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PREPROGLUCAGON (PPG) NEURONS INNERVATE NEUROCHEMICALLY IDENTIFIED AUTONOMIC NEURONS IN THE MOUSE BRAINSTEM

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Abstract—Preproglucagon (PPG) neurons produce glucagon-like peptide-1 (GLP-1) and occur primarily in the nucleus tractus solitarius (NTS). GLP-1 affects a variety of central autonomic circuits, including those controlling the cardiovascular system, thermogenesis, and most notably energy balance. Our immunohistochemical studies in transgenic mice expressing YFP under the control of the PPG promoter showed that PPG neurons project widely to central autonomic regions, including brainstem nuclei. Functional studies have highlighted the importance of hindbrain receptors for the anorexic effects of GLP-1. In this study, we assessed YFP innervation of neurochemically identified brainstem neurons in transgenic YFP–PPG mice. Immunoreactivity for YFP plus choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) and/or serotonin (5-HT) was visualised with two- or three-colour immunoperoxidase labelling using black (YFP), brown and blue-grey reaction products. In the dorsal motor nucleus of the vagus (DMV), terminals from fine YFP-immunoreactive axons closely apposed a small proportion of ChAT-positive and rare TH-positive/ChAT-positive motor neurons, mostly ventral to AP. YFP-immunoreactive innervation was virtually absent from the compact and loose formations of the nucleus ambiguus. In the NTS, some TH-immunoreactive neurons were closely apposed by YFP-containing axons. In the A1/C1 column in the ventrolateral medulla, close appositions on TH-positive neurons were more common, particularly in the caudal portion of the column. A single YFP-immunoreactive axon usually provided 1–3 close appositions on individual ChAT- or TH-positive neurons. Serotonin-immunoreactive neurons were most heavily innervated, with the majority of raphé pallidus, raphé obscurus and parapyramidal neurons receiving several close appositions from large varicosities of YFP-immunoreactive axons. These results indicate that GLP-1 neurons innervate various populations of brainstem autonomic neurons. These include vagal efferent neurons and catecholamine neurons in areas linked with cardiovascular control. Our data also indicate a synaptic connection between GLP-1 neurons and 5-HT neurons, some of which might contribute to the regulation of appetite. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: glucagon-like peptide 1, nucleus of the solitary tract, tyrosine hydroxylase, choline acetyltransferase, serotonin, green fluorescent protein.

INTRODUCTION

Glucagon-like peptide 1 (GLP-1) is an incretin that is released from enteroendocrine cells and facilitates absorption of nutrients (Holst, 2007). Like other gut peptides, such as cholecystokinin, GLP-1 is also synthesised by neurons within the central nervous system. GLP-1 is produced by post-translational processing of preproglucagon (PPG) and immunoreactivity for the products of PPG processing is found in many brain regions, with highest levels occurring in the dorsomedial (DMH) and paraventricular nucleus (PVN) of the hypothalamus and lowest levels in the cortex and hindbrain (Jin et al., 1988; Vrang et al., 2007; Tauchi et al., 2008). Somata capable of synthesising GLP-1, however, are restricted to the lower brainstem. The largest population of GLP-1-containing somata occurs in the caudal nucleus of the solitary tract (NTS) and there are also some cell bodies in the dorsomedial part of the medullary reticular nucleus (Jin et al., 1988; Larsen et al., 1997). Similarly, *in situ* hybridisation has revealed PPG mRNA only in the caudal NTS, the intermediate reticular nucleus (IRT) and the olfactory bulb (Merchenthaler et al., 1999). Retrograde tracing has confirmed that the hypothalamic axons containing GLP-1 arise from the cell bodies in the NTS and IRT (Larsen et al., 1997; Vrang et al., 2007).

Microinjection of GLP-1 or GLP-1 agonists into the brain has a multitude of effects, including suppression of food intake, control of blood glucose levels, nausea, changes in blood pressure and heart rate, as well as neuroprotection and effects on learning and memory

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Abbreviations: 5-HT, serotonin, 5-hydroxytryptamine; AP, area postrema; ChAT, choline acetyltransferase; DAB, diaminobenzidine; DMH, dorsomedial nucleus of the hypothalamus; DMV, dorsal motor nucleus of the vagus; GFP, green fluorescent protein; GLP-1, glucagon-like peptide 1; Ig, immunoglobulin; IRT, intermediate reticular nucleus; KLH, keyhole limpet haemocyanin; LPS, lipopolysaccharide; NA, nucleus ambiguus; NHS, normal horse serum; NTS, nucleus of the solitary tract; PPG, preproglucagon; PPY, parapyramidal region; RVLM, rostral ventrolateral medulla; TH, tyrosine hydroxylase; YFP, yellow fluorescent protein.

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(Tang-Christensen et al., 1996; Turton et al., 1996; Van Dijk et al., 1996; Thiele et al., 1998; Kinzig et al., 2002; Yamamoto et al., 2002; During et al., 2003; Cabou et al., 2008; Sandoval et al., 2008). Because of the functional importance of GLP-1 in the brain, we recently reinvestigated the distribution of central neurons capable of synthesising GLP-1 using a transgenic mouse in which GLP-1 neurons express yellow fluorescent protein (YFP) throughout their cytoplasm under the control of the PPG promoter (Reimann et al., 2008). This approach allowed us to visualise PPG neurons and their processes with an unprecedented level of detail. Our study revealed the full distribution of PPG cell bodies and dendrites within the medulla and demonstrated that axons were widespread throughout the brain with the notable exception of the cerebellum, hippocampus and cerebral cortex (Llewellyn-Smith et al., 2011). These observations matched well with the distribution of GLP-1 receptors within the brain (Shughrue et al., 1996; Merchenthaler et al., 1999). Notably, we found varicose axons in many central sites that are involved in regulating autonomic functions. GLP-1 released in these areas could contribute to the global and coordinated control of food intake, energy balance and maintenance of cardiovascular homoeostasis.

Recent functional studies have highlighted the importance of brainstem circuitry and brainstem GLP-1 receptors for physiological function (Yamamoto et al., 2003; Wan et al., 2007a,b; Hayes et al., 2008, 2009; Holmes et al., 2009; Williams et al., 2009; Barrera et al., 2011; see also (Trapp and Hisadome, 2011 for review). These findings, together with the fact that our immunohistochemical study revealed many more varicose immunoreactive axons in the brainstem than previously reported, prompted us to define the innervation targets of PPG neurons within the brainstem in more detail. Catecholamine neurons in the area postrema (AP) have been shown to express GLP-1 receptors (Yamamoto et al., 2003) and a subpopulation of cholinergic dorsal vagal motor neurons responds electrically to GLP-1 (Wan et al., 2007a,b; Holmes et al., 2009), consistent with these cell types receiving inputs from GLP-1 neurons.

In this study, we used transgenic YFP–PPG mice (Reimann et al., 2008) in order to take advantage of the strong YFP expression that occurs throughout the cytoplasm of PPG neurons, including their terminals (Hisadome et al., 2010; Llewellyn-Smith et al., 2011). To reveal GLP-1 innervation of cholinergic, catecholamine and serotonin neurons in the brainstem, we detected YFP-immunoreactivity in combination with immunoreactivity for choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) or 5-hydroxytryptamine (5-HT), using two-colour or three-colour immunoperoxidase staining.

EXPERIMENTAL PROCEDURES

These studies were performed on 11 adult male and 11 adult female mGLU-124 Venus YFP mice (Reimann et al., 2008), which will be referred to here as YFP–PPG mice. Mice were perfused at 12–16 weeks of age and weighed between 25 and 35 g with males consistently being heavier than females of the same age. Mice were bred at Imperial College, kept on a 12-h light:dark cycle and had unlimited access to food and water. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, with appropriate ethical approval.

Mice under halothane anaesthesia were given heparin (500 IU/l), flushed with phosphate-buffered saline to remove blood and perfused transcardially with 60 ml of phosphate-buffered 4% formaldehyde, pH 7.4. Brains were post-fixed intact for 3 days at room temperature on a shaker in the same fixative and then shipped to Flinders for sectioning and immunohistochemical staining.

Blocks containing the brainstem were trimmed from the post-fixed brains and infiltrated with sucrose. Three series of transverse 30-μm cryostat sections were cut from each block. Because we did not use a matrix to block the brains, the dorsoventral tilt of the sections varied amongst mice. Consequently, Bregma values could not be reliably assigned to the brainstem sections studied here.

Immunohistochemistry

Cryostat sections were first washed 3 × 10 min in 10 mM Tris, 0.9% NaCl, 0.05% thimerosal in 10 mM phosphate buffer, pH 7.4, (TPBS) containing 0.3% Triton X-100 and then exposed to TPBS-Triton containing 10% normal horse serum (NHS) for at least 30 min. TPBS-Triton containing 10% NHS was used to dilute primary antibodies; TPBS-Triton containing 1% NHS, to dilute secondary antibodies; and TPBS-Triton, to dilute the avidin-horseradish peroxidase complex. All steps in each protocol occurred on a shaker at room temperature and 3 × 10-min washes in TPBS were done after each incubation in an immunoreagent.

Titration was used to determine the working concentrations of primary antibodies. Optimal dilutions produced the maximum number of immunoreactive structures with minimal non-specific background staining. In the case of the anti-green fluorescent protein (GFP) antibody used to detect the YFP-expressing neurons, more dilute antibody was used to optimally visualise axons than for optimally visualising cell bodies (see Llewellyn-Smith et al., 2011). We have previously shown that mouse tissue lacking YFP-expressing neurons shows no staining after immunoperoxidase processing to reveal GFP (Llewellyn-Smith et al., 2011).

After completion of the double or triple-labelling immunoperoxidase protocol detailed below, sections were mounted in serial order onto chrome alum-gelatine coated slides, dried and dehydrated. Coverslips were applied with Permaslip mounting medium (Alban Scientific, St Louis MO, USA).

Two-colour Immunoperoxidase Labelling. Double immunoperoxidase labelling was used to localise YFP-immunoreactivity with a black reaction product and either ChAT-, TH- or 5-HT-immunoreactivity with a brown reaction product. After treatment with TBS-Triton and 10% NHS–TBS-Triton, the sections were transferred into 1:50,000 or 1:100,000 chicken anti-GFP (Catalogue #ab13970, Lot #623923; Abcam, Cambridge, UK) for 3–5 days. After washing, the sections were exposed overnight to 1:500 biotinylated donkey anti-chicken immunoglobulin (Ig) Y (Catalogue #703-065-155, Jackson ImmunoResearch, West Grove, PA) and then to 1:1,500 ExtrAvidin-peroxidase (Sigma–Aldrich, St Louis MO, USA) for 4–6 h. Structures containing YFP-immunoreactivity were stained black with a nickel-intensified diaminobenzidine (DAB) reaction in which peroxide was generated using glucose oxidase (Llewellyn-Smith et al., 2005). After a second blocking step in 10% NHS–TBS-Triton, sections

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