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SPINALLY PROJECTING PREPROGLUCAGON AXONS PREFERENTIALLY INNERVATE SYMPATHETIC PREGANGLIONIC NEURONS

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Abstract—Glucagon-like peptide-1 (GLP-1) affects central autonomic neurons, including those controlling the cardiovascular system, thermogenesis, and energy balance. Preproglucagon (PPG) neurons, located mainly in the nucleus tractus solitarius (NTS) and medullary reticular formation, produce GLP-1. In transgenic mice expressing glucagon promoter-driven yellow fluorescent protein (YFP), these brainstem PPG neurons project to many central autonomic regions where GLP-1 receptors are expressed. The spinal cord also contains GLP-1 receptor mRNA but the distribution of spinal PPG axons is unknown. Here, we used two-color immunoperoxidase labeling to examine PPG innervation of spinal segments T1–S4 in YFP-PPG mice. Immunoreactivity for YFP identified spinal PPG axons and perikarya. We classified spinal neurons receiving PPG input by immunoreactivity for choline acetyltransferase (ChAT), nitric oxide synthase (NOS) and/or Fluorogold (FG) retrogradely transported from the peritoneal cavity. FG microinjected at T9 defined cell bodies that supplied spinal PPG innervation. The deep dorsal horn of lower lumbar cord contained YFP-immunoreactive neurons. Non-varicose, YFP-immunoreactive axons were prominent in the lateral funiculus, ventral white commissure and around the ventral median fissure. In T1–L2, varicose, YFP-containing axons closely apposed many ChAT-immunoreactive sympathetic preganglionic neurons (SPN) in the intermediolateral cell column (IML) and dorsal lamina X. In the sacral parasympathetic nucleus, about 10% of ChAT-immunoreactive preganglionic neurons received YFP appositions, as did occasional ChAT-positive motor neurons throughout the rostrocaudal extent of the ventral horn. YFP appositions also occurred on NOS-immunoreactive spinal interneurons and on spinal YFP-immunoreactive neurons. Injecting FG at T9 retrogradely labeled many YFP-PPG cell bodies in the medulla but none of the spinal YFP-immunoreactive neurons. These results show that brainstem PPG neurons innervate spinal autonomic and somatic motor neurons. The distributions of spinal PPG axons and spinal GLP-1 receptors correlate well. SPN receive the densest PPG innervation. Brainstem PPG neurons could directly modulate sympathetic outflow through their spinal inputs to SPN or interneurons.

Key words: choline acetyltransferase, green fluorescent protein, glucagon-like peptide-1, nucleus of the solitary tract, parasympathetic preganglionic neurons, retrograde tracing.

INTRODUCTION

Glucagon-like peptide 1 (GLP-1) is released postprandially from enteroendocrine cells in the gut and facilitates absorption of nutrients (Holst, 2007). GLP-1 is also a potent satiety signal (Turton et al., 1996) and treatment with GLP-1 analogs results in sustained weight loss in humans (Drucker and Nauck, 2006; Buse et al., 2009; Holst, 2013). Furthermore, studies using microinjections of GLP-1 receptor antagonists into specific brain regions in rodents indicate that, within the central nervous system (CNS), endogenous GLP-1 can also produce satiety (Hayes et al., 2009; Williams et al., 2009; Barrera et al., 2011; Dossat et al., 2011; Alhadeff et al., 2012).

In the CNS preproglucagon (PPG) is processed post-translationally to produce GLP-1, GLP-2, and oxyntomodulin (Holst, 2007). The highest levels of these PPG products occur in the dorsomedial hypothalamus (DMH) and hypothalamic paraventricular nucleus (PVN) and the lowest levels are found in the cortex and hindbrain (Jin et al., 1988; Vrang et al., 2007; Tauchi et al., 2008). The medulla oblongata contains most of the brain's GLP-1-synthesizing neurons. The majority of GLP-1-producing somata in the brainstem occur in the caudal nucleus tractus solitarius (NTS). Some GLP-1 cell bodies are also located in the dorsomedial region of the medullary reticular nucleus (Jin et al., 1988; Larsen et al., 1997). Retrograde

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Abbreviations: AP, area postrema; CAA, central autonomic area; ChAT, choline acetyltransferase; DAB, diaminobenzidine; DMH, dorsomedial nucleus of the hypothalamus; DMV, dorsal motor nucleus of the vagus; Ex-4, exendin-4; FG, Fluorogold; GFP, green fluorescent protein; GLP-1, glucagon-like peptide 1; ICN, intercalated nucleus; IML, intermediolateral cell column; IRT, intermediate reticular nucleus; L, lumbar; LE, lumbar enlargement; NHS, normal horse serum; NOS, nitric oxide synthase; NTS, nucleus of the solitary tract; PPG, preproglucagon; PVN, paraventricular nucleus of the hypothalamus; S, sacral; SPN, sympathetic preganglionic neuron; T, thoracic; YFP, yellow fluorescent protein.

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tracing has verified that the GLP-1 axons in the hypothalamus arise from these two subgroups of GLP-1 neurons (Larsen et al., 1997; Vrang et al., 2007). Consistent with these immunocytochemical data, the only brain regions that contain detectable levels of PPG mRNA are the caudal NTS, the intermediate reticular nucleus (IRT) and the olfactory bulb (Merchenthaler et al., 1999). Using transgenic mice in which expression of yellow fluorescent protein (YFP) is controlled by the glucagon promoter (Reimann et al., 2008), we have previously described the full distribution of PPG cell bodies and dendrites within the medulla and shown widespread GLP-1 innervation throughout the brain (Llewellyn-Smith et al., 2011). This distribution of YFP-PPG axons within the brain parallels the distribution of brain GLP-1 receptors (Shughrue et al., 1996; Merchenthaler et al., 1999). Taken together, these anatomical findings suggest that GLP-1 release from PPG axons within the brain could influence homeostatic responses and help to coordinate energy balance, food intake and cardiovascular function.

More recently, we have demonstrated that PPG neurons directly innervate cranial parasympathetic preganglionic neurons in the dorsal motor nucleus of the vagus (DMV), again using YFP-PPG mice (Llewellyn-Smith et al., 2013). We also showed that populations of ventral medullary neurons that have been implicated in central sympathetic control received PPG input. Although the spinal cord has not yet been examined for the presence of PPG mRNA, perikarya expressing mRNA for GLP-1 receptors occur in laminae V–X throughout the rat spinal cord (Merchenthaler et al., 1999). Hence, our observations raised the possibility that GLP-1 neurons in the brainstem might also provide direct input to spinal autonomic neurons.

Here, we demonstrate that there is GLP-1 innervation of neurons in the spinal cord that contain immunoreactivity for the enzymes that synthesize acetylcholine and nitric oxide. As in previous work (Hisadome et al., 2010; Llewellyn-Smith et al., 2011, 2013), we used transgenic YFP-PPG mice in order to take advantage of the strong YFP expression that occurs throughout the cytoplasm of PPG neurons, including their terminals. We detected spinal PPG axons by the presence of YFP-immunoreactivity and identified their innervation targets using immunoreactivity for choline acetyltransferase (ChAT), nitric oxide synthase (NOS), and/or Fluorogold (FG) retrogradely transported from the peritoneal cavity. We determined the source of the spinal GLP-1 innervation by retrograde transport of FG from injections into thoracic (T) spinal segment T9.

EXPERIMENTAL PROCEDURES

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and had the required ethics approvals. We used a total of 21 adult mGLU-124 Venus YFP mice (Reimann et al., 2008), which we call YFP-PPG mice here. Bred at Imperial College, the 13 male and eight female mice received food and water *ad libitum* and were kept on a 12-h light:12-h dark cycle. When perfused at 12–16 weeks after birth, the mice weighed 25–35 g, with females being lighter than males of the same age.

Retrograde tracing with FG

Intraperitoneal injections. Three male YFP-PPG mice had FG (0.2% in distilled water, 40 μl) injected into the peritoneal cavity as in Anderson and Edwards (1994).

Spinal injections. Three male and two female YFP-PPG mice were anesthetized with ketamine (75 mg/kg; i.m.) and medetomidine (0.3 mg/kg; i.m.) and placed in a stereotaxic frame. The thoracic spinal cord (segment T9) was carefully exposed by retracting the intervertebral space between vertebrae T6–T7 followed by removal of the yellow ligament. The dura mater was pierced with a 19-g needle and the intermediolateral cell column (IML) was unilaterally targeted with a microinjection of FG (2%, 50 nl) placed 0.2 mm lateral to the midline and 0.5 mm ventral from the dorsal surface of the spinal cord. Anesthesia was reversed with atipamezol (1 mg/kg; i.m.) and postoperative analgesia was given for 4 days (buprenorphine, 1 mg/kg). Mice recovered normally without abnormalities in locomotion. All mice with FG injections were perfused transcardially 7 days after surgery. Correct targeting of the area with FG and its spread within the spinal cord was confirmed postmortem by analysis of spinal cord sections immunoperoxidase stained for FG.

Perfusion and tissue preparation

YFP-PPG mice under ketamine and medetomidine anesthesia were heparinized (500 IU/l) and their blood was removed with a flush of phosphate-buffered saline. The mice were then transcardially perfused with 60 ml of phosphate-buffered 4% formaldehyde. After 3 days of post-fixation, brains and spinal cords still in their vertebral columns were shipped to Flinders for sectioning and immunohistochemical processing.

Spinal cords were removed from their vertebral columns, post-fixed at room temperature on a shaker for 2–3 days in phosphate-buffered 4% formaldehyde and then divided into segments. The rostral edge of the dorsal root entry zone was considered to mark the rostral boundary of each segment. For horizontal sections, cords from two male YFP-PPG mice were divided into three lengths (T1–T7, T8–L3 and L4–S4). For transverse sectioning, thoracic and upper lumbar cords from 19 mice were divided into T1–3 and then two-segment length blocks through to segment L3; segments L4–S4 were left as one piece. The blocks containing T1–L3 from each mouse were embedded together in albumin gelatine (Llewellyn-Smith et al., 2007) to form a single block. Segments L4–S4 from two to three mice were embedded together in a single block of albumin gelatine. The albumin gelatine blocks for transverse sectioning and the cord lengths for horizontal sectioning were infiltrated with 20% then 30% sucrose. Blocks for transverse sectioning were cut at 25 μm on a cryostat. Cord segments for horizontal cryostat sections were cut at 30 μm.

The medullas of mice with injections of FG at T9 were blocked without the use of a brain matrix, infiltrated with sucrose and cut into three series of transverse 30-μm