CCK Stimulation of GLP-1 Neurons Involves α₁-Adrenoceptor-Mediated Increase in Glutamatergic Synaptic Inputs

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OBJECTIVE—Glucagon-like peptide 1 (GLP-1) is involved in the central regulation of food intake. It is produced within the brain by preproglucagon (PPG) neurons, which are located primarily within the brain stem. These neurons project widely throughout the brain, including to the appetite centers in the hypothalamus, and are believed to convey signals related to satiety. Previous work demonstrated that they are directly activated by leptin and electrical activity of the afferent vagus. Another satiety hormone, cholecystokinin (CCK), has also been linked to activation of brain stem neurones, suggesting that it might act partially via centrally projecting neurones from the nucleus tractus solitarius (NTS). The aim of this study was to investigate the neuronal circuitry linking CCK to the population of NTS-PPG neurones.

RESEARCH DESIGN AND METHODS—Transgenic mice expressing yellow fluorescent protein (Venus) under the control of the PPG promoter were used to identify PPG neurones in vitro and to record their electrical and pharmacological profile.

RESULTS—PPG neurones in the NTS were excited by CCK and epinephrine, but not by the melanocortin receptor agonist melanotan II. Both CCK and epinephrine acted to increase glutamatergic transmission to the PPG neurones, and this involved activation of α₁-adrenergic receptors. Inhibition of adrenergic signalling abolished the excitatory action of CCK.

CONCLUSIONS—CCK activates NTS-PPG cells by a circuit involving adrenergic and glutamatergic neurones. NTS-PPG neurones integrate a variety of peripheral signals that indicate both long-term energy balance and short-term nutritional and digestion status to produce an output signal to feeding and autonomic circuits.

Glucagon-like peptide 1 (GLP-1) is a hormone produced by specialized endocrine cells in the intestinal epithelium (1) and a population of preproglucagon (PPG) neurons in the nucleus tractus solitarius (NTS) (2–5). Its physiological effects include the modulation of gastric emptying, glucose homeostasis, and appetite control, with both central and peripheral mechanisms likely contributing to its satiety evoking effects (7–15). Activation of central GLP-1 receptors seems likely to require release of GLP-1 from PPG neurones. In support of this hypothesis, a number of findings have suggested the involvement of the brain stem PPG neurones in appetite control. The immediate early gene cFOS, for example, is activated in PPG cells by peripheral satiety signals, such as gastric distension or systemically administered leptin (16,17).

The recent development of transgenic mice expressing eYFP (Venus) under the control of the PPG promoter (18) has enabled identification of this cell population in brain slice preparations and allowed the first characterization of PPG neurone activity in vitro (19). PPG neurones were shown to be directly regulated by leptin but were not affected by GLP-1 or peptide YY, two hormones that are released from enteroendocrine L-cells after a meal and have been shown to act as peripheral satiety signals.

Another peripheral satiety signal that might affect activity of PPG neurones is cholecystokinin (CCK). CCK, which is released postprandially from enteroendocrine L-cells, inhibits food intake and was actually the first gut-derived satiety hormone to be identified (20,21). It is well established that CCK acts on receptors in the periphery located on vagal afferent neurones that project to the nucleus of the solitary tract (22–24). Intraperitoneal application of CCK-8 induces cFOS immunoreactivity in pro-opiomelanocortin (POMC), catecholaminergic, and GLP-1-producing neurones in the vagal complex (25,26). However, recent studies on brain slice preparations have established that there are also direct effects of CCK within the lower brain stem (27). Although those experiments did not address the question of whether central CCK is released in a postprandial fashion, it is clear that microinjection of CCK-8 directly into the NTS suppresses food intake (28), thus suggesting that CCK acts locally within the NTS on neurones that integrate and relay satiety signals.

In this study, we examined the effect of CCK on the activity of PPG neurones, identified using the PPG-eYFP mouse strain (18). CCK-triggered activity was observed to be indirect, involving a complex neuronal network of catecholaminergic and glutamatergic signaling.

MATERIALS AND METHODS

Transgenic animals. Transgenic mice were used that expressed a modified yellow fluorescent protein (YFP; Venus) under the control of the PPG promoter (18). Two founder strains, mGlu-V23-124 and mGlu-V50-144, created using mouse BACs, were used interchangeably, since we observed no difference in the pattern of YFP expression in the brain stem. Animals were bred as heterozygotes on a C57/B6 background and were genotyped as described previously (18) before experimental use. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, with appropriate ethical approval.

Single-cell RT-PCR. Samples for RT-PCR and single-cell RT-PCR were harvested and amplified in a multiplex and nested PCR protocol as described previously (29), using primers listed in Table 1. Reverse-transcribed samples were split in half, with one half used to test for PPG and the other half used to test for CCKAR and CCKBR in a multiplex first PCR and individual nested PCRs. The first PCR reaction product (5 µL) was used as a template for the nested PCR. As negative controls for single-cell RT-PCR, reactions were
performed with solution from pipettes that were inserted into the slice without recording from a cell and with samples that were not reverse-transcribed. For positive controls, PCRs were performed on a 1:100 dilution of mouse brain stem cDNA.

**Electrophysiology.** Coronal (200 μm) brain stem slices were obtained from adult (>8 weeks) transgenic mice of either sex after halothane anesthesia and dissection in ice-cold low Na+ solution containing (in mmol/L): 200 sucrose, 2 DIABETES diabetes.diabetesjournals.org

### TABLE 1

| Primer           | PPG                        | CCK<sub>A</sub> receptor | CCK<sub>B</sub> receptor |
|------------------|----------------------------|---------------------------|--------------------------|
| Outer primer     |                            | TCAGTGGTGGGAAACGGCCTT     | TCTGGGACTGAGGCGGACGAG    |
| Inner primer     |                            | ACCTGGTCATAGGCCCCGAGC     | GAAGGACGGCCACGGTTGG     |
| Forward          | ATGAGACCGTTTATACGGTGCCT    | TGGTGCAAGGTTATCGAGA       |                          |
| Reverse          | CTGTGGAACAGGTTGCTGCCT      |                           |                          |
| Forward          | ACCAAGGGAACCGGGAACCCA     | TCTTGAAGTGCCGTGGCAAA      | GCCATCTGGAGACCACTGCAA    |
| Reverse          | CCAAGGTTCTTACGCTATGCGG    | CACCTGGCAACAGGGAGGC       | CCCCGTTGGTTCTGGAGCCG     |
| Product size (bp)| Outer                      | 492                       | 886                      |
|                  | Inner                      | 186                       | 285                      |

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established under DIC optics as described previously (19). All experiments for this study were performed on the spontaneously active population of PPG neurons only. Any burst-firing cells were discarded (19). In current clamp (perforated-patch configuration) spontaneously active cells had a resting potential of $-47 \pm 1$ mV ($n = 29$) and fired action potentials at a frequency of $1.5 \pm 0.2$ Hz ($n = 28$).

Bath application of 100 nmol/L CCK octapeptide, sulfated (CCK-8s), had no significant effect on the resting membrane potential or the input resistance (data not shown) but led to a reversible increase in action potential frequency by 104 ± 34%, in 7 of 15 cells tested (Fig. 1), whereas no changes were observed in the remaining cells. This CCK-8s-induced increase in firing rate was abolished by bath application of 20 μmol/L DNQX, a non-NMDA glutamate receptor antagonist, in 5 of 5 cells tested, suggesting that CCK-8s activated PPG cells via an increase in glutamatergic synaptic activity, rather than directly by activation of postsynaptic CCK receptors on PPG neurons. In agreement with these results, single-cell RT-PCR analysis demonstrated that m-RNA for the CCK receptors CCKAR and CCKBR is absent from PPG neurons ($n = 8$) but present in cDNA from mouse brain stem (Fig. 1E). These results indicated the presence of central CCK receptors, which might reside on either vagal afferent terminals or another cell population within the brain stem, but not on PPG neurons.

**CCK modulation of spontaneous EPSC activity.** The previous results suggested that CCK might exert its effects via enhancement of glutamatergic synaptic inputs. To explore this possibility further, voltage-clamp recordings were performed to isolate EPSCs. EPSCs had a frequency of $2.3 \pm 0.3$ Hz ($n = 53$) and a mean amplitude of $25 \pm 2$ pA. These were predominantly glutamatergic, since DNQX (10 μmol/L) inhibited spontaneous synaptic activity by 91 ± 5% (Fig. 2; $n = 6$). Application of 100 nmol/L CCK-8s caused a reversible increase in sEPSC frequency in ~60% PPG neurons tested (18 of 29; Fig. 2). The remaining cells showed no response.

Suppression of food intake by CCK-8s has previously been reported to be mediated by picrotoxin (30), and additionally CCK-8s has been shown to modulate γ-aminobutyric acid (GABAergic) activity in the rat NTS (31). To verify that CCK activation of PPG cell activity is not because of the inhibition of an ongoing inhibitory input from GABAergic or glycinergic neurons, the effects of the GABA<sub>A</sub> and glycine receptor antagonist picrotoxin were tested on sEPSC frequency. Bath application of 50 μmol/L picrotoxin under control conditions had no significant effect on sEPSC frequency, thus demonstrating the absence of a tonic inhibition of glutamatergic input ($n = 5$).
FIG. 1. CCK increases spontaneous activity of PPG neurons. A: Current-clamp recording demonstrating that bath application of 100 nmol/L CCK-8s led to an increase in spontaneous action potential firing frequency of this PPG neuron. B: A plot of the firing frequency for the recording shown in A (the part of the recording shown at top is indicated by gray background). C: Short segments of the original current-clamp recording from B at time points indicated by i, ii, iii, and iv. D: Mean data for firing frequency from experiments as depicted in A and B. CCK-8 (100 nmol/L) significantly increased firing rate. This effect of CCK-8s was occluded in the presence of the non-NMDA glutamate receptor antagonist DNQX. CCK had no effect on resting membrane potential (E_m) or resting input resistance (R_i). Number of recordings for each condition is given above the bars. *P < 0.05. E: Typical single-cell RT-PCR analysis for preproglucagon (ppg) and the CCK receptors (CCKAR, CCKBR) for three PPG neurons and controls. Agarose gel (2%) demonstrating that the 186-bp PCR product for PPG, the 285-bp PCR product for CCKAR, and the 341-bp product for CCKBR can be obtained from brain stem cDNA (1:100 dilution; positive control; indicated by arrows) with the primers specified in Table 1. In contrast, cytoplasm extracted from single cells showing eYFP fluorescence (cell1, cell2, cell3) was only positive for ppg, but not CCKAR or CCKBR (only bands for primers visible). Negative (neg) control: pipette solution without cytoplasm extracted from cell (negative control). Molecular weight ladder shows bands at 100-bp intervals. (A high-quality digital representation of this figure is available in the online issue.)
Furthermore, picrotoxin failed to affect the stimulatory effect of 100 nmol/L CCK-8s on EPSC frequency (Fig. 2; \(n=5\)), thus precluding the involvement of inhibitory interneurons at any stage of the CCK pathway of PPG cell activation.

We previously demonstrated that the majority of NTS PPG neurons receive direct (monosynaptic) glutamatergic input from vagal afferent fibers (19). As the cell bodies of primary afferent neurons in the solitary tract are lost in the slice preparation, their nerve terminals should not fire action potentials; if they were responsible for the tonic glutamatergic tone on PPG neurons, this should be TTX independent. However, bath application of 0.5 \(\mu\)mol/L TTX reduced the frequency of EPSCs by 63 \(\pm\) 7\% \((n=5;\) Fig. 2), thus indicating that sEPSC activity is partially a result of action potential–dependent electrical activity within the in vitro brain stem slice preparation. Preincubation with 0.5 \(\mu\)mol/L TTX also completely prevented the stimulatory effect of CCK-8 on sEPSC frequency \((n=5;\) Fig. 2).

**Postsynaptic effects of melanocortin receptor agonist on PPG neurons.** POMC neurons within the NTS were shown previously to be activated by peripherally administered CCK, as determined by cFOS staining (25,32). Furthermore, CCK administration failed to suppress food intake in the hyperphagic melanocortin receptor 4 (MC-4) knock-out mouse model (25). Consequently, we tested whether PPG neurons are electrically stimulated by MC-4 receptor activation. Bath application of the melanocortin receptor agonist MT-II (100 nmol/L) had no significant effect on firing rate \((1.5 \pm 0.7 \text{ Hz} vs. 1.7 \pm 0.8 \text{ Hz};\) Fig. 3), membrane potential \((-52 \pm 1 \text{ mV} vs. -52 \pm 2 \text{ mV}), or input resistance of PPG neurons \((n=7),\) thus making it unlikely that the observed effects of CCK on PPG cells are mediated via NTS POMC neurons.

**Postsynaptic effects of catecholamines on PPG neurons.** It has been shown previously that systemically applied CCK activates noradrenergic cells in the NTS (33). To investigate the hypothesis that these neurons might mediate the effects of CCK on PPG cells, catecholamines were applied to PPG neurons in current-clamp recordings. Epinephrine \((10 \mu\text{mol/L})\) reversibly increased the firing rate from 1.6 \(\pm\) 0.3 to 2.6 \(\pm\) 0.6 Hz in five of six PPG neurons tested (Fig. 3) and had no effect on the remaining cell. Similarly, norepinephrine at a concentration of 100 \(\mu\text{mol/L},\) but not 10 \(\mu\text{mol/L},\) increased the action potential frequency by 85 \(\pm\) 37\% in six of eight cells tested (Fig. 3). Dopamine \((10 \mu\text{mol/L})\), by contrast, had no effect on firing rate \((control 2.2 \pm 0.4 \text{ Hz};\) dopamine 2.1 \(\pm\) 0.5 Hz; \(n=5\)). None of the catecholamines tested had a significant effect on membrane potential or input resistance, suggesting that the action of epinephrine and norepinephrine was presynaptic. In support of this notion, the effect of 10 \(\mu\text{mol/L}\) epinephrine on action potential frequency was suppressed by 10 \(\mu\text{mol/L}\) DNQX (Fig. 3). Thus, similar to CCK, epinephrine and norepinephrine appear to activate PPG neurons by increasing glutamatergic input.

**Epinephrine increases spontaneous synaptic transmission.** The ionotropic glutamate receptor antagonist kynurenic acid \((1 \mu\text{mol/L})\) reduced the frequency of synaptic events by 93 \(\pm\) 4\% \((n=5;\) Fig. 4), whereas epinephrine \((10 \mu\text{mol/L};\) \(n=12)\) significantly increased the frequency of EPSCs (Fig. 4). However, in the presence of 1 \(\mu\text{mol/L}\) kynurenic acid the effect of epinephrine was occluded \((n=4)\), thus verifying that epinephrine acted by modulating the glutamatergic input onto the PPG cells.

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**FIG. 2.** CCK stimulation of sEPSCs is sensitive to TTX but not picrotoxin. A: The vast majority of sEPSCs in PPG neurons are glutamatergic as demonstrated by their inhibition by 10 \(\mu\text{mol/L}\) DNQX in this voltage-clamp recording at a holding potential of –70 mV. Bottom traces: Overlay of 15 consecutive 500 ms traces from the recording above under control conditions (top) and in the presence of DNQX (bottom). B: CCK-8s, bath-applied at 100 nmol/L, led to an increase in sEPSC frequency. C: Mean normalized effects of CCK on sEPSC frequency in the presence or absence of various drugs. The mean sEPSC frequency in presence of the drug \((freq_d)\) as a fraction of the frequency in the absence of any drug \((freq_c)\) is plotted. The excitatory CCK effect is not reduced by the GABA and glycine receptor antagonist picrotoxin \((30 \mu\text{mol/L})\) but prevented by TTX \((0.5 \mu\text{mol/L})\). *P < 0.05 compared with control; **P < 0.01 compared with control; #P < 0.05 compared with CCK. Numbers of cells tested are given above the bars.
...effect of CCK-8s on EPSC frequency (n = 5; Fig. 5). These results suggest that CCK acts on PPG neurons via modulation of adrenergic inputs.

**DISCUSSION**

Application of CCK led to a significant increase in the frequency of spontaneous glutamatergic EPSCs in ~50% of PPG neurons tested. This effect was sufficient to increase the firing frequency of these neurons and is therefore likely to be of physiological relevance. It is not clear whether the lack of response in the remaining PPG cells was caused by severed connections in the acute slice preparation or whether it reflects the existence of different subpopulations of PPG neurons that differ in their responsiveness to CCK. Similarly, ~20% PPG neurons failed to respond to epinephrine or norepinephrine with a change in electrical activity.

It is well established that CCK activates vagal primary afferents, interacting with CCK receptors in the periphery (22,23,34,35), but potentially also directly within the dorsal vagal complex (27,31,36). It has been shown that neurons of the NTS centralis were stimulated by CCK-8 via an increase in spontaneous glutamatergic but not GABAergic synaptic transmission (37), similar to the observation made here. In contrast with our current observations, however, Baptista et al. (37) found that the effect of CCK persisted in the presence of TTX, suggesting a direct presynaptic effect of CCK. In their study ~50% of those cells that responded to CCK were tyrosine hydroxylase immunoreactive, and thus catecholaminergic. Catecholaminergic NTS neurons tend to be second order neurons (38), but a follow-up study by Baptista and colleagues (27) showed that the CCK response persisted in vagally deafferented rats, thus suggesting that CCK does not necessarily act on vagal terminals within the NTS. Our present study also indicates that although PPG neurons are mainly second order neurons (19), CCK-8 has no effect on the vagal afferents impinging directly onto these cells, but likely involves the activation of catecholaminergic cells, which modulate the release of glutamate onto the PPG neurons (Fig. 6).

Although CCK-8 also activates POMC neurons in the NTS (25,32), our finding that the melanocortin receptor agonist Melanotan II had no effect on PPG neuron activity suggests that CCK stimulation of PPG neurons does not involve activation of MC-3 or MC-4 receptors.

**α2-Adrenergic modulation of PPG cell activity.** In the hypothalamus, norepinephrine action can elicit either eating or satiety, mirroring the receptor type involved (39). Within the PVN, activation of α1-adrenoceptors seems to elicit satiety (40) and activation of α2-adrenoceptors causes hyperphagia (41,42). The findings that the satiety factor CCK activates adrenergic/noradrenergic (A2) NTS neurons (37,43) suggest that these transmitters act as anorectic signals at the level of the NTS. This is supported by our finding that epinephrine/norepinephrine, like CCK and leptin (19), have an excitatory effect on PPG neurons (Fig. 6).

Resembling what is seen with CCK, epinephrine, or norepinephrine had no direct effect on PPG neurons, but modulated glutamatergic synaptic input to cause an increase in firing frequency. Similar to the findings in PVN, we observed that PPG neurons, which are likely to convey anorectic signals, were activated by the α1-receptor agonist phenylephrine but not by the α2-receptor agonists clonidine and dexmedetomidine. Intraperitoneal application of yohimbine, which is widely used as an α2-adrenergic receptor antagonist has, however, been reported to suppress feeding...
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FIG. 4. Epinephrine acts on α1-adrenoreceptors and increases the frequency of spontaneous glutamatergic EPSCs. A: Voltage-clamp recording from a PPG neuron at a holding potential (VH) of −70 mV demonstrating the effects of epinephrine on sEPSCs. B: Overlay of 15 consecutive 500 ms traces from the recording shown in A under control conditions (top) and in the presence of epinephrine (bottom). C: Overlay of 15 consecutive 500 ms traces under control conditions and in the presence of phenylephrine or clonidine, respectively, as indicated above each overlay. Phenylephrine, but not clonidine, led to an increase in sEPSC frequency. D: Mean normalized effects of epinephrine and selective α1- (phenylephrine) and α2- (clonidine, dexmedetomidine) adrenoreceptor agonists on sEPSC frequency. The mean sEPSC frequency in presence of the drug (frequD) is plotted as a fraction of the frequency in the absence of any drug (frequC). The effect of epinephrine is blocked by the glutamate receptor antagonist kynurenic acid (Kyn) and by the α-adrenergic receptor antagonist yohimbine. Yohimbine also blocked the effect of phenylephrine. Kyn itself blocked the majority of sEPSCs. *P < 0.05, **P < 0.01, compared with control. Numbers of cells tested are given above the bars.

Physiological relevance. In addition to the A1/C1 and A2/C2 neurons in ventrolateral medulla and NTS, respectively, catecholaminergic neurons have been found in the area postrema of rat. These neurons have been shown to express GLP-1 receptors and have been hypothesized to act as a link between circulating GLP-1 and activation of hypothalamus-projecting NTS PPG neurones (45). Our results presented here, however, indicate that there is no direct catecholaminergic input onto PPG neurons and instead that these cells are excited indirectly by adrenoreceptor activation. Although we showed previously that GLP-1 had no effect on PPG cell activity in the same brain stem slice configuration (19), our results are not compatible with the idea that the adrenergic population found here to modulate PPG neurones are themselves GLP-1 responsive. Because we recently demonstrated that NTS-PPG neurones send varicose axons into the action potential (5), an alternative explanation for the GLP-1 receptor–positive catecholaminergic cells in the action potential might be that they are themselves responsive to GLP-1 released from NTS-PPG neurones.

Although it is clear that gut-derived, as well as centrally injected, CCK causes satiety (20,28); at present, we do not know whether CCK receptors within the lower brain stem would be activated by peripherally released CCK that crosses the blood brain barrier or whether CCK released as a transmitter from central neurons or from vagal afferents serves these receptors. A number of studies have demonstrated CCK-immunoreactive fibers and even cell bodies in the caudal NTS of rodents (46–48). It has been suggested that a large fraction of these cell bodies are catecholaminergic (48), but it is unclear whether the CCK-immunoreactive fibers found in the NTS are of local origin (47) or represent vagal afferent fibers, as suggested by Palkovits et al. (46). In any case, it remains to be established whether the release of CCK within the NTS is in fact linked to food ingestion. It is, however, clear that CCK receptor activation within the NTS causes a reduction in food intake (28). Furthermore, intraperitoneal application of CCK-8 induces c-FOS immunoreactivity in the vagal complex, and immunohistochemical characterization of these c-FOS–positive cells revealed that they include POMC, catecholaminergic, and GLP-1-producing neurons (25,26).
A physiological role for PPG neurons in conveying anorectic signals is supported by the findings that they exhibit c-Fos immunoreactivity in response to leptin, CCK, and gastric distension (16,17,26) and are activated directly by leptin (19) and indirectly by CCK and epinephrine/norepinephrine in vitro. This suggests that NTS-PPG neurons integrate a variety of peripheral signals that indicate both long-term energy balance and short-term nutritional and digestive status to produce an output signal to feeding and autonomic circuits to optimize digestion and assimilation of nutrients and regulate caloric intake.

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K.H. researched data and edited the manuscript. F.R. and F.M.G. contributed to the study design and edited the manuscript. S.T. designed the study and wrote the manuscript.

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FIG. 6. Schematic representation of synaptic inputs to PPG neurons. PPG neurons (yellow) express leptin receptors (Hisadome et al. [19]) and receive direct glutamatergic input both from the tractus solitarius (TS; TTX-insensitive; Hisadome et al. [19]) and local glutamatergic neurons (green; TTX-sensitive). Input from adrenergic/noradrenergic neurons (pink) is indirect via $\alpha_2$-adrenergic receptors, activation of which enhances glutamatergic input to PPG neurons. CCK enhances the activity of PPG neurons. Its effect is occluded by either $\alpha_2$-adrenergic receptor antagonists or non-NMDA glutamate receptor antagonists. Thus, it acts either on (nor)adrenergic cells or presynaptic from those, as suggested by Baptista et al. (37). (A high-quality digital representation of this figure is available in the online issue.)
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