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Gastric afferents project to the aldosterone-sensitive HSD2 neurons of the NTS

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Abstract

The HSD2 (11- β -hydroxysteroid dehydrogenase - type 2 enzyme) containing neurons of the nucleus tractus solitarius (NTS) become activated during low-sodium and high-aldosterone states such as hypovolemia. This response may be due to hormonal and/or neural signals. Hormonal signals may activate neurons in the area postrema that innervate the HSD2 neurons. The vagus nerve projects directly to the HSD2 neurons and this could be another route whereby these neurons receive information about systemic sodium/aldosterone status. The peripheral sites of origin that contribute to this vagal projection remain unknown, and in the present study, we injected the transganglionic tracer, cholera toxin β -subunit-horseradish peroxidase (CTb-HRP), into wall of various gastrointestinal organs (stomach, small and large intestine) or liver of rats. Confocal microscopy of brainstem sections stained by a double immunohistochemical procedure was used to analyze whether the HSD2 neurons received axonal contacts from specific gastrointestinal structures. The major source of afferents arose from the stomach, mainly from its pyloric antrum, but a weaker input originated from the fundus region. A trace amount originated from the duodenum. The terminal part of the small intestine and large intestine did not contribute to this projection. Similarly, no afferent inputs from the liver or portal vein were found. In conclusion, HSD2 neurons receive an input mainly from the stomach and these results are considered as potential sites affecting sodium intake.

Keywords

aldosterone; hepatic portal region; liver afferents; nucleus tractus solitarius; sodium balance; stomach; transganglionic tracer; vagus; visceral afferents

1. Introduction

The nucleus tractus solitarius (NTS) contains a unique set of neurons that express 11- β -hydroxysteroid dehydrogenase -type 2 (HSD2) – a glucocorticoid inactivating enzyme which allows these neurons to become sensitive to physiological levels of aldosterone. These neurons become activated, as demonstrated by c-Fos expression, during low-sodium and high-aldosterone states induced by dietary sodium deprivation and hypovolemia (Geerling et al., 2006a). Chemical signals may reach the HSD2 neurons by two routes: 1)

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activation of neurons in the nearby area postrema and/or 2) activation of vagal afferents. Area postrema projects to the HSD2 neurons (Sequeira et al., 2006), and thus, neurons in this region could serve as a chemical-neural transducer. The vagus nerve projects directly onto the HSD2 neurons (Shin et al., 2009), and these sensory fibers may carry feedback information regarding sodium/aldosterone status from the gastrointestinal tract or liver. In the latter situation, the site(s) of origin responsible for this innervation remains unknown. Thus, the objective of the present study was to determine whether selected regions of the gastrointestinal tract and/or liver innervate the HSD2 neurons. Various abdominal organs were injected with the transganglionic tracer - cholera toxin β -subunit-horseradish peroxidase (CTb-HRP), and after several days survival, the rats were killed and their brainstems processed for histochemistry. Sections through the NTS were stained by a double immunohistochemical procedure and examined by confocal microscopy to determine whether the HSD2 neurons were innervated by specific gastrointestinal regions. As shown here, only a selective input from the stomach was identified and the physiological significance of these findings is discussed.

2. Results

Table 1 presents a summary of the CTb-HRP experiments performed in the present study. The main source of putative vagal innervation of the HSD2 neurons originates from the pyloric antrum of the stomach, and with a lesser input from the fundus of the stomach and a trace contribution from the duodenum. The other sites, including hepatic structures, did not contribute to this projection.

All cases involving CTb-HRP injections into the wall of antrum of the stomach resulted in axonal labeling in the ipsilateral medial and commissural areas of the NTS. As shown in Figure 1, the axonal labeling was most dense in the medial NTS, with lesser amounts in the commissural nucleus. Some contralateral axonal labeling was present, and it was considerably weaker than what was observed on the ipsilateral side. The vagal afferents originating from the antrum also provided a relatively light input of the region containing the HSD2 neurons. Our sample included 115 ± 24.5 (mean $\pm$ standard error of the mean) HSD2 neurons per case (range 65–164, $n=4$); these were from the ipsilateral side. About 38% of these neurons received one or more close contacts from the antrum (43.0/case). Our analysis revealed that the HSD2 neurons with one or more close contacts from the stomach antrum had an average of 119.8 contacts per cell. The distribution was as follows: 78% (93.5/case) were axo-dendritic and 22% (26.3/case) were axo-somatic (Figure 2). Among HSD2 neurons that received any vagal apposition, the average number of axo-dendritic and axo-somatic contacts per cell was 2.9 ± 0.5 and 1.8 ± 0.3 , respectively. In addition, CTb-HRP injection in the antrum wall resulted in extensive bilateral, retrograde labeling throughout the dorsal motor nucleus of the vagus nerve (DMX).

Figure 3 shows the distribution of axonal labeling in the NTS after CTb-HRP was injected to the wall of the stomach at the level of the fundus. Just like the antrum cases, axonal labeling was found mainly in the ipsilateral medial, commissural, and HSD2 regions of the NTS. When compared to the antrum case, the overall density of axonal labeling was weaker in the fundus experiments. Our sample included 140 ± 25.5 HSD2 ipsilateral neurons per case (range 68–188, $n=4$). About 30% of HSD2 neurons received one or more close contacts following CTb-HRP injections in the fundus region (40.0 neurons/case). Approximately 8% (11.3 neurons/case) of the HSD2 neurons received axo-somatic close contacts and 26% (35.0 neurons/case) received axo-dendritic close contacts of the HSD2 neurons (Figure 4). Within this population, there were about 1.4 ± 0.2 axo-somatic contacts/neuron while the average number of axo-dendritic contacts was 2.0 ± 0.1 /neuron. Within this set of HSD2 neurons receiving contacts from the fundus, the average total number of contacts was 86.5/

case. These results suggest that ~30% (40.0 neurons/case) of HSD2 neurons in the NTS receive input from the stomach fundus and among all contacts on HSD2 neurons, ~82% (70.0/case) were axo-dendritic and ~18% (16.3/case) were axo-somatic. Fundus injections also resulted in extensive retrograde labeling throughout the dorsal motor nucleus of the vagus nerve bilaterally in all cases.

Injections of CTb-HRP into the duodenum wall resulted in very few bouton like structures in close apposition with the HSD2 neurons. The overall amount of labeling was considerably less than found for the fundus and antrum cases described above. Most of the close contacts were found on the rostral HSD2 neurons, namely, those lying rostral to the level of the area postrema. Since the numbers of putative innervated HSD2 neurons was very low, we did not quantify these findings. In addition, we found very little evidence for afferent labeling in the NTS. However, cell body labeling was found in the dorsal vagal nucleus. As noted in Table 1, no axonal labeling was present in the NTS after CTb-HRP injections into the wall of the terminal ileum.

The distribution of CTb-HRP axonal labeling in the NTS after injections to the cecum were similar to those described earlier (Altschuler et al., 1991). Dense axonal labeling was found in medial NTS, but only weak axonal labeling was seen in commissural region, and only an occasional fiber was seen in area where the HSD2 neurons were distributed. None of the HSD2 neurons were innervated by afferents arising from the cecum.

As shown in Table 1, none of the liver experiments produced axonal labeling in the NTS. Also, there was no retrograde cell body labeling in the dorsal motor nucleus of the vagus. The latter negative finding is consistent with an earlier report (Sugitani et al., 1991).

3. Discussion

Results from the present study show that the major source of vagal inputs to the HSD2 neurons originates from the stomach, especially its pyloric antrum region. A weaker input from the fundus region was observed, and a few fibers originating from the duodenum was noted. Contrary to our *a priori* expectations, no evidence was found for an input from the liver or its related portal vein, and the discrepancy between our anatomical findings and data from the physiological literature is discussed below.

3.1 Technical issues

CTb-HRP was used here as a transganglionic tracer and this decision was based on data that suggest this particular lectin-conjugate is currently regarded as the best reagent for this type of neuroanatomical analysis (Wang et al., 1998). The superiority of CTb-HRP over other transganglionic tracers (wheat germ agglutinin and isolectin B4 from *Griffonia simplicifolia*) was demonstrated using the urinary bladder system (Wang et al., 1998). Wang and associates found that CTb-HRP labeled more dorsal root ganglion cells than either of the two other lectin-conjugate tracers and also resulted in more extensive transganglionic axonal labeling patterns within the spinal cord. CTb-HRP was transported mainly by small dorsal root ganglion (DRG) neurons which possessed unmyelinated axons, and the majority of the neurons expressed either immunoreactive substance P or calcitonin gene-related peptide (Wang et al., 1998).

The selection of an appropriate post-injection survival period used in the present experiment was based on previously published information where others demonstrated that vagal afferents originating from stomach and cecum are successfully labeled in the NTS by 24–72 hours, and the 72 hour time point was the optimal time for demonstration of the maximal amount of transganglionic axonal labeling in the NTS (Shapiro and Miselis, 1985;

Altschuler et al., 1991). One other point should be noted and that is, in the experiments presented here that yielded negative results in terms of transganglionic axonal labeling in the NTS (viz., terminal ileum and liver), we used considerably more CTb-HRP than has been used previously. Thus, we are not certain why these particular sites failed to show any vagal afferent fiber labeling in the NTS.

3.2 Transganglionic labeling of vagal afferents

The subdiaphragmatic vagal trunks and their various branches are made up of mainly sensory fibers, accounting for ~75–90% of the total fiber population (Asala and Bower, 1986; Prechtl and Powley, 1990). Most of the fibers are unmyelinated, and they originate from neurons in the nodose ganglion. Well over 50% of these neurons contain substance P or calcitonin gene-related peptide (Helke and Hill, 1988). Due to the similarities of the vagal afferent system and the urinary bladder afferent system (see above), we reasoned that CTb-HRP may also be an efficient transganglionic tracer for studying the central NTS projections that arise from various gastrointestinal organs, just as it was used by Wang and associates (1998) to the central visceral projections of the bladder.

Earlier we reported that following injections of biotinylated dextran amine (BDA) into the nodose ganglion of rats resulted in a sparse input to the HSD2 neurons (Shin et al., 2009). Approximately 80% of the ipsilateral HSD2 neurons had at least one ‘close contact’ from a BDA-labeled vagal fiber. Most of the contacts were axo-dendritic (80%), with the remaining 20% being axo-somatic appositions. Thus, the HSD2 neurons do receive information from the vagus nerve, but it represents only a minority of the synaptic information these neurons receive.

The present study represents an extension of this work (Shin et al., 2009) and here we were able to show that only a selected part of the gastrointestinal tract (viz., stomach) provides an input to the HSD2 neurons. When we began this study, we had hypothesized that we would find a hepatic portal afferent projection to the NTS since there was physiological evidence that these afferents activate the NTS (Kahrilas and Rogers, 1984; Morita et al., 1997) and perhaps these afferents would project directly to the HSD2 neurons. No evidence for such a projection was found.

3.3 Hepatic nerve and innervation of the hepatic portal region

Several anatomical tracing studies have suggested that the common hepatic branch of the vagus nerve (which we refer to here as the ‘common hepatic nerve’ and in some instances as the ‘hepatic nerve’ for brevity) provides a dense input to the NTS (Rogers and Hermann, 1983; Norgren and Smith, 1988); these studies were based on using a transganglionic tracer applied to the central end of the common hepatic nerve and allowing tracer to move from the periphery into the NTS. While positive data were obtained, the interpretation of their findings was based on the assumption that the common hepatic nerve only carries sensory information from the liver or portal vein to the NTS. Later work showed that the bulk of the sensory fibers contained within the common hepatic nerve originates from distal gastric antrum, duodenum, pylorus, and pancreas, and not the liver, portal vein, or bile ducts (Phillips et al., 1997; Berthoud, 2004).

An important correlative study revealed that when DiI – a highly sensitive carbocyanine fluorescent axonal tracer – was injected into the nodose ganglion of rats, no labeled vagal fibers were found in the liver parenchyma and only a limited number of labeled axons were seen along the wall of the portal vein and in other tissues, including the hepatic hilus and intrahepatic bile ducts (Berthoud et al., 1992). When both of these anatomical findings are taken together, a strong case can be made that the common hepatic nerve is not a major

sensory nerve of the liver, but in reality is primarily carrying afferent information from the stomach (pyloric antrum) and duodenum to the NTS. Given these anatomical findings, a case can be made to re-examine some of the earlier studies which suggested the existence of a vagally mediated system of osmo- and sodium receptors lying in the liver. Several reviews have summarized these early studies (Haberich, 1968; Sawchenko and Friedman, 1979; Latt, 1980).

A more challenging issue is the significance of the vagal innervation of the portal vein which appears to be relatively weak in comparison to the bile ducts (Berthoud et al., 1992; Berthoud, 2004). A more robust innervation originates from the dorsal root ganglia (Barja and Mathison, 1984; Goehler and Sternini, 1996), and this may subserve an alternative osmoreceptive afferent pathway (Vallet and Baertschi, 1982; Stoppini et al., 1984).

The human liver transplant literature suggests that there are no apparent long-term disruptions in sodium balance in humans who have had liver transplants (Colle et al., 2004). Of course, evaluation of these patients is highly complicated due to post-transplant drug regimens.

In rats, hepatic denervation causes increases in arterial blood pressure (Carlson et al., 1998), decreases renal sympathetic activity producing diuresis and urinary sodium excretion (Carlson et al., 1998), and increases sodium balance when rats are fed diets of high NaCl food (Morita et al., 1995). Hepatic nerve lesions, however, do not selectively denervate the liver, and as explained above, these nerve transactions also disrupt the vagal sensory afferents that originate from the pyloric antrum and duodenum. So many of the functions ascribed to osmo- and sodium receptors of the hepatic portal system may be functions actually carried out by the stomach and duodenum. Indeed, sodium/osmosensitive vagal receptors have been documented in the antrum and duodenum (Clarke and Davison, 1978; Mei, 1983; Mei and Garnier, 1986; Wu et al., 2005), and additional evidence is accumulating that these receptors may serve as an early detector of ingested NaCl, thus, could potentially be a key afferent system that triggers the thirst response (Manesh et al., 2006).

3.4 Conclusions

The present study has demonstrated the major source of putative vagal afferent innervation of the aldosterone-sensitive HSD2 originates from the stomach, and in particular, from the pyloric antrum region. The duodenum provides a weak input. Contrary to our expectations, no evidence for inputs from the hepatic portal region was found.

4. Experimental procedures

Experimental protocols were approved by the Washington University School of Medicine Animal and Biological Safety Committees, and conformed to NIH guidelines. The surgical experiments and perfusions were performed on male and female Sprague-Dawley rats (160–300 g; Harlan Laboratories, Indianapolis, IN) under general anesthesia (sodium pentobarbital, 50 mg/kg, intraperitoneal).

The rats were caged individually and underwent dietary sodium deprivation - a treatment that increases the intensity of immunofluorescence labeling for HSD2 in a specialized group of aldosterone-sensitive neurons in the NTS for 7–9 days prior to gastrointestinal injections with a transganglionic tracer (see below). These animals were maintained on low sodium chow (0.01% Na⁺ content, product #85292, Harlan-Teklad, Madison, WI) and distilled water. The bedding material from each cage was changed daily to prevent the salt-deprived

rats from any additional sodium intake related to eating their own urinary or fecal excretions.

4.1 CTb-HRP Injections

Sodium-deprived rats were anesthetized and specific regions of the gastrointestinal tract were surgically exposed. Microinjections of 2% cholera toxin β -subunit-horseradish peroxidase (CTb-HRP, product #D0564, Sigma, St. Louis, MO) made in distilled water were made (see Table 1). A few crystals of the vital dye Fast Green (product # F-7252, Sigma Chemical, St. Louis, MO) were added to the solution. During the injection, the organs were kept moisturized with saline-soaked gauze and then, at the end of the injection period, the injected organ was carefully washed with saline to eliminate any tracer not bound to the tissue. The injections were made with a glass micropipette (50 μ m tip) that was connected to a hand controlled air pressure system where the movement of the meniscus was measured via a calibrated reticule in an operating microscope. Fast Green served as an excellent tissue marker, and photographs were taken of each injected organ. Table 1 summarizes the experiments.

In other experiments, the hepatic nerve was isolated and 2% CTb-HRP was applied to the cut central end (Table 1). Similarly, CTb-HRP was injected around the wall of the portal vein. A second group of rats received intravenous injections of CTb-HRP in the ileocolic vein, so there would be widespread distribution throughout the liver (Blei et al., 1984). Because negative results were obtained with these two procedures, two additional sets of experiments were performed: 1) direct injections into the hepatic parenchyma and 2) injections into the hilus region of the liver.

After 3 or 4 days survival, the rats were anesthetized and perfused through the aorta with saline, followed by 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.4). Brains were removed and stored in fixative 1–5 days.

4.2 WGA-HRP Injections

An additional set of transneuronal tracing experiments (n=20) was performed using the transganglionic tracer – 2% wheat germ agglutinin-horseradish peroxidase (WGA-HRP, product #L-3892, Sigma). The volume of tracer injected was similar to what was used in the CTb-HRP experiments. These injections were made in either the antrum or fundus regions of the stomach, and the rats were killed 3, 5, and 7 d later by vascular perfusion (as above). While retrograde cell body labeling was found in the dorsal vagal nucleus, no axonal labeling was identified in the NTS and hence, the use of this tracer was discontinued.

4.3 Histology

The dorsal portion of the brainstem was cut in the horizontal plane at 200 μ m on a freezing microtome, and the sections collected in serial order in 0.1 M sodium phosphate buffer. The sections were transferred to glass staining plates and agitated for 4 h in a 1% Triton X-100 (product # X-100; Sigma) solution made in 0.01M potassium phosphate buffered saline (KPBS; pH = 7.4) at room temperature. The sections washed two times in KPBS, and then, incubated to a mixture of two primary antibodies: a rabbit polyclonal CTb antiserum raised against the β subunit of cholera toxin (1:25,000; #B65927R; Biodesign, Saco, ME) and an affinity-purified sheep polyclonal antibody to HSD2 (1:40,000; product # 1296; Chemicon International, Temecula, CA). The two antibodies were added to a KPBS solution (0.01M, pH 7.4) containing 5% donkey serum and 0.3% Triton X-100 (Sigma). The sections were reacted for 40–48 h in this solution at room temperature on a shaker, and then, washed (2 $\times$, 10 min each) in KPBS, and transferred into a solution of two secondary antibodies: Cy2-conjugated donkey anti-sheep (1:500; Jackson ImmunoResearch, West Grove, PA) and Cy3-

conjugated donkey anti-rabbit (1:500; Jackson) for 5h. After two washes in KPBS, the sections were mounted on gelatin coated glass slides, air dried, and coverslipped using a fade-retardant glycerol mounting solution containing sodium azide and n-propyl gallate.

For the WGA-HRP experiments, a biotinylated goat anti-WGA (1:1000; BA0024, Vector Labs, Burlingame, CA) was used in conjunction with the HSD2 antibody (as described above), and visualized using Cy3-streptavidin (1:500; Jackson). The optimal dilutions of this antibody and the secondary antibody were determined in preliminary tests taken from rat brains which had WGA-HRP injections in the cerebral cortex.

The HSD2 antibody used here was raised against a recombinant protein generated from nucleotides 385–1204 of the rat HSD2 gene (Gomez-Sanchez et al., 2001). A dilution of 1:40,000 produced optimal signal-to-noise for staining of the putative HSD2 neurons in the NTS as well as cells in the subcommissural organ and neurons in the ventrolateral division of the ventromedial hypothalamic nucleus (Geerling et al., 2006b); these data correlate with in situ hybridization studies of HSD2 mRNA localization in the rat brain (Roland et al., 1995).

4.4 Data analysis and photomicrographs

The sections were imaged using an Olympus Fluoview FV500b laser-scanning confocal microscope. Montage images were acquired using a 20× oil objective (1.17 numerical aperture, NA) in 1 µm z-steps. For these displays, 90–120 z-frames from were collapsed and aligned using Adobe Photoshop (San Jose, CA). Some photos were taken with a 60× oil objective (1.4 NA) in 0.4–0.5 µm z-steps. These images were compiled of 20–25 z-frame steps; all appositions were verified by examining single optical planes. Manipulation of the stacks and z-frame compression data were performed with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Pseudo-coloration of the images and uniform brightness and contrast adjustment were performed in Adobe Photoshop.

Cell counts were performed using the MetaMorph program and 90–120 z-frames were examined from 200 µm sections from images taken at a 20× magnification. Inherent to confocal imaging of labeled animal tissue is a signal-to-noise problem in which scattered individual background pixels may obfuscate analysis. To prevent this possible problem during the counts, CTb-HRP labeled contacts were only counted if they appeared to be continuous with a CTb-HRP axon (*en passant*).

The terms “apposition” and “close contact” are used here to indicate a labeled bouton (~2–6 pixels in diameter, or 1–3 µm) immediately adjacent to an HSD2-labeled soma or dendrite. The bouton was apposed within the X–Y plane of one or more single optical planes with no unlabeled pixels between the two structures.

The sample consisted of HSD2 neurons from a series of consecutive sections from the ipsilateral NTS side. Only neurons that had a complete cell body with a least one dendrite were included in the sample.

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Figure 1.

A horizontal section through the NTS showing the regions that receive an afferent input from the pyloric antrum of the stomach. CTb-HRP labeled axons (red) are seen in the ipsilateral medial and commissural areas (com) NTS regions and they overlap with HSD2 neurons (green). The densest area of innervation was localized to the medial NTS. Panel (A) shows the full 200 µm depth of this section as one compressed plane. Panel (B) shows a line drawing from panel (A). The red lines are CTb-HRP labeled axons and green dots represent the HSD2 neurons. AP, area postrema; com, commissural nucleus; 4V, fourth ventricle.

Figure 2.

Examples of axo-somatic (A–C) and axo-dendritic (D–F) close contacts between CTb-HRP labeled boutons (red) and HSD2 labeled neurons (green) from the antrum of stomach case. Regions of apparent apposition are highlighted with white arrows. D–F: CTb-HRP labeled axonal fibers wrap around an HSD2 dendrite. The partial overlap between some boutons and cells in these images is due to the fact that they represent a 2D projection of 20–25 confocal planes extending ~10 µm in the z-axis, combined in order to show more detail (individual contacts typically do not exhibit any overlapping pixels when examined in individual confocal planes).

Figure 3.

A horizontal section through the NTS shows the NTS regions that receive an afferent input from fundus part of the stomach. Axonal labeling was found mainly in the ipsilateral medial and commissural (com) NTS, including near the HSD2 neurons. The overall density of axonal labeling was weaker in these experiments compared to the antrum cases (Figure 1). Panel (A) shows the full 200 µm depth of this section as one compressed plane. Panel (B) shows the illustration drawing of panel (A). The red lines are CTb-HRP labeled axons and green dots are HSD2 neurons. Abbreviations, see Figure 1.

Figure 4.

Examples of axo-somatic (A–C) and axo-dendritic (D–F) close contacts between CTb-HRP labeled boutons (red) and HSD2 labeled neurons (green) from the fundus case. Regions of apparent apposition are highlighted with white arrows. The partial overlap between some boutons and cells in these images is due to the fact that they represent a two-dimensional projection of 20–25 confocal planes extending ~10 µm in the z-axis, combined in order to show more detail (individual contacts typically do not exhibit any overlapping pixels when examined in individual confocal planes).

Table 1

CTb-HRP Injections

Case No.	Injection Site	Total Volume (μl)	No. of Injections	Each injection (μl)	Survival Period (Days)	Axon Labeling in NTS	Axonal Contacts on HSD2 Neurons	Neuronal labeling in dorsal vagal nucleus
STOMACH								
2368	Fundus	10	9	0.8 – 1.5	3	+	+	+
2371	Fundus	10	8	0.8 – 1.5	3	+	+	+
2380	Fundus	10	10	0.8 – 1.5	3	+	+	+
2381	Fundus	10	10	0.8 – 1.5	3	+	+	+
2336	Antrum	10	10	0.8 – 1.5	3	+	+	+
2337	Antrum	10	8	0.8 – 1.5	3	+	+	+
2343	Antrum	10	7	0.8 – 1.5	3	+	+	+
2344	Antrum	10	10	0.8 – 1.5	3	+	+	+
SMALL TESTINE								
2408	Duodenum	25	30	0.5 – 1	3	+	+	+
2428	Duodenum	25	33	0.5 – 1	3	+	+	+
2429	Duodenum	25	26	0.5 – 1	3	+	+	+
2411	Terminal Ileum	25	34	0.5 – 1	4	–	–	+
2412	Terminal Ileum	25	39	0.5 – 1	4	–	–	+
2413	Terminal Ileum	25	32	0.5 – 1	4	–	–	+
LARGE TESTINE								
2389	Cecum	80	9	8 – 12	3	+	–	+
2390	Cecum	80	10	8 – 12	3	+	–	+
2410	Cecum	80	21	8 – 12	4	+	–	+
LIVER								
2774	Hepatic Nerve	500	1	500	3	–	–	+
2775	Hepatic Nerve	500	1	500	3	–	–	+
2777	Hepatic Nerve	500	1	500	3	–	–	+
2780	Hepatic Nerve	500	1	500	3	–	–	+

Case No.	Injection Site	Total Volume (μl)	No. of Injections	Each injection (μl)	Survival Period (Days)	Axon Labeling in NTS	Axonal Contacts on HSD2 Neurons	Neuronal labeling in dorsal vagal nucleus
2781	Hepatic Nerve	500	1	500	3	—	—	+
2463	Ileo-Colic Vein	80	1	80	3	—	—	+
2465	Ileo-Colic Vein	80	1	80	3	—	—	+
2465	Ileo-Colic Vein	80	1	80	3	—	—	+
2466	Ileo-Colic Vein	80	1	80	3	—	—	+
3326	Portal Vein Wall	500	1	500	3	—	—	+
3327	Portal Vein Wall	500	1	500	3	—	—	+
3328	Portal Vein Wall	500	1	500	3	—	—	+
3329	Portal Vein Wall	500	1	500	3	—	—	+
3330	Portal Vein Wall	1000	2	500	3	—	—	+
3331	Portal Vein Wall	1000	2	500	3	—	—	+
3495	Hepatic Hilus	1	1	1	3	—	—	—
3496	Hepatic Hilus	1	1	1	3	—	—	—
3497	Hepatic Hilus	1	1	1	3	—	—	—
3498	Hepatic Hilus	2	2	1	3	—	—	—
3499	Hepatic Hilus	1	1	1	3	—	—	—
3500	Hepatic Parenchyma	2	2	1	3	—	—	—
3501	Hepatic Parenchyma	5	5	1	3	—	—	—