases synaptic transmission via the blockade of potassium conductances and subsequent neuronal depolarization (reviewed in ⁵³) verified the viability of presynaptic nerve terminals in T3-SCI rats and provided electrophysiological support to the immunohistochemical and *in vivo* observations that CCK-8s signal does not fully reach the NTS neuron. The lack of response of NTS neurons to CCK-8s perfusion likely results in an impairment of the synaptic inputs to DMV neurons and, by consequence, the vagal motor output to the stomach.

Ultimately, the metabolic state of the organism initiates or modulates a complex interaction of neuropeptides and neurotransmitters acting upon vagal afferents, thereby activating vago-vagal gastrointestinal reflexes. It is known that feeding modulates vagal afferent expression of cannabinoid ⁵⁴, melananin-concentrating hormone ⁵⁵, and neuropeptide YY ⁵⁶ receptors. This modulation occurs through a CCK1-mediated effect ⁵⁴⁻⁵⁶. CCK1 receptor expression in the nodose ganglion of intact rats is, however, unchanged following a 48 hr fast or consumption of a high fat diet ⁵⁷. Therefore, it is unlikely that our observations are a consequence of a CCK1 receptor expression change induced by the hypophagia and weight loss we observed after SCI.

Functional interactions between CCK and neuroactive peptides such as ghrelin or leptin have been reported. For example, a synergistic interaction between subthreshold concentrations of leptin and CCK occurs in vagal afferent neurons in primary cell culture ⁵⁸. Conversely, vagal afferent discharge in response to CCK is antagonized by pretreatment with ghrelin ⁵⁹. Those results would suggest that diminished leptin or elevated ghrelin release in our SCI groups reduced CCK-mediated activation in the NTS. This is unlikely, however, since groups in the present study were fasted overnight. Additionally, separate studies of control and injured animals reveal an appropriate increase in serum ghrelin in response to overnight fasting ⁶⁰.

Previous reports have suggested that peripheral CCK-8s activates vago-splanchnic and splanchno-splanchnic gastric reflexes ⁶¹. However, the integrity of a vago-splanchnic reflex circuit is unlikely given the histological evidence regarding the severity of our injury model. Additionally, we have demonstrated that celiac ganglionectomy does not alter post-SCI gastroparesis, suggesting that our model does not involve a splanchnic-mediated component ²⁸. Finally, while the potential for a splanchnic-mediated effect of peripheral CCK-8s on gastric reflex function remains, the effects of vagotomy following central CCK-8s did not support a major role for activation of splanchnic neural circuits ¹⁸.

Taken together, our data suggest that a diminished pre-synaptic response to the model peptide CCK-8s, as well as ghrelin ⁶⁰, may reflect a generalized failure of vagal afferent neurotransmission to respond properly to gastrointestinal peptides in a T3-SCI model highly reminiscent of the gastrointestinal clinical presentation of neurotrauma patients. While visceral hypersensitivity is recognized as a critical component in gastrointestinal pathobiology ⁶², evidence of vagal afferent hyposensitivity has also been identified ^{63,64}. In particular, Xue and colleagues suggest that part of the inflammation-induced diminished afferent sensitivity is mediated through an inducible NOS mechanism ⁶⁴.

In conclusion, our data suggest that gastroparesis following T3-SCI is due, in part, to diminished sensitivity of pre-synaptic NTS inputs to CCK-8s. Vago-vagal neurocircuitry remains anatomically intact following SCI. Therefore, we propose that rather than post-injury loss of a spinosolitary circuit, compromised vagal sensory input affects the gain of vagally-mediated reflexes. Changes in viscerosensory drive from vagal afferents offers a more straightforward, and powerful, explanation for the reduced mechano- and

chemosensory drive of vago-vagal gastric reflexes following SCI. Furthermore, reduced viscerosensory competence is consistent with our previous observation that vagally-mediated gastric relaxation is diminished in T3-SCI rats²⁸. The attenuated action of CCK-8s may reflect a global reduction in the sensitivity of gastric neurocircuitry to gastrointestinal peptides.

Acknowledgments

The authors wish to acknowledge their gratitude to Cesare and Zoraide Travagli and W. Nairn Browning for their support and encouragement. MT, EQC & GMH contributed to the design, generation, analysis, and presentation of *in vivo* data. KNB & AT contributed to the design, generation, analysis, and presentation of *in vitro* data. All authors wrote portions of the text in proportion to their background and effort.

Funding

This work was supported by National Institutes of Health Grants NINDS 49177 (G.M. Holmes) and NIDDK 55530 (R. A. Travagli).

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Figure 1.

Left: Luxol-stained white matter (left column) is highlighted as gray in computer rendering (right column) from T3 spinal cords of control, 3 day (*middle*) and 3 week (*bottom*) SCI rats. The lesion epicenter 3D after T3-SCI shows minimal luxol-staining of white matter limited to the peripheral rim of the tissue section (closed arrowheads; background pseudo-colored pink to provide contrast). At 3 weeks, cross-sectional area and luxol staining is limited to the extreme periphery of the tissue (open arrowheads. Calibration 500 μ m.

Right: Graphic summary of cross section area and spared white matter. (* $p < 0.05$ vs. control, † $p < 0.05$ vs 3D SCI).

Figure 2.

CCK-8s i.p. increases *c-fos* immunoreactivity in NTS of control but not T3-SCI rats

A. Representative micrograph of *c-fos* immunoreactivity in control (left) and T3-SCI (right) following CCK-8s i.p. Note that the *c-fos* immunoreactivity in all areas of the NTS is higher in control vs T3-SCI rats. Measurements indicate distance from calamus scriptorius.

AP=area postrema; NTS= nucleus tractus solitarius; TS= tractus solitarius; cc= central canal.

B. Summary graphic of the *c-fos* immunoreactive neurons in NTS and AP. Note that SCI reduces the *c-fos* positive neurons in NTS but not in AP. (* $p < 0.05$ vs. control).

Figure 3.

Microinjection of sulfated cholecystikinin (CCK-8s) in the left dorsal vagal complex (DVC) induces a vagally mediated decrease in gastric tone in controls but not in T3-SCI rats 3 days after injury.

A: representative photomicrograph (*left*) of brain stem injection site with the injection region circled and schematic representation (*right*) of effective injection areas in 3 day $\circ$ and 3 week subjects $\blacklozenge$. For clarity, representative samples of injection sites are depicted. AP, area postrema; NTS, nucleus tractus solitarius; DMV, dorsal motor nucleus of the vagus; CC, central canal; XII, hypoglossus. Calibration 250 μ m.

B: representative original polygraph records of gastric motility and tone from fasted control and T3-SCI animals after unilateral microinjection of 450 pmol of CCK-8s (PBS injection data not shown). Note the significantly smaller decrease in gastric tone upon CCK-8s microinjection in T3-SCI vs control rats.

C: Graphic summary of gastric relaxation induced by CCK-8s. Note the significant reduction of the CCK -8s-induced gastric relaxation in T3-SCI vs. control rats. (* $p < 0.05$ vs. control).

Figure 4.

Diminished gastroinhibitory actions of CCK-8s after T3-SCI remain following maximal activation of peripheral, post-ganglionic, muscarinic receptors.

A: top left: representative traces of gastric motility and tone following microinjection of 450pmoles of CCK-8s in the DVC. CCK-8s induced a mild gastric relaxation in an intact control. *top right:* gastroinhibitory action following CCK-8s microinjection was readily distinguished during continual infusion of the cholinergic agonist bethanechol ($50 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ iv). *bottom left:* CCK-8s induced a brief gastric relaxation in a T3-SCI 3 days post-op. *bottom right:* Bethanechol infusion did not reveal an immediate effect of subsequent CCK-8s microinjection. Note that both before and after bethanechol infusion the decrease in gastric tone upon CCK-8s microinjection is significantly smaller in T3-SCI vs control rats. *B:* graphic summary of change in gastric tone produced by CCK-8s alone, continual intravenous bethanechol alone, and CCK-8s during continual intravenous bethanechol. Note that the effects of microinjection of CCK-8s in the DVC were significantly attenuated in T3-SCI vs. control animals.

(* $p < 0.05$ vs. control, † $p < 0.05$ vs. pre-bethanechol control).

Control:

Figure 5.

CCK-8s increased the frequency of spontaneous excitatory postsynaptic currents (sEPSC) in NTS neurons of control but not T3-SCI rats.

A) representative trace of sEPSC (top trace) in an NTS neuron from a control rat, note that perfusion with 100nM CCK-8s increased the frequency of sEPSC (bottom trace).

B) representative trace of sEPSC (top trace) in an NTS neuron from a T3-SCI rat, note that perfusion with 100nM CCK-8s did not increase the frequency of sEPSC (mid trace). The lack of responsiveness to CCK-8s in T3-SCI NTS neurons was specific to a rearrangement to gastrointestinal peptides such as CCK since the response to 4-AP was preserved (bottom trace)

Table 1
Reduced brainstem vagal sensitivity after 3 week recovery from SCI

	Control	T3-SCI
CCK-8s	-0.42±0.06g	-0.07±0.02g [*]
Bethanechol	0.74±0.08g	0.6±0.12g
CCK-8s + Bethanechol	-0.43±0.02g	-0.05±0.04g [†]

^{*}
p<0.05 vs. 3w control CCK-8s

[†]
p<0.05 vs. 3w control CCK-8s+bethanechol