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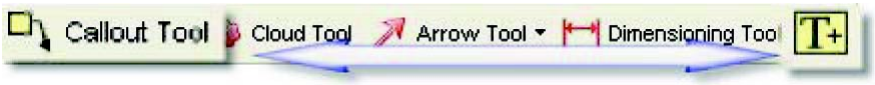

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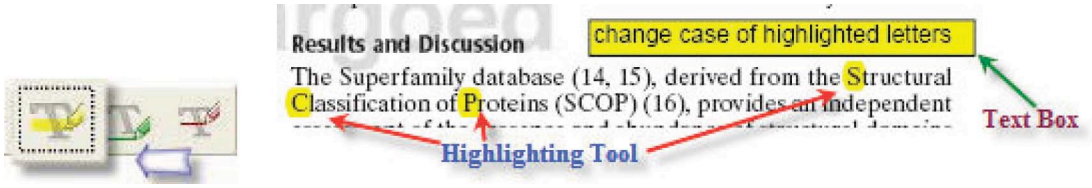

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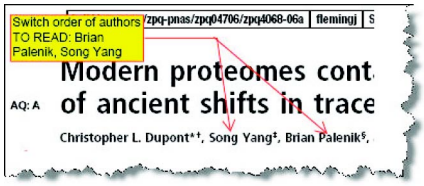

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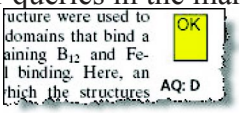

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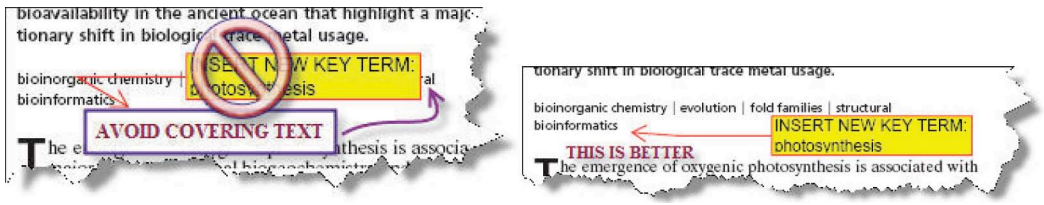

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Review

PPG neurons of the lower brain stem and their role in brain GLP-1 receptor activation

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Trapp S, Cork SC. PPG neurons of the lower brain stem and their role in brain GLP-1 receptor activation. *Am J Physiol Regul Integr Comp Physiol* 309: R000–R000, 2015. First published August 19, 2015; doi:10.1152/ajpregu.00333.2015.—Within the brain, glucagon-like peptide-1 (GLP-1) affects central autonomic neurons, including those controlling the cardiovascular system, thermogenesis, and energy balance. Additionally, GLP-1 influences the mesolimbic reward system to modulate the rewarding properties of palatable food. GLP-1 is produced in the gut and by hindbrain preproglucagon (PPG) neurons, located mainly in the nucleus tractus solitarii (NTS) and medullary intermediate reticular formation. Transgenic mice expressing glucagon promoter-driven yellow fluorescent protein revealed that PPG neurons not only project to central autonomic control regions and mesolimbic reward centers, but also strongly innervate spinal autonomic neurons. Therefore, these brain stem PPG neurons could directly modulate sympathetic outflow through their spinal inputs to sympathetic preganglionic neurons. Electrical recordings from PPG neurons in vitro have revealed that they receive synaptic inputs from vagal afferents entering via the solitary tract. Vagal afferents convey satiation to the brain from signals like postprandial gastric distention or activation of peripheral GLP-1 receptors. CCK and leptin, short- and long-term satiety peptides, respectively, increased the electrical activity of PPG neurons, while ghrelin, an orexigenic peptide, had no effect. These findings indicate that satiation is a main driver of PPG neuronal activation. They also show that PPG neurons are in a prime position to respond to both immediate and long-term indicators of energy and feeding status, enabling regulation of both energy balance and general autonomic homeostasis. This review discusses the question of whether PPG neurons, rather than gut-derived GLP-1, are providing the physiological substrate for the effects elicited by central nervous system GLP-1 receptor activation.

appetite; brain stem; glucagon-like peptide-1; hippocampus; neuroanatomy

FOR TWO DECADES, IT HAS BEEN known that glucagon-like peptide (GLP-1) reduces food intake by acting within the central nervous system (95). That first study showed through the use of the GLP-1 receptor antagonist exendin-9 (Ex9) that endogenous GLP-1 has the ability to suppress food intake and that this effect is dependent on the feeding state of the animal. While unequivocally showing that GLP-1 has a physiological role in brain, the source of centrally acting GLP-1 remains less clear. Does postprandially released GLP-1 from the enteroendocrine L-cells reach the central nervous system (CNS) from the circulation, despite its short half-life in the blood, or does the small number of preproglucagon (PPG) neurons in the lower brain stem (37, 58, 65) provide sufficient GLP-1 for the plethora of brain regions that express its receptor? Although this question remains largely unanswered, it is clear that central GLP-1 is integral to many more processes linked to energy homeostasis than simply food intake. This review provides a detailed account of the different actions of GLP-1 in the brain, with a particular emphasis on the potential role of the PPG neurons in these processes.

In the brain, the highest levels of GLP-1 were found within the hypothalamus and thalamus, followed by the lower brain stem (87). This led to the hypothesis that GLP-1 might be synthesized as a neurotransmitter within the central nervous system, and subsequent immunohistochemistry has located GLP-1 immunoreactivity in cell bodies in the lower brain stem and axon terminals in hypothalamic nuclei (53). The relative distribution of GLP-1 between the brain stem and hypothalamus seems to reflect that GLP-1 synthesis occurs in the cell body before transport to axon terminals, where it is stored ready for release (111). GLP-1-producing neurons have been identified either by GLP-1 immunoreactivity and were, thus, called GLP-1 neurons, or through the presence of preproglucagon mRNA (which is translated and cleaved to proglucagon, the peptide precursor for GLP-1) and were named PPG neurons. Consequently, these two cell populations are expected to be identical, because proglucagon is processed to GLP-1 and GLP-2 rather than glucagon in the brain (85). For the purpose of this review, we use the term PPG neurons to refer to the

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GLP-1-producing cells in the brain no matter how they were identified, and only differentiate if it is of specific significance.

Gut-Derived GLP-1 and the Brain

When assessing the importance of brain-derived GLP-1, the first question to address is whether peripheral GLP-1 reduces food intake. There is strong evidence suggesting it does (22, 29, 81, 82, 102, 103), and the weight loss resulting from bariatric surgery appears to depend on this (62). The greatly reduced length of the upper intestinal tract in Roux-en-Y bypass patients means that nutrients reach the L-cells in the ileum much faster and at higher concentrations leading to increased GLP-1 release. Additionally, a recent study suggests that binge-eating episodes may be linked to reduced circulating GLP-1 levels in bulimia nervosa patients. However, the increased "fullness" experienced by patients with purging disorders appears not to be linked to increased postprandial GLP-1 release (14).

While peripheral GLP-1 undoubtedly reduces food intake (102), dissecting the pathways involved turns out to be difficult. The three main possibilities of how peripheral GLP-1 might exert central effects are the following. *1*) Circulating GLP-1 might have unrestricted access across the blood-brain barrier (BBB) and act directly on brain GLP-1 receptors (GLP-1R). *2*) Circulating GLP-1 might only access areas of the brain that express an incomplete BBB, and, thus, GLP-1R expressed in the area postrema (AP) and the subfornical organ (11, 65) might act as relays to generate an electrical signal in brain linked to peripheral GLP-1. *3*) Peripheral GLP-1 might bind to GLP-1R on vagal afferent neurons, for example, at the portal vein, and generate an electrical signal to the brain. Given that circulating GLP-1 is rapidly degraded by the enzyme DPP-IV, the prospect of intestinally released GLP-1 reaching the brain in sufficient concentration to cross the BBB is questionable. Most rodent studies have used the infusion of exogenous GLP-1 or GLP-1 analogs to assess GLP-1 effects. This usually involved supraphysiological concentrations, and, depending on both the route of administration and the dose given, pathways that are not relevant under physiological conditions might have been recruited. Consequently, results vary substantially between studies and some suggest that GLP-1 in the circulation can reach any part of the brain, whereas others claim that GLP-1 is restricted to areas of the brain with a leaky BBB (44, 72). The latter view was further substantiated by the finding that lesioning the area postrema blocked the effect of hepatoportal vein GLP-1 infusion (76). Additionally, in most rodent studies peripherally administered GLP-1 retained its satiety effect after vagotomy or vagal deafferentation (33, 108). One study demonstrated that the satiety effect of intraperitoneal GLP-1, but not intravenous GLP-1, depends on an intact vagal nerve (83). In contrast, in humans, intravenous GLP-1 seems to be dependent on an intact vagal nerve to reduce food intake (75).

To clarify the importance of vagal afferents compared with area postrema GLP-1Rs, a recent study addressed the question of whether a standard meal raises plasma GLP-1 levels sufficiently to have a physiological effect in brain. Their findings suggest that the permeability of the BBB might be irrelevant for postprandial release of GLP-1 because rats that consumed a 3-g meal within 5–6 min, showed significant elevations in plasma GLP-1 only in the hepatoportal vein and not in the vena cava (76). This would suggest that under physiological conditions, GLP-1 receptors within the CNS, except those outside the BBB, are only reached by brain-derived GLP-1, suggesting a fundamental role for the PPG neurons.

Treatment with GLP-1 Analogs

The considerations for the physiological situation as described above are unlikely to apply when considering the action of systemically administered stable GLP-1 analogs used clinically in the treatment of Type 2 diabetes mellitus. Given that GLP-1 analogs are resistant to DPP-IV inactivation, it seems feasible that they cross the BBB and exert their actions directly on GLP-1R in the brain. Indeed, both exendin-4 (Ex4) and liraglutide retained their food intake inhibitory effect after vagotomy, suggesting that they act directly in the brain (43). Similarly, another study found no effect of AP lesioning on food intake reduction by Ex4 (7). A recent elegant study by Secher et al. (86) has gone some way further to address this question. First, animals that had undergone either subdiaphragmatic vagotomy or lesion of the AP maintained the weight-limiting effects of intraperitoneal liraglutide to a similar degree as sham-operated controls, suggesting that these "entry points" do not significantly contribute to the therapeutic effects of liraglutide. Furthermore, fluorescently labeled liraglutide (liraglutide^{750}) could access multiple sites located behind the BBB, such as the hypothalamic paraventricular nucleus (PVN) and the arcuate nucleus (ARC), as well as in all circumventricular organs. Collectively, the data strongly suggest that liraglutide has the ability to cross the BBB and interact with CNS GLP-1R directly. Thus, the use of GLP-1 analogs is likely to mimic the full activation of both the peripheral (enteroendocrine) and the central (neuronal) GLP-1 system at the same time. Whether this also occurs in a physiological situation is currently unclear.

Inputs to PPG Neurons

Given that PPG neurons are the likely physiological source of GLP-1 to reach brain GLP-1Rs, it is imperative to know what signals, and particularly satiety signals, activate these neurons. Interestingly, PPG neurons do not express GLP-1R but receive direct synaptic input from the vagus nerve (37). CCK and leptin, short- and long-term satiety peptides, respectively, increased the electrical activity of PPG neurons, while ghrelin, an orexigenic peptide, and the melanocortin receptor agonist melanotan II had no effect (36, 37). Leptin receptors have been localized to PPG neurons (25, 40) and leptin application to the lower brain stem causes an increase in PPG mRNA transcription in mouse (26). Consequently, it seems likely that leptin signaling is directly responsible for the correlation between PPG mRNA and fat mass. In that respect, it is surprising that leptin has no direct effect on PPG neurons in rat (40, 61). However, a synergistic effect on food intake has been described, and intracerebroventricular Ex9 attenuated the food intake suppression by hindbrain leptin delivery (109).

Their location, inputs, and projections suggest PPG neurons are ideally suited to turn a postprandial rise in hepatoportal GLP-1 into release of GLP-1 within the brain. This idea is further supported by the observation that gastric distension, another satiety signal that activates vagal afferent neurons, elicited elevated c-Fos expression in GLP-1-immunoreactive

neurons in rat (100). This makes the PPG neurons an important initial relay that links peripheral satiety signals to the brain circuits regulating food intake.

However, more controversy arises when considering the further signaling pathway. Hayes et al. (32) demonstrated that blockade of hindbrain GLP-1R reduced gastric distension, but not duodenal nutrient infusion-induced satiety. If it is assumed that PPG neurons are activated by both of these signals, we would have to conclude that Hayes' observations reflect the existence of distinct subpopulations of PPG neurons that project locally (those activated by gastric distension), and those that project elsewhere (those activated by duodenal nutrient infusion), with both of these populations being involved in the regulation of food intake.

In the absence of definitive answers to these questions, this review focuses on what we do know about the PPG neurons and the action of GLP-1 specifically within the CNS, and addresses the following question instead: Could the small population of PPG neurons located in the lower brain stem account for all functions ascribed to GLP-1 in the CNS? While the most prominent function of GLP-1 in the brain is reduction of food intake (81, 91, 94, 95, 97) and general metabolic control, the additional effects are wide reaching. Evidence suggests involvement in energy expenditure by influencing thermogenesis from brown adipose tissue (9, 60) and blood glucose control (47, 84). In addition, effects on cardiovascular control (106), presumably via the modulation of autonomic outflow, cognitive function (18), as well as a general neuroprotective effect (18) have been reported. We will consider these, in turn, and explore how PPG neurons might be in a position to fulfill these diverse functions (Fig. 1). F1

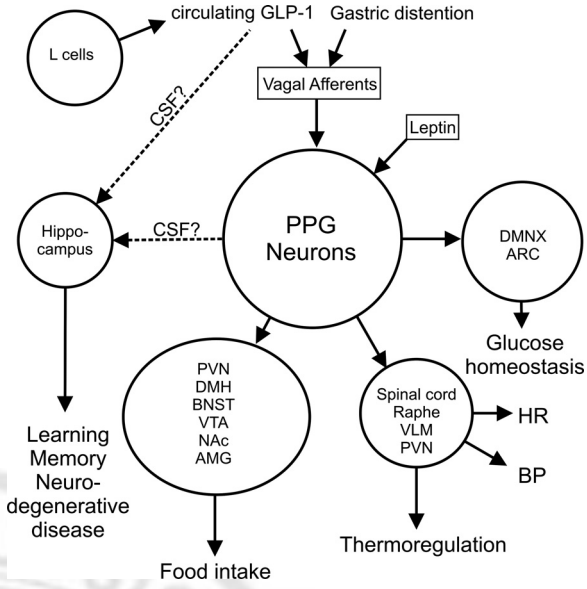

Fig. 1. Functions assigned to glucagon-like peptide (GLP-1) receptor activation in different parts of the central nervous system and how preproglucagon (PPG) neurons might be linked to these functions. All areas indicated, except for the hippocampus, receive some level of direct innervation from PPG neurons. In the absence of innervation, hippocampal GLP-1 receptors might be accessible to GLP-1 in the cerebrospinal fluid (CSF). However, the physiological relevance of CSF GLP-1 has not been established yet. Should there be a role for CSF GLP-1, this could also reach other GLP-1Rs throughout the CNS. ARC, arcuate nucleus; AMG, amygdala; BNST, bed nucleus stria terminalis; BP, blood pressure; DMH, dorsomedial hypothalamus; DMNX, dorsal motor nucleus of the vagus; HR, heart rate; NAc, nucleus accumbens; PPG, preproglucagon; PVN, paraventricular hypothalamic nucleus; VLM, ventrolateral medulla; VTA, ventral tegmental area.

PPG Neuron Anatomy: Projection Targets

To date, we have a very good understanding of the anatomical distribution of PPG cell projections throughout the CNS and some insight into the distribution of GLP-1 receptors within the brain. This knowledge allows us to assess whether the PPG cells would be in a position to deliver GLP-1 to the sites in the CNS where it is expected to exert its specific actions.

The anatomy of PPG neurons has been studied most extensively in rat and mouse (53, 56–58), but other species, including nonhuman primates (98) and a limited study in humans (110), confirm a conserved pattern of distribution for the cell bodies in the nucleus of the solitary tract and the intermediate reticular nucleus of the lower brain stem. In addition, PPG cell bodies have been located in the olfactory bulb in rat and mouse, as well as in the piriform cortex and lumbrosacral spinal cord of mouse (57, 58, 65).

In both rat and mouse, the PPG axons and terminals are distributed widely throughout the CNS, with major innervation, signified by varicose axons, not only in the hypothalamic nuclei involved in feeding, but also in the amygdala, large parts of the limbic system, and various nuclei involved in autonomic control (41, 53, 56–58) (Fig. 2). It is assumed that virtually the entire distribution of PPG axons within the brain originates from the PPG neurons in nucleus tractus solitarius (NTS) and intermediate reticular formation (IRT) (Fig. 2). This is based on the assumption that PPG cells in the olfactory bulb are local interneurons (65) and the finding that spinal PPG neurons do not send ascending axons to the brain (57). This leaves only the possibility that the small number of PPG neurons within the piriform cortex might contribute to the PPG axons observed throughout the brain. Studies in which retrograde tracers were injected into areas with PPG axon terminals demonstrated labeling of NTS and IRT PPG neurons, with no obvious topographical mapping that would suggest the existence of projection-specific subpopulations of PPG neurons in the brain stem (5, 16, 53, 57, 92, 99). Similarly, spinal PPG projections, thus far, only characterized in mouse, originate from both NTS and IRT PPG cells (57). These spinal projections target predominantly preganglionic sympathetic neurons located in the central autonomic area (CAA) and the intermediolateral cell column (IML) from thoracic to lumbar levels (57). F2

Brain GLP-1 and Food Intake

Action on hypothalamic sites consistently seems to reduce food intake linked to satiety, whereas action within the amygdala and brain stem appears to be linked additionally to the feeling of visceral malaise and its consequential reduction in food intake (28, 43, 45, 64, 97). Individual studies have claimed either a role in conditioned taste aversion for the amygdala (45) and a homeostatic regulation for the brain stem (32), or vice versa (28, 43, 97). In contrast, GLP-1 in the ventral tegmental area (VTA) or the nucleus accumbens (NAc), appears to selectively reduce the appeal of highly palatable food as reflected in a reduced meal size, rather than meal frequency (15, 67) or reduced motivation to work for this

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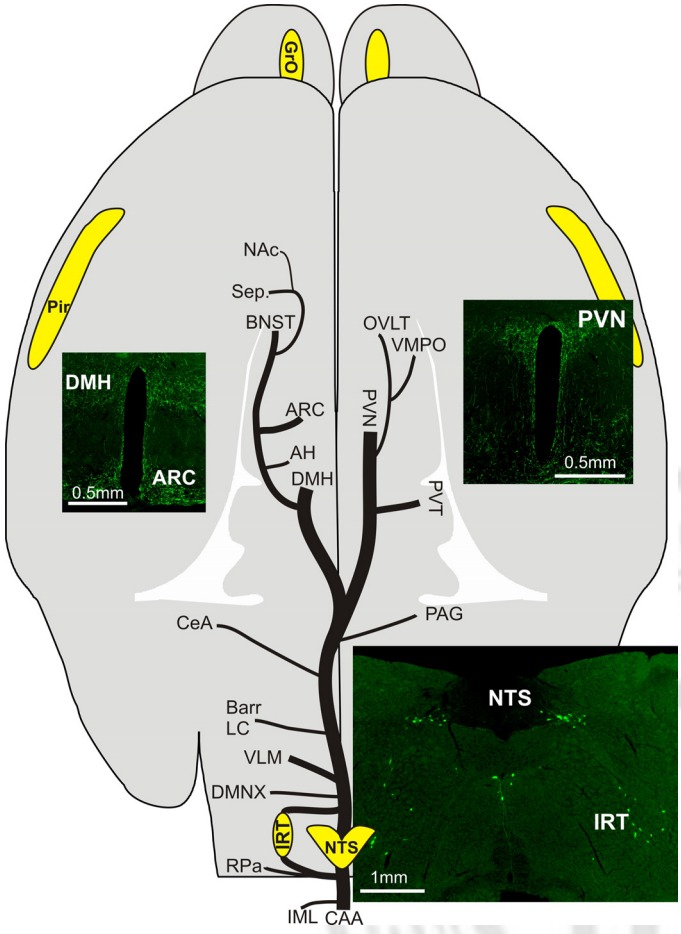

Fig. 2. PPG neuron populations and their main projection targets throughout the CNS. Schematic indicates the location of the PPG cell bodies (areas in yellow) and the projections originating from these sites. Line thickness gives an indication of the relative strength of this innervation. PPG neurons in the GrO are assumed to be local interneurons, and projections from PPG neurons in the piriform cortex have not been examined to date. Photomicrographs show the PPG cell bodies in the lower brain stem and the axon terminals in hypothalamic target areas. AH, anterior hypothalamic area; ARC, arcuate nucleus; Barr, Barrington's nucleus; BNST, bed nucleus stria terminalis; CAA, central autonomic area; CeA, central amygdala; DMH, dorsomedial hypothalamus; DMNX, dorsal motor nucleus of the vagus; GrO, granule cell layer of the olfactory bulb; IML, intermediolateral cell column; IRT, intermediate reticular nucleus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus tractus solitarii; OVLT, organum vasculosum of the lamina terminalis; PAG, periaqueductal gray; Pir, piriform cortex; PVN, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus; RPa, raphe pallidus; Sep, Septum; VLM, ventrolateral medulla; VMPO, ventromedial posterior nucleus.

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food (13) (for review see Ref. 89), although studies without the choice of highly palatable food also reported a reduction in food intake upon injection of GLP-1 into NAc (16) or Ex-4 into the VTA (13). Given that the limbic system is central for the rewarding properties of behavior, substances, and food (59), this is hardly surprising. In fact, several studies have concluded that activation of GLP-1R in the limbic system can attenuate the reward from the consumption of cocaine (19, 27) or amphetamines (19, 21), and even lead to reduced consumption of narcotics, such as alcohol (20, 88). These observations also go hand in hand with the presence of GLP-1 receptor mRNA (65) and PPG projections to the VTA, bed nucleus of the stria terminalis (BNST), amygdala, nucleus accumbens (NAc), and lateral septum (LS). In mouse, projections to BNST and LS are substantially more numerous than to VTA and NAc. Whether this is also the case in rat remains to be determined. The latest additions to brain regions implicated in the regulation of food intake by GLP-1 are the parabrachial nucleus and the hippocampus (4, 39, 78). Notably, the hippocampus, the entire cerebellum, and most of the cortex, with the exception of the piriform cortex, are devoid of PPG axons (58). Except for these small regional discrepancies, there is a good correlation throughout the brain between the presence of fibers from PPG neurons and GLP-1 receptors, or GLP-1 action.

Medullary Projections of PPG Neurons

A detailed investigation of the neurochemical identity of the projection targets of PPG neurons within the brain stem revealed strong projections to catecholaminergic neurons in the ventrolateral medulla (56). This region of the brain stem and particularly adrenergic/noradrenergic cells in this area have been shown to be critically involved in central respiratory and cardiovascular control (2, 63). This innervation could, thus, contribute to the effects of central GLP-1 on blood pressure, heart rate, and more general sympathetic activity (10, 34, 106). Additionally, a recent study in rat has demonstrated that activation of GLP-1R in the NTS can reduce hedonic food intake. Activation of NTS GLP-1R through microinjection of the GLP-1R agonist Ex4 eliminated the preference for peanut butter over chow. NTS Ex4 increased dopamine-β-hydroxylase expression in the NTS, as well as tyrosine-hydroxylase and dopamine receptor 2 expression in the VTA, suggesting that GLP-1 acts on NTS catecholaminergic neurons to elicit this effect (77). Interestingly, these catecholaminergic A2 cells in the NTS are also activated by food intake, and in contrast to PPG neurons, which are only activated by satiating meals, A2 neurons are also activated by smaller amounts of food intake (50), suggesting that this activation is independent of GLP-1 released from PPG neurons.

A somewhat smaller percentage of catecholaminergic neurons in the NTS also received close appositions from PPG neurons, whereas catecholaminergic neurons in the area postrema (AP) had very sparse innervation from PPG neurons (56). These catecholaminergic AP cells have been shown to express GLP-1 receptors (11, 105), and this finding would suggest that the majority of AP GLP-1 receptors are, indeed, geared toward sampling blood GLP-1 (72), or ventricular GLP-1 (39), rather than receiving synaptic inputs from PPG neurons.

Of the cholinergic cell populations found in the brain stem, only the dorsal vagal neurons received a significant level of close appositions from PPG axons. This mirrors functional studies from rat, which demonstrated that about 50% of pancreas-projecting dorsal vagal neurons respond electrically to GLP-1 in vitro (101) and that Ex4 injection into the dorsal vagal complex reduces gut motility in vivo (38). Thus, PPG axons innervating dorsal vagal neurons would be in a prime position to affect both gut function and, thus, potentially indirectly appetite, and pancreatic endocrine secretions and, thereby, blood glucose homeostasis. Additionally, given recent evidence that 50% of brain stem PPG neurons project directly to sympathetic preganglionic neurons in the spinal cord (57), it is also feasible to suggest a direct sympathetic mechanism,

mediated either through inhibition of hepatic glucose production or increased absorption into muscle. Furthermore, hindbrain PPG neurons are also known to send ascending projections to forebrain areas, for example, the ARC (58), known to have connections with presympathetic nuclei, such as the PVN. This provides a further possible indirect mechanism through which CNS GLP-1 is able to regulate plasma glucose. Injection of GLP-1 into the arcuate nucleus, for example, significantly reduced hepatic glucose production following insulin infusion (84).

A third population of brain stem neurons that were found to receive intense innervation from PPG axons were the serotonergic neurons in the raphe pallidus and the parapyramidal area, with numerous PPG axon varicosities making contact with individual 5-HT immunoreactive neurons (56). Like PPG neurons, serotonin neurons have been shown to be involved in the regulation of food intake (30, 51), and both cell populations are activated by visceral malaise (48, 52, 80). Similarly, both cell populations have been implicated in thermoregulation (34, 70). A hint that GLP-1 and 5-HT pathways might converge stems from the observation that mice lacking 5-HT2C receptors fail to exhibit satiation upon peripheral administration of GLP-1 (6). This suggests that 5-HT might be required downstream of GLP-1 for satiation. If the 5-HT that would normally bind to these 5-HT2C receptors were released from the 5-HT neurons in the raphe pallidus and parapyramidal area, then GLP-1 receptors on these neurons could be the ones targeted in chronically decerebrate rats to cause hypothermia and hypophagia (34).

Both the catecholaminergic cells in the ventrolateral medulla and the serotonergic raphe neurons that receive innervation from PPG neurons are potentially presympathetic neurons, which might project directly to preganglionic sympathetic neurons located in the IML and the CAA of the spinal cord, and would, thus, constitute another feasible pathway for PPG neurons to modulate sympathetic outflow. This would also constitute the "normal" pathway for NTS neurons to exert sympathetic effects. However, recently it was discovered in mouse that PPG neurons also send direct projections down into the spinal cord. In fact ~50% of brain stem PPG neurons appear to do so (57). These direct spinal projections target primarily preganglionic sympathetic neurons in the IML and CAA, but also, to a lesser degree, parasympathetic preganglionics in the lumbar cord and relatively small numbers of motor neurons. This innervation provides a previously unknown pathway for PPG neurons to directly modulate sympathetic outflow and, thereby, processes such as heart rate, blood pressure, and thermoregulation by brown adipose tissue.

Forebrain Projections of PPG Neurons

Detailed knowledge of the specific cell types receiving close appositions from PPG neurons is more sparse beyond the brain stem. Electrophysiological data from the VTA and the NAc suggests that dopaminergic neurons in these regions do not receive direct close appositions, but that glutamatergic input to these neurons is modulated by GLP-1 (66, 67). In the parabrachial nucleus of mouse, close appositions of PPG axons on calcitonin gene-related peptide neurons have been reported (78). Within the PVN, a study in rat characterized cell types receiving innervation from PPG fibers. These included oxytocin and corticotropin-releasing hormone-producing cells, as well as primarily the parvocellular subset of vasopressin neurons (92).

If GLP-1 receptors in the brain are activated by brain-derived GLP-1, it would be expected that the projection pattern would be mirrored by the expression pattern for GLP-1R. The distribution of GLP-1 receptors in the brain has been analyzed in detail by in situ hybridization in the rat (65) and using a genetic tag in mouse (11). Recently, two mouse lines have been developed that express Cre-recombinase in GLP-1R-expressing cells, or a FLAG-tagged humanized GLP-1 receptor, respectively (42, 79). The distribution of PPG projections in mouse (56–58) shows a high degree of overlap with the expression of GLP-1R in both mouse and rat (11, 65). However, notable differences also exist. For example, GLP-1R is highly expressed in the CA3 region of the caudal hippocampus (11, 65), a region devoid of PPG innervation and GLP-1 immunoreactivity (41, 58). This raises two possible scenarios: *1*) GLP-1R expressed in these regions act in a presynaptic manner, i.e., they are located on terminals, which are potentially in distal sites where PPG axons occur; or *2*) GLP-1R in these regions receive GLP-1 from nonneuronal sources.

Hippocampus

This discrepancy raises the question of whether hippocampal neurons, indeed, express GLP-1 receptors, and if so, what is the source of the GLP-1 that the hippocampus detects. In the absence of reliable antibodies to detect GLP-1R (17), the answer was sought primarily by producing functional data from the hippocampus. For instance, the observation that GLP-1 elicits increases in firing rate in dissociated hippocampal neurons (71) led to the speculation that hippocampal GLP-1 may be involved in learning, memory, and even neuroprotection.

Mice deficient for the GLP-1R gene ($Glp1r^{-/-}$) showed an impaired ability to perform learning and memory tasks (1, 18), whereas overexpression of the GLP-1R in the hippocampus improved learning and memory (18). Furthermore, GLP-1 has been shown to elicit neuroprotective effects within the hippocampus. Pretreatment with GLP-1 in dissociated hippocampal neurons has been shown to be protective against glutamate-induced excitotoxicity, and oxidative stress-induced apoptosis (24, 107). This has led to a volume of work examining the potential for GLP-1-based therapeutics for the treatment of cognitive impairment and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (1, 3, 23, 55, 104).

In a mouse model of Alzheimer's disease, administration of the GLP-1 analog liraglutide significantly improved memory retention compared with vehicle-dosed controls (31). Furthermore, chronic administration of liraglutide in these mice resulted in significantly higher numbers of CA1 neurons compared with both wild-type and vehicle controls, suggesting liraglutide may confer proliferative capabilities in these neurons (31).

Obesity and Type 2 diabetes have long been known to contribute to cognitive decline (12, 104). Type 2 diabetes has been associated with an increase in the levels of Alzheimer's disease-associated hyperphosphorylated tau in the hippocampus (104). In a rat model of Type 2 diabetes, systemic administration of the GLP-1 analog Ex4 significantly reduced the

levels of hyperphosphorylated tau, compared with saline-treated controls (104). Furthermore, mice on a high-fat diet showed impaired cognitive scores, which was significantly improved during treatment with the GLP-1 analog lixisenatide (54). The mechanisms governing these pathologies are unclear; however, for obesity-induced cognitive decline, glycemic status appears to contribute (69). What remains to be clarified is whether the improvement in cognition and hyperphosphorylated tau levels is due to direct GLP-1 actions within the hippocampus, or indirectly through improved glycemic control.

The question of whether GLP-1 can exert direct actions within the hippocampus was addressed recently. Electrophysiological recordings in acute slices from rat hippocampus demonstrated that application of GLP-1 and Ex4 increased the amplitude and frequency of postsynaptic inhibitory currents in hippocampal CA3 pyramidal neurons (49). Additionally, a new transgenic mouse model expressing Cre-recombinase under the promoter for GLP-1R (79) demonstrated that GLP-1Rs are functional in a subset of hippocampal pyramidal cells (11), in support of the in situ hybridization data from the rat (65) and nonhuman primate (35).

In the absence of PPG fibers entering the hippocampus, this leaves open the question as to what is the source of GLP-1 to reach hippocampal GLP-1Rs under physiological conditions? Kanoski and colleagues (39) have proposed that PPG neurons might release GLP-1 into the cerebroventricular space and that hippocampal neurons might respond to GLP-1 in the cerebrospinal fluid. While these authors were able to detect GLP-1 in the cerebrospinal fluid, the question of the source of this GLP-1 remains open and raises speculations over the mode of GLP-1 release from PPG neurons.

PPG Neurotransmitter

The studies described above have demonstrated close appositions of PPG varicosities and terminals on other neurons, indicative of synaptic connections. However, while this provides a good indication as to whether cells receive inputs from PPG neurons, it cannot be taken for granted that this input is the synaptic release of GLP-1 onto those cells. It has recently been demonstrated in the rat that PPG neurons exhibit immunoreactivity for both GLP-1 and the vesicular glutamate transporter vGlut2 in their axon varicosities in the PVN and dorsomedial hypothalamic nuclei, suggesting that glutamate is a cotransmitter at synapses within the hypothalamus (111). Similarly, we have analyzed PPG axon varicosities in the spinal cord of mice expressing yellow fluorescent protein (YFP) in PPG neurons (PPG-YFP mice) and also detected the vesicular glutamate transporter vGlut2 inside these varicosities (Fig. 3), confirming the similarity between rat and mouse. These findings suggest that PPG neurons would primarily release glutamate, and the observed expression pattern for their axon and their innervation of specific cell types might be most relevant for their glutamatergic phenotype. F3

Neuropeptide release has been studied most extensively in the neurosecretory neurons of the hypothalamus. Here, neuropeptide containing dense core vesicles were rarely found at presynaptic sites but were more often observed throughout the axon shaft and somatodendritic complex (68). Furthermore, stimulation of these neurosecretory fibers showed putative neuropeptide release at nonsynaptic sites, such as cell bodies, axon shafts, and dendrites, in preference to synaptic complexes (reviewed in detail in Ref. 96). Once released, neuropeptides have the ability to diffuse through the interstitial space to targets distal from its site of release. This so-called "volume transmission"; whereby signals act through less defined spatiotemporal constraints, is predicated by the high affinity of neuropeptides to their receptors (picomolar-nanomolar range) compared with traditional synaptic receptors [nanomolar-micromolar range (90)]. So far, little attention has been paid to the mechanism of GLP-1 release. Electrophysiological studies have shown that GLP-1R have the affinity to respond to GLP-1 in the picomolar and nanomolar range (11, 49), suggesting they may respond to low levels of GLP-1 in the interstitial space.

The electron microscopy data provided by Zheng et al. (110, 111) demonstrates clearly that GLP-1 and glutamate are present in the same terminals; however, the study offers little insight into how the GLP-1 might be released. No clear dense core vesicles were observed in the cells analyzed by electron-microscopy, and thus, it remains unclear whether GLP-1 vesicles are released in a strictly synaptic fashion opposite postsynaptic densities or whether they might be released in a paracrine fashion, while only glutamate is released synaptically. AQ: 4

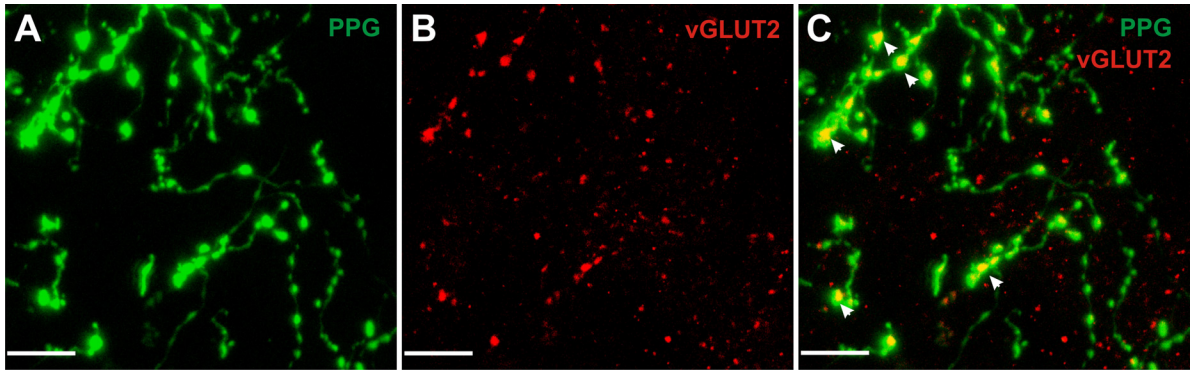

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Fig. 3. PPG neurons are glutamatergic. Confocal photomicrographs are shown from spinal cord sections obtained from PPG-YFP mice. *A*: YFP-immunofluorescence (green) revealing PPG axons in thoracic spinal cord. *B*: vGLUT2 immunofluorescence (red) revealed the presence of glutamatergic vesicles and, thereby, a glutamatergic cell phenotype, in the same spinal cord section as *A*. *C*: overlay of photomicrographs *A* and *B* reveals that vGLUT2 immunoreactivity is present inside PPG axon varicosities visualized in this section, as evident from colocalization of red and green signal (yellow; arrowheads). This suggests that spinally projecting mouse PPG neurons are glutamatergic (immunostaining produced by I. J. Llewellyn-Smith). Scale bar: 10 μm.

PPG Cells Outside the Lower Brain Stem

Both PPG neurons, specifically located in the granule cell layer (93), and GLP-1Rs, located to various cell populations (65), have been found within the olfactory bulb. Similarly, PPG neurons have been identified in the piriform cortex, where they appear to form a small subset of pyramidal neurons (58). These findings might indicate a further role for GLP-1 in olfactory signal processing (73). It is attractive to consider that GLP-1 could modulate the gain of olfactory pathways and, thus, the importance assigned to olfactory cues. While in a hungry state, the smell of foods could be perceived as intense and enticing, and yet, the same smell when satiated could have a significantly reduced effect on conscious thought. However, whether GLP-1 indeed plays any role in olfactory processing is currently purely speculative.

Most recently a population of PPG neurons has been discovered in the lower lumbar and upper sacral spinal cord (57). While the function of this population is currently unknown, ~20% of these cells received close appositions from PPG axon varicosities, and about 20% had close appositions from cholinergic axons. Around 15% of those cells with close appositions from cholinergic axons also had close appositions from PPG axons. These neurons were not ascending to the brain and did not leave the spinal cord like sympathetic or parasympathetic preganglionic neurons. Given their location within the spinal cord, it might be likely that they influence lumbar-sacral parasympathetic outflow, but this requires experimental validation.

Manipulation of PPG Cells In Vivo

While the data discussed thus far make a compelling argument for a significant role for PPG neurons in the physiological activation of GLP-1 receptors in the CNS, the ultimate proof that this is, indeed, the case is still missing, mainly because of difficulties in dissecting functional effects mediated by these neurons from those of systemic or exogenous GLP-1. The way forward here seems to be the selective manipulation of PPG neurons in vivo. Barrera et al. (8) led the way by using lentiviral mediated RNA interference to reduce the transcription of PPG in the NTS of rat. By stereotaxic injection of this virus, they managed to reduce PPG mRNA levels in the NTS by 50% while leaving PPG mRNA levels in the IRT unchanged. This treatment reduced the density of GLP-1 immunoreactive fibers in the PVN by ~30% and caused nocturnal hyperphagia leading to increased body weight. These results confirmed that PPG neurons clearly play a physiological role in the regulation of appetite. On the other hand, these authors failed to demonstrate any effect of NTS PPG knockdown on energy expenditure, including locomotor activity. Similarly, glucose tolerance was not altered independently of body weight. The latter findings were surprising, given the fact that GLP-1 injection into the CNS affects energy expenditure and glucose tolerance (46, 47, 84) and suggest that more research is needed to clarify whether these findings were a result of insufficient knockdown or genuinely indicate that PPG neurons are not involved in these processes. In any case, the above results from Barrera et al. (8) have confirmed a clear role for PPG neurons in the regulation of short-term food intake, and their finding of a correlation between NTS PPG mRNA levels and fat mass further suggests a role for central GLP-1 in the long-term control of body weight. This finding ties in with the observation in mouse that leptin activates these neurons (37).

Perspectives and Significance

At the beginning of this review, the question was posed: Could the small population of PPG neurons located in the lower brain stem account for all functions ascribed to GLP-1 in the CNS? The definitive answer is not there yet, but the anatomical and functional evidence reviewed here clearly support the notion that it is likely. What is needed now are more experiments along the lines started by Barrera et al. (8): studies that interfere directly with the activity of the PPG neurons in vivo. In fact, the recent development of a transgenic mouse strain that expresses Cre recombinase under the control of the glucagon promoter (74) has now opened the door for further manipulation of the activity of PPG neurons in vivo, and in addition to the approach taken by Barrera et al. (8), this mouse allows the simultaneous modulation of the release of GLP-1 and the cotransmitter glutamate from these cells. Given that neuropeptides often have a modulatory role rather than acting as the main transmitter, this approach may finally reveal the true importance of the PPG neurons in the regulation of energy balance.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.T. conception and design of research; S.T. and S.C.C. interpreted results of experiments; S.T. and S.C.C. prepared figures; S.T. and S.C.C. drafted manuscript; S.T. and S.C.C. edited and revised manuscript; S.T. approved final version of manuscript; S.C.C. performed experiments; S.C.C. analyzed data.

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AQ: 5

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