

Glucagon-Like Peptide 1 Excites Hypocretin/Orexin Neurons by Direct and Indirect Mechanisms: Implications for Viscera-Mediated Arousal

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Glucagon-like peptide 1 (GLP-1) is produced by neurons in the caudal brainstem that receive sensory information from the gut and project to several hypothalamic regions involved in arousal, interoceptive stress, and energy homeostasis. GLP-1 axons and receptors have been detected in the lateral hypothalamus, where hypocretin neurons are found. The electrophysiological actions of GLP-1 in the CNS have not been studied. Here, we explored the GLP-1 effects on GFP (green fluorescent protein)-expressing hypocretin neurons in mouse hypothalamic slices. GLP-1 receptor agonists depolarized hypocretin neurons and increased their spike frequency; the antagonist exendin (9–39) blocked this depolarization. Direct GLP-1 agonist actions on membrane potential were abolished by choline substitution for extracellular Na^+ , and dependent on intracellular GDP, suggesting that they were mediated by sodium-dependent conductances in a G-protein-dependent manner. In voltage clamp, the GLP-1 agonist Exn4 (exendin-4) induced an inward current that reversed near -28 mV and persisted in nominally Ca^{2+} -free extracellular solution, consistent with a nonselective cationic conductance. GLP-1 decreased afterhyperpolarization currents. GLP-1 agonists enhanced the frequency of miniature and spontaneous EPSCs with no effect on their amplitude, suggesting presynaptic modulation of glutamate axons innervating hypocretin neurons. Paraventricular hypothalamic neurons were also directly excited by GLP-1 agonists. In contrast, GLP-1 agonists had no detectable effect on neurons that synthesize melanin-concentrating hormone (MCH). Together, our results show that GLP-1 agonists modulate the activity of hypocretin, but not MCH, neurons in the lateral hypothalamus, suggesting a role for GLP-1 in the excitation of the hypothalamic arousal system possibly initiated by activation by viscera sensory input.

Key words: feeding; neuropeptide; lateral hypothalamus; gut; NTS; interoceptive; narcolepsy

Introduction

Glucagon-like peptide 1 (GLP-1) is synthesized in the brain (Han et al., 1986; Jin et al., 1988; Larsen et al., 1997a), in which it may act as a neuromodulator (Kreymann et al., 1989). GLP-1 has been detected in the caudal brainstem in a subset of neurons in the nucleus of the solitary tract (NTS), a key relay of interoceptive information from the gut (Larsen et al., 1997a; Merchenthaler et al., 1999; Saper, 2002). GLP-1-containing axons ramify widely throughout the hypothalamus, particularly in the paraventricular nucleus (PVN), arcuate, and lateral hypothalamic (LH) regions (Han et al., 1986; Jin et al., 1988; Rinaman, 1999). In parallel, high levels of GLP-1 receptor mRNA have been found in the hypothalamus (Merchenthaler et al., 1999). GLP-1 receptors belong to the G-protein-coupled receptor family (Thorens, 1992) and have been postulated to act by increasing intracellular cAMP levels via the stimulatory G-protein G_s (Drucker et al., 1987); the GLP-1

receptor has also been reported to couple with the inhibitory $G_{i/o}$ G-protein (Hallbrink et al., 2001).

The NTS, the primary source of GLP-1-containing neurons in the CNS, receives sensory information from the gut via the vagus nerve, and neurons here respond to a variety of gut sensory signals that relay information from gut stretch receptors and from gut fat and glucose sensors (Powley, 2000; Saper et al., 2002). GLP-1 neurons projecting to the hypothalamus may modulate blood pressure and heart rate (Yamamoto et al., 2002, 2003) and may initiate anxiety responses associated with interoceptive stress (Rinaman, 1999; Seeley et al., 2000; Kinzing et al., 2003). GLP-1 has also been postulated to reduce food intake (Tang-Christensen et al., 1996; Turton et al., 1996). CNS administration of GLP-1 inhibits food intake and increases Fos expression in the hypothalamus (Tang-Christensen et al., 1996; Turton et al., 1996; Larsen et al., 1997b). Repeated intracranial injections of GLP-1 or the GLP-1 receptor agonist exendin-4 (Exn4) cause a reduction in body weight (Meeran et al., 1999).

Hypocretin and melanin-concentrating hormone (MCH) are expressed by different cell populations in the LH (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998). Hypocretin enhances arousal (Hagan et al., 1999), potentially related to metabolic state (Yamanaka et al., 2003), and loss of hypocretin peptide or its receptors results in narcolepsy (Chemelli et al., 1999; Lin et al.,

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1999; Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). MCH neurons may also be involved in the regulation of energy homeostasis. MCH injections into the brain cause a robust increase in food intake (Qu et al., 1996; Rossi et al., 1999); blockade of the MCH receptor reduces body weight (Borowsky et al., 2002).

Although functional studies have shown many interesting effects of GLP-1 on behavior, we are unaware of any work on the cellular actions of GLP-1 in the CNS. Here, we studied the action of GLP-1 on the activity of hypocretin identified by green fluorescent protein (GFP) expression in hypothalamic slices. GLP-1 agonists consistently excited hypocretin neurons by direct postsynaptic, sodium-dependent mechanisms. GLP-1 enhanced release of glutamate from axons innervating hypocretin cells by a presynaptic mechanism. In contrast, GLP-1 agonists had no detectable effect on MCH neurons. These results suggest that GLP-1 acts selectively on the components of the LH that activate the hypothalamic arousal system.

Materials and Methods

Animals. To identify hypocretin cells, hypothalamic slices were prepared from transgenic mice [from Dr. T. Sakurai (University of Tsukuba, Tsukuba, Japan)] that selectively express GFP in hypocretin neurons (Li et al., 2002; Yamanaka et al., 2003). MCH-containing neurons were identified by selective GFP expression after recombinant adeno-associated virus (AAV-MCH) infection *in vivo* or by using transgenic mice that selectively express GFP in MCH cells (van den Pol et al., 2004); the electrophysiological properties of MCH neurons were similar with each method of identification. We used 2- to 3-week-old animals maintained in a normal 12 hr light/dark cycle. The Yale University Committee on Animal Care and Use approved all of the procedures used in this study.

Slice preparation. All of the slice preparation procedures were performed as previously described (Li et al., 2002; Acuna-Goycolea et al., 2004). Briefly, animals were deeply anesthetized (sodium pentobarbital, 100 mg/kg), and then the brain was rapidly removed and placed in ice-cold oxygenated (95% O₂–5% CO₂) solution that contained (in mM): 220 sucrose, 2.5 KCl, 6 MgCl₂, 1 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose, pH 7.4, with HCl. A block of tissue containing the hypothalamus was dissected, and coronal slices (200–300 μm) were cut in cold sucrose-artificial CSF (ACSF) with a vibratome. The hypothalamic slices were equilibrated with 95% O₂ and 5% CO₂ and incubated for 1–2 hr in an equilibrium chamber with standard ACSF containing (in mM): 124 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.23 NaH₂PO₄, 26 NaHCO₃, and 10 glucose, pH 7.4, with HCl. Then, a slice containing the LH was moved to a recording chamber mounted on an Olympus Optical (Tokyo, Japan) BX51WI upright microscope equipped with video-enhanced infrared-differential interference contrast (DIC) and fluorescence. Tissue was continuously superfused with a standard ACSF solution. The ACSF temperature in the chamber was maintained at 34–36°C using a dual-channel heater controller (Warner Instruments, Hamden, CT). Neurons were visualized with an Olympus Optical 40× water-immersion lens.

Electrophysiology. Low-resistance patch pipettes (4–6 MΩ) made from borosilicate glass tubing (World Precision Instruments, Sarasota, FL) using a PP-83 vertical puller (Narishige, Tokyo, Japan) were used for whole-cell current- and voltage-clamp recordings. Recording pipettes were filled with a pipette solution that contained (in mM): 145 KMeSO₄ (or KCl for IPSCs), 1 MgCl₂, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, and 0.5 Na₂-GTP, pH 7.3, with KOH. In the experiments in which calcium currents were studied, a CsCH₃SO₃-based pipette solution was used. This solution contained (in mM): 130 CsCH₃SO₃, 1 MgCl₂, 10 HEPES, 11 EGTA-Cs, 2 Mg-ATP, and 0.5 Na₂-GTP, pH 7.3, with CsOH. The bath solution used in these experiments contained (in mM): 78.5 NaCl, 40 TEA-Cl, 3 KCl, 2 MgCl₂, 5 BaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10

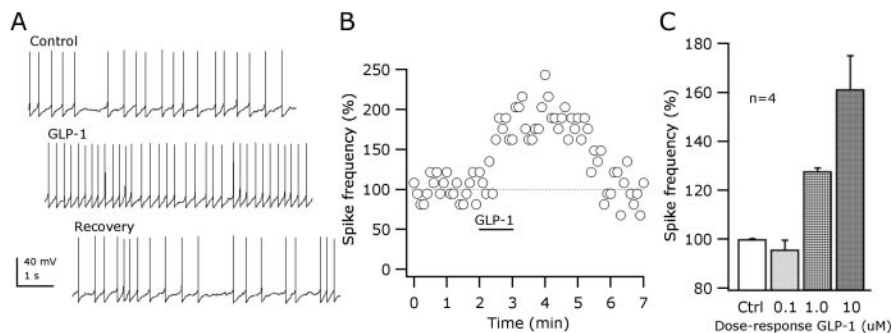


Figure 1. GLP-1 excites hypocretin neurons. *A*, Representative traces showing the excitatory effects of GLP-1 on the spike frequency. *B*, Time course of GLP-1 actions on firing rate in a typical hypocretin neuron. *C*, Bar graph shows the effect of different concentrations of GLP-1 on the firing rate of hypocretin neurons.

glucose, pH 7.4, with HCl. The hypocretin or MCH-expressing neurons were identified by the GFP fluorescence, and then DIC was used to achieve the cell-attached configuration in these cells. The whole-cell configuration was obtained after gentle application of negative pressure. Slow and fast compensation of the capacitance were automatically performed using Pulse software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Access resistance was continuously monitored during the experiments. Only those cells in which access resistance (changes <10%) was stable were included in the analysis. An EPC9 amplifier and Pulse software were used for data acquisition (HEKA Elektronik). PulseFit (HEKA Elektronik), Axograph (Axon Instruments, Foster City, CA), and Igor Pro (WaveMetrics, Lake Oswego, OR) software were used for analysis.

Event detection and statistical analysis. Excitatory and inhibitory synaptic currents as well as miniature activity were detected using Axograph software (Clements and Bekkers, 1997). The detection was performed comparing the spontaneous events with a simulated template, which was generated based on an exponential function as described previously (Gao and van den Pol, 2001). Briefly, a template was generated based on a double-exponential function in which the time course (rise and decay) as well as the width of a typical synaptic current was used. Then, the template function was moved along the recorded trace, and every single event was compared with the template. To distinguish the synaptic events from background noise, only those events with amplitude >5 pA were included in the analysis.

Statistical analyses were performed using one-way ANOVA test for between-group (i.e., control, treatment, recovery) comparisons. To detect pairwise differences, ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons was used. The nonparametric Kolmogorov–Smirnov test was used for comparison of the cumulative fractions of the amplitude before and after drug applications. A value of $p < 0.05$ was considered statistically significant; data are reported as means \pm SEM throughout.

Reagents. Some of the reagents used were purchased from Sigma (St. Louis, MO) and include the following: DL-2-amino-5-phosphonovaleric acid (AP-5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and bicuculline methiodide (BIC). Glucagon-like peptide 1 (7–36) amide was obtained from Bachem Bioscience (King of Prussia, PA), Exn4 from American Peptide Company (Sunnyvale, CA), exendin (9–39) amide (Exn_{9–39}) from Phoenix Pharmaceuticals (Belmont, CA), and tetrodotoxin (TTX) from Alomone Labs (Jerusalem, Israel).

Results

Glucagon-like peptide 1 excites hypocretin neurons

Using whole-cell current clamp, we evaluated the effect of GLP-1 on the spike frequency of hypocretin neurons in hypothalamic slices *in vitro*. Application of 1 μM GLP-1 significantly increased the frequency of action potentials, returning to control levels 4–10 min after peptide washout (Fig. 1*A,B*). Representative traces showing the GLP-1 effect on the firing rate of hypocretin neurons are presented in Figure 1*A*. The time course of the GLP-1 effect is shown in Figure 1*B*. The excitatory effect was

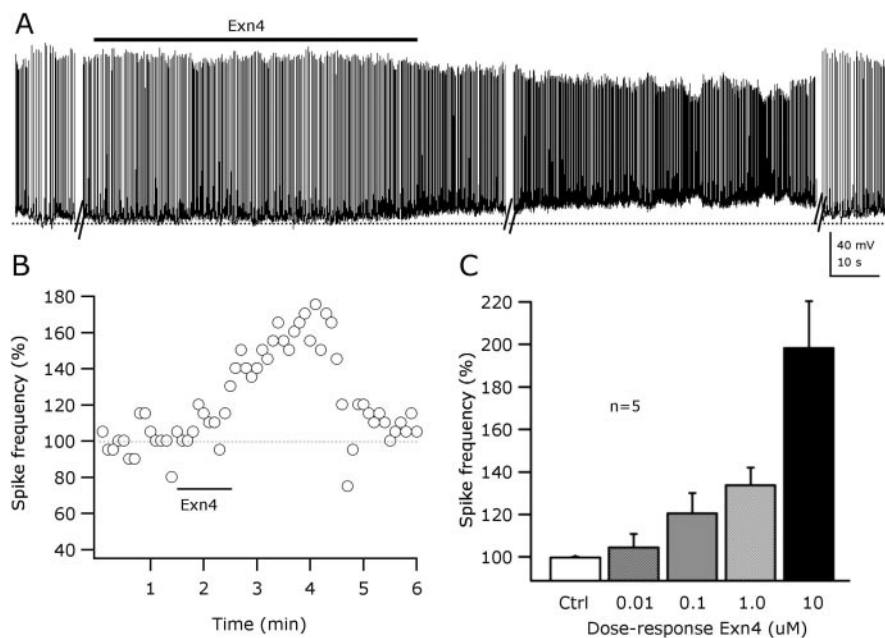


Figure 2. The protease-resistant long-acting GLP-1 receptor agonist Exn4 depolarizes and enhances spike frequency of hypocretin neurons. *A*, Exn4 depolarized the membrane potential and increased the spike frequency of hypocretin cells. *B*, Time course of the Exn4 actions on the frequency of action potentials in a typical cell. *C*, The Exn4 effects on spike frequency were dose dependent.

concentration dependent, with a mean increased spike rate induced by GLP-1 of $27.8 \pm 1.2\%$ at $1 \mu\text{M}$ and $61.3 \pm 13.7\%$ at $10 \mu\text{M}$ compared with pretreatment levels ($n = 4$; $p < 0.05$; ANOVA) (Fig. 1C). No significant effect on spike frequency was detected with 100 nM GLP-1 (Fig. 1C) ($n = 5$; $p = 0.83$; ANOVA).

Native mammalian GLP-1 is very sensitive to degradation by proteases (Goke et al., 1993; Thorens et al., 1993; Mentle, 1999; Yamamoto et al., 2002, 2003); for this reason, we additionally evaluated the effect of the protease-resistant long-acting GLP-1 receptor agonist Exn4 (Eng et al., 1992; Goke et al., 1993; Gallwitz et al., 2000) on the frequency of action potentials in hypocretin neurons. Exn4 ($1 \mu\text{M}$) induced a robust depolarization of the membrane potential and increased the firing rate of hypocretin neurons (Fig. 2A). The time course of the Exn4 effect is shown in Figure 2B. These GLP-1 receptor agonist actions were dose dependent, as revealed by the concentration–response analysis (Fig. 2C). Lower doses of Exn4 (10 nM) did not significantly affect the firing rate of hypocretin neurons ($4.8 \pm 5.9\%$ increase in the spike frequency in comparison with the control; $n = 5$; $p = 0.50$; ANOVA), whereas 0.1 , 1 , and $10 \mu\text{M}$ doses of Exn4 significantly increased firing ($n = 5$; $p < 0.05$; ANOVA). Together, these results are consistent with the view that the activation of GLP-1 receptors excites hypocretin neurons.

Direct actions of the GLP-1 receptor agonist in hypocretin neurons

G-protein-dependent direct action of GLP-1

The excitatory effect of the GLP-1 receptor activation described above might be explained by direct actions on membrane properties or by indirect modulation of the excitatory synaptic inputs to hypocretin neurons. We first evaluated the direct actions of $1 \mu\text{M}$ Exn4 in the presence of TTX in the bath. When $0.5 \mu\text{M}$ TTX was applied to the recorded cell under current clamp, a complete suppression of spontaneous action potentials was observed, consistent with the idea that action potentials in hypo-

cretin neurons are attributable to the activation of voltage-dependent sodium channels ($n = 6$; data not shown). In the presence of TTX, Exn4 was delivered to the hypocretin cells for 1–2 min. Under these conditions, Exn4 resulted in a robust and long-lasting depolarization of the membrane potential in hypocretin cells (Fig. 3A, trace 1). The membrane potential was depolarized by $7.8 \pm 2.5 \text{ mV}$ in the presence of Exn4 and returned to pretreatment levels after 3–10 min of Exn4 washout (Fig. 3B). These effects were statistically significant (ANOVA; $n = 6$; $p < 0.05$). In voltage clamp, holding the cell at -60 mV with TTX ($0.5 \mu\text{M}$) in the bath, Exn4 induced an inward current ($n = 8$; data not shown). The mean peak amplitude of the Exn4-induced current ($25.7 \pm 5.4 \text{ pA}$) was reached $\sim 1 \text{ min}$ after peptide application. Recovery was observed after 5–15 min of Exn4 washout.

We performed control experiments with the GLP-1 receptor antagonist Exn_{9–39} (Eng et al., 1992; Raufman et al., 1992) in the bath solution. All of the experiments were done with TTX ($0.5 \mu\text{M}$) in the external solution. Exn_{9–39} ($1 \mu\text{M}$) was added to the bath 3–7 min before Exn4 application. In the presence of Exn_{9–39}, Exn4 did not depolarize the membrane potential of hypocretin cells (Fig. 3A, trace 2). In nine cells tested under these conditions, Exn4 ($1 \mu\text{M}$) shifted the membrane potential by $0.7 \pm 1.4 \text{ mV}$, a nonsignificant effect (Fig. 3B) ($p > 0.05$; ANOVA followed by a Bonferroni procedure for multiple comparisons). These results suggest that the direct depolarizing Exn4 actions are dependent on the specific activation of GLP-1 receptors in hypocretin neurons.

GLP-1 receptors have been suggested to couple to the G_s-mediated signal transduction pathway (Drucker et al., 1987). To address this issue, we studied the GTP dependence of the Exn4 depolarizing effects by using the nonhydrolyzable GDP analog GDPβS in the recording pipette. These experiments were performed in the presence of TTX ($0.5 \mu\text{M}$) to block spike-dependent synaptic activity, and AP-5 ($50 \mu\text{M}$), CNQX ($10 \mu\text{M}$), and BIC ($30 \mu\text{M}$) to reduce the possibility of detecting an Exn4 effect on presynaptic glutamate and/or GABA terminals. After 5–10 min of dialysis and with GDPβS included in the pipette solution, Exn4 did not evoke significant depolarizing responses in hypocretin cells (Fig. 3A, trace 3;B) (change in membrane potential, $1.5 \pm 0.9 \text{ mV}$; $n = 6$; $p = 0.74$; ANOVA). These experiments confirm that hypocretin cells express functional GLP-1 receptors on their somatodendritic region and that GDPβS blocks the actions of Exn4, suggesting that activation by Exn4 depolarizes the membrane potential of hypocretin neurons in a GTP-dependent manner, acting via G-proteins.

Na⁺, K⁺, and Ca²⁺ channels

To study the Na⁺ contribution to the Exn4-induced depolarization in hypocretin neurons, we performed experiments in which NaCl was replaced by an equimolar concentration of choline chloride in the bath solution in the presence of TTX (Shen and North, 1992; Liu et al., 2002). When choline was substituted for sodium, the Exn4-induced depolarization was almost completely

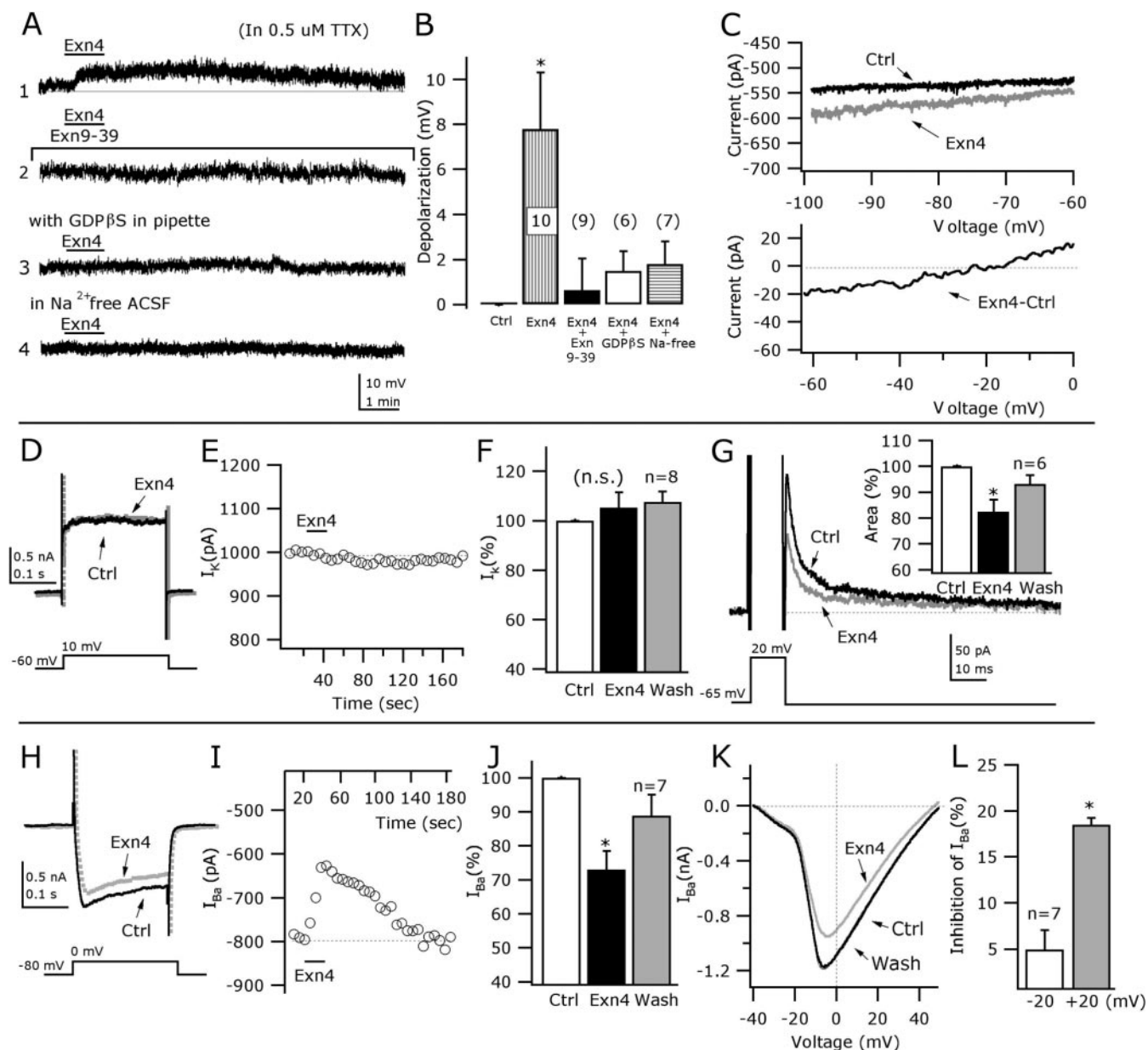


Figure 3. Direct actions of GLP-1 agonist on hypocretin neurons. *A*, Trace 1, With $0.5 \mu\text{M}$ TTX in the bath, Exn4 depolarized the hypocretin cell membrane potential. Trace 2, In the presence of the GLP-1 receptor antagonist Exn $_{9-39}$ in the external solution, Exn4 failed to depolarize the membrane potential. Trace 3, The depolarizing actions of Exn4 were blocked by intracellular GDP βS . Trace 4, When extracellular NaCl was replaced by an equimolar concentration of choline chloride, the depolarizing effects of Exn4 were depressed. *B*, Bar graph summarizing the Exn4 action on the membrane potential in normal, Exn $_{9-39}$ -containing ACSF, low intracellular GTP, and Na $^{+}$ -free extracellular conditions. *C*, Exn4 ($1 \mu\text{M}$) induced an inward current at voltages between -100 and -60 mV (top graph). The Exn4-induced current showed a reversal potential consistent with a nonspecific cationic conductance (trace from one cell shown in bottom graph). *D–F*, Exn4 did not evoke a detectable change on potassium currents activated by a voltage step from -80 to 0 mV for 200 msec. n.s., Not significant. *G*, Exn4 depressed afterhyperpolarization currents in hypocretin neurons. In the presence of Exn4, the time integral of I_{AHP} was reduced by 17.5% (bar graph). *H–L*, Exn4 depressed calcium currents in hypocretin neurons. *H*, Exn4 depressed the current responses evoked by a voltage step from -80 to 0 mV for 200 msec. The time course and mean effect of Exn4 on calcium currents are shown in *I* and *J*, respectively. * $p < 0.05$. *K*, Current response in the presence and the absence of Exn4 in a typical hypocretin neuron after a voltage ramp from -40 to $+50$ mV. *L*, When the relative inhibitory effect of Exn4 on calcium currents was compared at -20 and $+20$ mV, a significant difference was detected suggesting that the GLP-1 agonist actions were voltage dependent. Ctrl, Control; Wash, washout. *Statistically significant, $p < 0.05$.

abolished in all of the cells tested (Fig. 3*A*, trace 4; *B*) ($n = 7$). With choline chloride in the extracellular solution (Na $^{+}$ -free solution), Exn4 changed the membrane potential by only 1.8 ± 1.1 mV ($p = 0.76$; ANOVA; not significant). When Exn4 depolarizing effects in the presence of NaCl or choline chloride in the bath were compared, a statistically significant difference was detected, suggesting that the Exn4 actions on the membrane potential were primarily dependent on extracellular Na $^{+}$ ($p < 0.05$; ANOVA). Some excitatory peptidergic sodium-dependent actions have

been associated with activation of nonspecific cationic conductances (Liu et al., 2002; Yang et al., 2004). As indicated above, Exn4 induced an inward current when hypocretin cells were held at -60 mV under voltage clamp. To determine the reversal potential of this Exn4-induced current, slow voltage ramp protocols (from -100 to 0 mV for 5 sec) were delivered to the hypocretin cells. These experiments were done in the presence of TTX ($0.5 \mu\text{M}$) in the bath and using Cs-based pipette solution to block the voltage-dependent K $^{+}$ currents that could be activated by these

depolarizing protocols. The application of Exn4 resulted in a consistent inward current ($n = 5$) (Fig. 3C, top graph), which showed a mean reversal potential near -28.4 ± 3.1 mV ($n = 5$) (Fig. 3C, bottom graph), consistent with a nonselective cationic current. In additional experiments, we tested a nominally calcium-free–high-magnesium (5 mM) extracellular solution to evaluate the extracellular calcium contribution to the Exn4-induced excitation in hypocretin cells. In the Ca^{2+} -free–high- Mg^{2+} ACSF, we still observed the Exn4-induced current in hypocretin cells ($n = 6$; data not shown). No decrease in the size of the Exn4-induced current was detected under these conditions, consistent with the view that Exn4 alters a nonselective cationic conductance in hypocretin cells (Formenti et al., 2001; Liu et al., 2002).

To determine whether Exn4 might modulate voltage-dependent potassium currents (I_K), we studied the current responses of hypocretin neurons after a voltage step from -60 to $+10$ mV (Gao and van den Pol, 2001). All of these experiments were done in the presence of TTX ($0.5 \mu\text{M}$) and CdCl_2 ($200 \mu\text{M}$) in the bath to block the voltage-dependent sodium and calcium channels, respectively. The application of Exn4 to the bath did not significantly modify the K^+ current, as shown in the traces of Figure 3D. A time course graph showing the lack of Exn4 effect on the peak I_K is presented in Figure 3E. In the presence of Exn4, the normalized amplitude of potassium currents were changed by $5.4 \pm 6.2\%$ compared with the control levels, a nonsignificant effect (Fig. 3F) ($n = 8$; $p = 0.81$; ANOVA). Additionally, the modulation of net whole-cell K^+ currents was studied with voltage ramp protocols from -100 to $+40$ mV in the presence of TTX and CdCl_2 in the bath. Under these conditions, the application of Exn4 ($1 \mu\text{M}$) did not modify the current response of eight hypocretin cells tested (data not shown), suggesting that the activation of GLP-1 receptors does not induce any detectable effect on the voltage-dependent K^+ currents studied.

Another important determinant of the excitability of many neurons is the afterhyperpolarization (AHP), a hyperpolarizing shift in the membrane potential at the end of an action potential (Sah, 1996). Some of the K^+ current that underlies this hyperpolarization of the membrane potential is mediated by voltage-insensitive Ca^{2+} -activated K^+ channels and may be modulated by a number of neurotransmitters, including some agonists of G_s -protein-coupled receptors (Stocker et al., 1999). To study the possible Exn4 modulation of hyperpolarizing currents (I_{AHP}) in hypocretin neurons, we used voltage-clamp protocols holding the cells at -65 mV. The I_{AHP} was detected after a voltage step from the holding potential to $+20$ mV for 10 msec (Fig. 3G, bottom). This protocol has been reported to induce a single unclamped action potential (Faber and Sah, 2002), allowing us to study in a quantitative manner the currents underlying single-spike hyperpolarization. After the voltage step, an outward current was detected with a mean amplitude of 93.4 ± 4.7 pA ($n = 6$) (Fig. 3G). The application of Exn4 to the bath resulted in a consistent decrease in the time integral of the I_{AHP} with no evident change in its kinetics. In the presence of Exn4 ($1 \mu\text{M}$), the time integral of the I_{AHP} was reduced by $17.5 \pm 4.5\%$ compared with the pretreatment value, a statistically significant effect (Fig. 3G, bar graph) ($n = 6$; $p < 0.05$; ANOVA). Together, these experiments suggest that the activation of GLP-1 receptors may increase the activity of hypocretin neurons, in part by reduction of the AHP current in these cells.

We also evaluated the Exn4 actions on voltage-dependent calcium currents, using CsCH_3SO_3 pipettes. In these experiments, TEA-Cl (40 mM)-containing ACSF was used as the extracellular

solution to inhibit potassium currents (the same amount of NaCl was removed to preserve the osmolarity in the bath), BaCl_2 was substituted for CaCl_2 to increase the conductance at the calcium channels, and the voltage-dependent sodium currents were blocked by addition of TTX ($0.5 \mu\text{M}$) to the bath. To activate the I_{Ba} , a voltage step from -80 to 0 mV for 200 msec was delivered to the hypocretin cells (Fig. 3H, bottom). Under these conditions, the application of Exn4 resulted in a consistent decrease in the I_{Ba} , as shown in the representative traces of Figure 3H (also I, J). The time course of the Exn4 actions on I_{Ba} is presented in Figure 3I; the maximum effect was reached ~ 30 sec after peptide application. In seven cells tested, Exn4 decreased the I_{Ba} by $27 \pm 5.4\%$, a statistically significant effect (Fig. 3J) ($p < 0.05$; ANOVA). Application of CdCl_2 ($200 \mu\text{M}$) to the recorded cells resulted in a complete abolition of the current response to the voltage steps, confirming that they were attributable to the activation of calcium channels ($n = 7$; data not shown). We next evaluated the voltage dependence of the Exn4 effects on the calcium channels. To address this issue, a voltage ramp command (from -60 to $+50$ mV for 100 or 300 msec) was delivered to the hypocretin cell, and the current responses in the presence and the absence of Exn4 were determined. The presence of Exn4 in the bath resulted in a consistent decrease in the current response of the hypocretin cells after the voltage ramp protocol (Fig. 3K). The relative effects of Exn4 on I_{Ba} showed some voltage dependence as suggested by the comparison of the Exn4 effect at potentials of -20 and $+20$ mV (Fig. 3K, L) ($n = 6$; $p < 0.05$; ANOVA). Together, these results support the view that the activation of GLP-1 receptors modulates, in a voltage-dependent manner, calcium channels in hypocretin cells.

GLP-1 enhances excitatory synaptic transmission in hypocretin neurons

Parallel to their direct actions on the membrane properties, the activation of GLP-1 receptors might modulate the synaptic inputs to the hypocretin cells. To address this question, we first evaluated the effect of Exn4 on the frequency of the spontaneous synaptic currents in hypocretin cells. Whole-cell voltage-clamp and KMeSO_4 pipettes were used in these experiments. Cells were held at -60 mV. Under these conditions, Exn4 ($1 \mu\text{M}$) reversibly increased the frequency of the postsynaptic currents in six cells tested, as shown in Figure 4A. These Exn4 actions were attributable to the GLP-1 receptor activation; in the presence of the GLP-1 receptor antagonist Exn_{9-39} ($1 \mu\text{M}$) in the external solution, no effect of Exn4 on the PSC frequency was detected (Fig. 4A, filled circles). Raw traces showing the typical Exn4 effect on the frequency of the PSCs in normal and Exn_{9-39} -containing ACSF are presented in Figure 4, B and C, respectively. The application of Exn_{9-39} to the recorded cells did not alter the PSC frequency, suggesting a lack of tonic GLP-1 receptor activation (Fig. 4D) ($n = 6$; $p > 0.05$; ANOVA and Bonferroni *post hoc* test). In $1 \mu\text{M}$ Exn4, the frequency of PSCs increased by $23.9 \pm 8.3\%$ compared with the pretreatment control level (range, 13.9 – 41.9%) (Fig. 4D). This effect was reversible and statistically significant ($n = 11$; $p < 0.05$; ANOVA). With Exn_{9-39} in the bath, Exn4 changed the PSC frequency by $3.4 \pm 2.3\%$, a nonsignificant effect (Fig. 4D) ($n = 9$; $p > 0.05$; ANOVA and Bonferroni *post hoc* test). These results are consistent with the view that specific activation of GLP-1 receptors can indirectly alter the activity of hypocretin neurons by increasing transmitter release from axons in synaptic contact with hypocretin neurons.

To test the hypothesis that the activation of GLP-1 receptors enhances the glutamate-mediated synaptic inputs to hypocretin

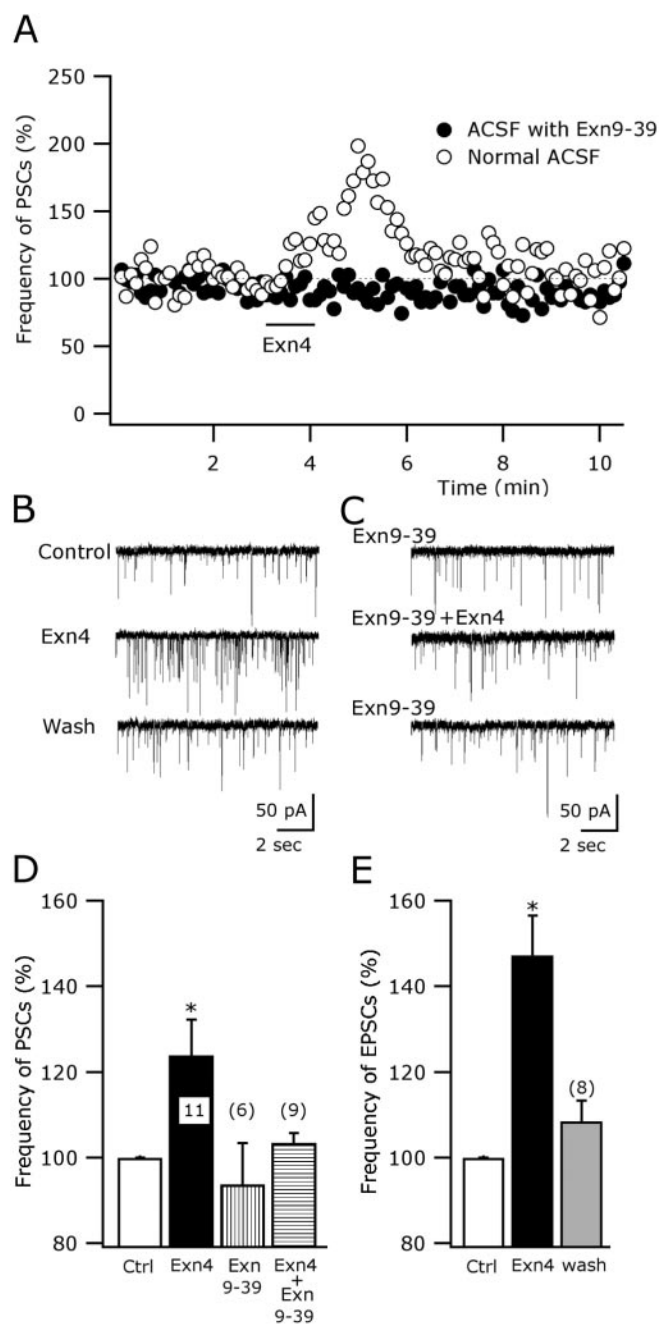


Figure 4. GLP-1 modulates excitatory synaptic inputs to hypocretin neurons. *A*, Time course of Exn4 effects on the frequency of hypocretin postsynaptic currents in normal (unfilled circles) and Exn₉₋₃₉-containing (filled circles) ACSF. In this experiments, the cells were held at -60 mV. *B*, Raw traces show the excitatory effect of Exn4 on the PSC frequency. *C*, With the GLP-1 receptor antagonist in the bath, Exn4 failed to increase the PSC frequency. *D*, This bar graph shows the mean effect of Exn4, Exn₉₋₃₉, and Exn4 plus Exn₉₋₃₉ on the PSC frequency. *E*, In the presence of $30 \mu\text{M}$ BIC in the bath and using KMeSO₄ pipettes, EPSCs were recorded. Exn4 increases the EPSC frequency, as shown in this bar graph. *Statistically significant. Ctrl, Control; Wash, washout.

neurons, we evaluated the effect of Exn4 on the frequency of the EPSCs. Recordings were done in voltage-clamp mode holding the cells at -60 mV. To selectively isolate the EPSCs, we bath applied the GABA_A receptor antagonist BIC ($30 \mu\text{M}$). Under these conditions, Exn4 ($1 \mu\text{M}$) reversibly enhanced the frequency of EPSCs, as shown in Figure 4*E*. One micromolar Exn4 significantly increased the EPSC frequency by $47.2 \pm 9.3\%$ ($n = 8$; $p < 0.05$;

ANOVA). After 5–10 min of peptide washout, the EPSC frequency returned to the control level (Fig. 4*E*). To confirm the glutamatergic nature of these excitatory currents, we then bath applied the AMPA and NMDA glutamate receptor antagonists CNQX ($10 \mu\text{M}$) and AP-5 ($50 \mu\text{M}$), respectively ($n = 7$; data not shown). After glutamate receptor blockade, a complete suppression of the synaptic activity was detected in all of the cells tested, suggesting that most of the postsynaptic excitatory currents in hypocretin cells are attributable to glutamate actions on their ionotropic receptors ($n = 7$; data not shown). The effect of the native GLP-1 on the frequency of EPSCs was also evaluated. The addition of $1 \mu\text{M}$ GLP-1 to the bath resulted in a substantial and reversible increase in the EPSC frequency (data not shown). The frequency of the EPSCs increased by $34 \pm 3.5\%$ compared with the pretreatment levels, a statistically significant effect ($n = 7$; $p < 0.05$; ANOVA). Together, our data show that the activation of GLP-1 receptors indirectly excites hypocretin neurons by increasing the excitatory, glutamate-mediated, tone onto these neurons.

Presynaptic modulation of excitatory transmission by GLP-1 agonists

To further evaluate the actions of GLP-1 and Exn4 on the glutamatergic axon terminals innervating hypocretin cells, we used TTX ($0.5 \mu\text{M}$) in the bath solution, and recorded the miniature EPSCs (mEPSCs) in the presence of $30 \mu\text{M}$ BIC. When AP-5 and CNQX were added to the medium, all of the excitatory miniature activity was abolished, confirming that these events are attributable to spontaneous glutamate release onto the recorded cells ($n = 7$; data not shown). Exn4 increased the frequency of the miniature excitatory activity (Fig. 5*A, B*). In some cells (Fig. 5*A*), we applied Exn4 twice to confirm its excitatory effects on the mEPSC frequency. Both the first and second application of Exn4 evoked an increase in the frequency of mEPSCs. The Exn4 effects were reversible, because the frequency of mEPSCs returned to pretreatment levels after 5–10 min of peptide washout (Fig. 5*C*). In five cells tested, Exn4 evoked a mean increase of $36.3 \pm 10.7\%$ in the mEPSC frequency ($n = 5$; $p < 0.05$; ANOVA). We further asked whether Exn4 could modulate the mEPSC amplitude. To address this question, the cumulative fractions of the amplitude in control and Exn4 conditions were compared. Kolmogorov–Smirnov analysis showed no significant change in the cumulative fractions of the amplitude after Exn4 ($1 \mu\text{M}$) application in the five cells tested ($p > 0.05$; Kolmogorov–Smirnov test) (Fig. 5*D*). Exn4 had no effect on mean mEPSC amplitude. The mean mEPSC amplitude was 24.7 ± 3.5 , 25.7 ± 2.1 , and 24.1 ± 2.9 pA in control, Exn4, and recovery conditions, respectively (ANOVA analysis; $p = 0.87$).

Additional experiments evaluating the effect of GLP-1 on both the mEPSC frequency and amplitude were conducted. Similar to Exn4, GLP-1 ($1 \mu\text{M}$) significantly enhanced the mEPSC frequency by $22.8 \pm 5.6\%$ (Fig. 5*E*) ($n = 8$; $p < 0.05$; ANOVA). After washout of the peptide, the frequency of the mEPSCs dropped back to control levels (Fig. 5*E*). Exn4 did not evoke any significant change in the cumulative distribution of the mEPSC amplitude in eight of eight cells (Fig. 5*F*) ($n = 8$; $p > 0.05$; Kolmogorov–Smirnov).

Because all of the excitatory synaptic activity was abolished by bath addition of the selective glutamate receptor antagonists AP-5 and CNQX, our results are consistent with the view that the activation of the GLP-1 receptors modulates the release of glutamate onto hypocretin cells, primarily by enhancing release from

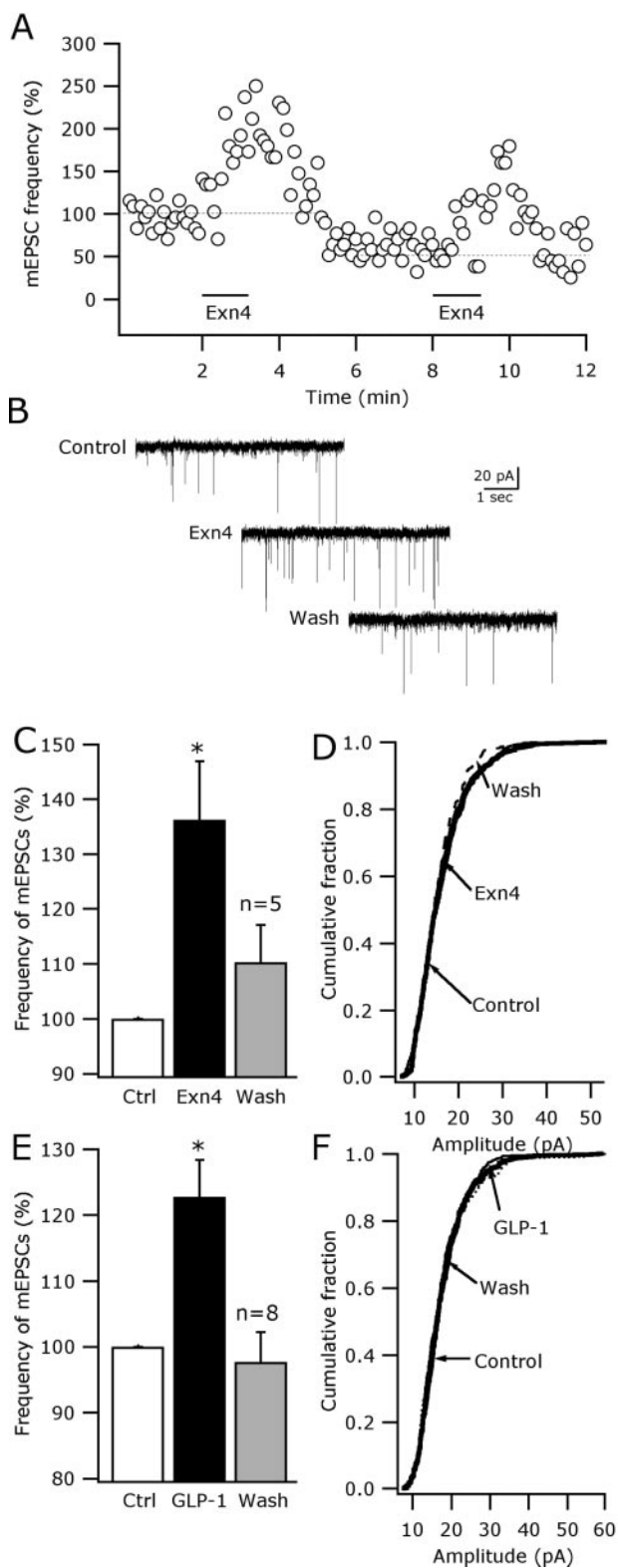


Figure 5. Presynaptic modulation of glutamate release by GLP-1 receptor agonists. *A*, Exn4 enhanced the frequency of mEPSCs. In this particular case, the GLP-1 agonist was applied twice to the recorded cell, evoking repeatable increases in the frequency of mEPSCs. *B*, Raw traces showing the excitatory effect of Exn4 on the frequency of mEPSCs. *C*, This bar graph shows the mean effect of Exn4 on the mEPSC frequency. *D*, The application of Exn4 to the bath failed to evoke a change in the cumulative distribution of the mEPSC amplitude in five cells tested. *E*, GLP-1 also enhanced the mEPSC frequency in hypocretin neurons, but did not modify the cumulative distribution of the mEPSCs amplitude (*F*). *Statistically significant. Ctrl, control; Wash, washout.

the presynaptic glutamatergic terminals that innervate hypocretin neurons.

Exendin-4 actions on inhibitory synaptic inputs to hypocretin cells

Most of the inhibitory synaptic transmission in the hypothalamus appears to be mediated by GABA (Decavel and van den Pol, 1990); we next studied the GLP-1 receptor modulation of the inhibitory, GABA-dependent, synaptic input to hypocretin neurons. AP-5 (50 μM) and CNQX (10 μM) were bath-applied to selectively suppress the glutamatergic synaptic transmission in hypocretin cells. To identify the IPSCs, KCl-filled pipettes were used. Under these conditions, IPSCs were detected as inward currents when the cells were held at -60 mV. Application of 1 μM Exn4 resulted in an increase in the frequency of the IPSCs in hypocretin neurons (Fig. 6*A*, raw traces). The Exn4 effect on IPSC frequency was reversible, because recovery was observed after peptide washout (Fig. 6*B*). The frequency of IPSCs was significantly increased by 26.4 ± 7.6% after Exn4 and returned to control levels when the peptide was washed out (*n* = 5; *p* < 0.05; ANOVA). The inhibitory activity was completely abolished by bicuculline (30 μM), confirming that it is attributable to the activation of GABA_A receptors (*n* = 8; data not shown). These results support the idea that GLP-1 receptors modulate the inhibitory, GABA_A receptor-dependent, synaptic inputs to hypocretin neurons.

To test whether Exn4 acted at the presynaptic GABAergic axon innervating hypocretin neurons, we recorded miniature IPSCs (mIPSCs) in the presence of 0.5 μM TTX to block the spike-dependent transmitter release, and AP-5 (50 μM) and CNQX (10 μM) to block the excitatory, ionotropic glutamate receptor-mediated, inputs to hypocretin cells. When Exn4 was applied to the bath, a significant and reversible increase in the mIPSC frequency was detected (Fig. 6*D*) (*n* = 4; *p* < 0.05; ANOVA). The frequency of mIPSCs was enhanced by 42.5 ± 14.3% in the presence of the GLP-1 agonist, returning to the control levels after Exn4 washout. Changes in the frequency of the miniature postsynaptic potentials have been taken as indications for presynaptic site modulation (Bekkers and Stevens, 1995). Additionally, we evaluated the Exn4 effects on the mIPSC amplitude. In the presence of Exn4 (1 μM), the cumulative distributions of the amplitude were significantly right-shifted in four cells tested, as shown in Figure 6*E* (*p* < 0.05; Kolmogorov-Smirnov), suggesting that Exn4 may postsynaptically modulate GABAergic actions. Previous studies have shown that G-protein, particularly G_s, activation can enhance GABA or glycine receptor-activated Cl⁻ channels (Song and Huang, 1990; Brandon et al., 2002), consistent with the present data.

GLP-1 receptor agonist actions on MCH and PVN neurons

MCH neurons are found in the LH near the hypocretin neurons and are postulated to play an important role in energy homeostasis (Qu et al., 1996; Shimada et al., 1998). GLP-1 receptor expression and GLP-1 immunoreactive fibers and boutons have been detected in this hypothalamic region, suggesting a possible role for GLP-1 in the modulation of the MCH neuron activity. To address this issue, we studied the GLP-1 agonist effects on the activity of MCH neurons in hypothalamic slices of transgenic or AAV-MCH-infected mice that show selective GFP expression in MCH neurons. We first evaluated the effect of Exn4 on spike frequency and membrane potential under current clamp. As described previously, the MCH neurons show very low or no spontaneous firing under resting conditions (Eggermann et al., 2003;

Gao et al., 2003; van den Pol et al., 2004). When Exn4 ($1 \mu\text{M}$) was added to the bath, no detectable change in the firing rate or the membrane potential of MCH neurons was observed ($n = 7$; $p > 0.05$; ANOVA) (Fig. 7A, trace 3), revealing little direct effect of the GLP-1 agonist on these cells. Similarly, Exn4 had no effect on the membrane potential in the presence of TTX ($0.5 \mu\text{M}$) (Fig. 7A, trace 3;B). In contrast, when glutamate ($50 \mu\text{M}$) was applied for control purposes to the recorded cells, a strong depolarization followed by a burst of action potentials was observed in all nine MCH neurons studied (Fig. 7A, trace 2). In the presence of TTX ($0.5 \mu\text{M}$), glutamate ($50 \mu\text{M}$) significantly depolarized the MCH membrane potential ($12.5 \pm 3.6 \text{ mV}$) (Fig. 7B) ($n = 8$; $p < 0.05$; ANOVA).

We next asked whether GLP-1 agonists might indirectly modulate MCH neurons. To address this issue, we studied the effect of Exn4 on the frequency of PSCs under voltage clamp, holding the GFP-expressing cells at -60 mV , and using KMeSO_4 in the pipette. The application of Exn4 to the slice did not significantly modify the frequency of PSCs in MCH neurons (Fig. 7C). In the presence of Exn4 in the bath, the normalized PSC frequency was changed by $4.4 \pm 4.9\%$ compared with the control, a nonsignificant effect ($n = 6$; $p > 0.05$; ANOVA). To evaluate the direct Exn4 action on the presynaptic axons innervating MCH neurons, we determined its actions on the frequency of miniature PSCs (mPSCs) in the presence of TTX ($0.5 \mu\text{M}$) in the bath. Under these conditions, no detectable changes in the frequency or the amplitude of mPSCs were observed after the addition of Exn4 ($1 \mu\text{M}$) to the recorded cells. The frequencies of the mPSC were 96.9 ± 11.2 and $95.4 \pm 25.3\%$ compared with the control (100%), in Exn4 and recovery conditions, respectively ($n = 5$; $p > 0.05$) (Fig. 7D). In five cells, we determined the Exn4 actions on the cumulative distribution of the mPSC amplitude. When the pre- and post-Exn4 cumulative fractions were compared in these cells, no significant alterations in the cumulative distributions of the mPSC amplitude were detected (Fig. 7E) ($n = 5$; $p > 0.05$; Kolmogorov–Smirnov). Together, these results suggest that the activation of GLP-1 receptors exerts no detectable effect on the synaptic inputs to MCH.

Additionally, we evaluated the GLP-1 agonist actions on PVN neurons, because high levels of expression of GLP-1 receptors have been reported in this hypothalamic region (Merchenthaler et al., 1999), and in parallel, GLP-1-positive fibers have been found in the PVN (Rinaman, 1999). To determine the effect of the GLP-1 agonist Exn4 on PVN neurons, whole-cell current-clamp experiments were conducted in mouse hypothalamic slices. Under DIC, the PVN was easily identified lateral to the third ventricle. Because a high density of GLP-1 receptors has been detected in the magnocellular subdivision (Merchenthaler et al., 1999), the recordings were done from neurons in the ventrolateral magnocellular region of the PVN.

Exn4 evoked three different types of response in PVN neurons

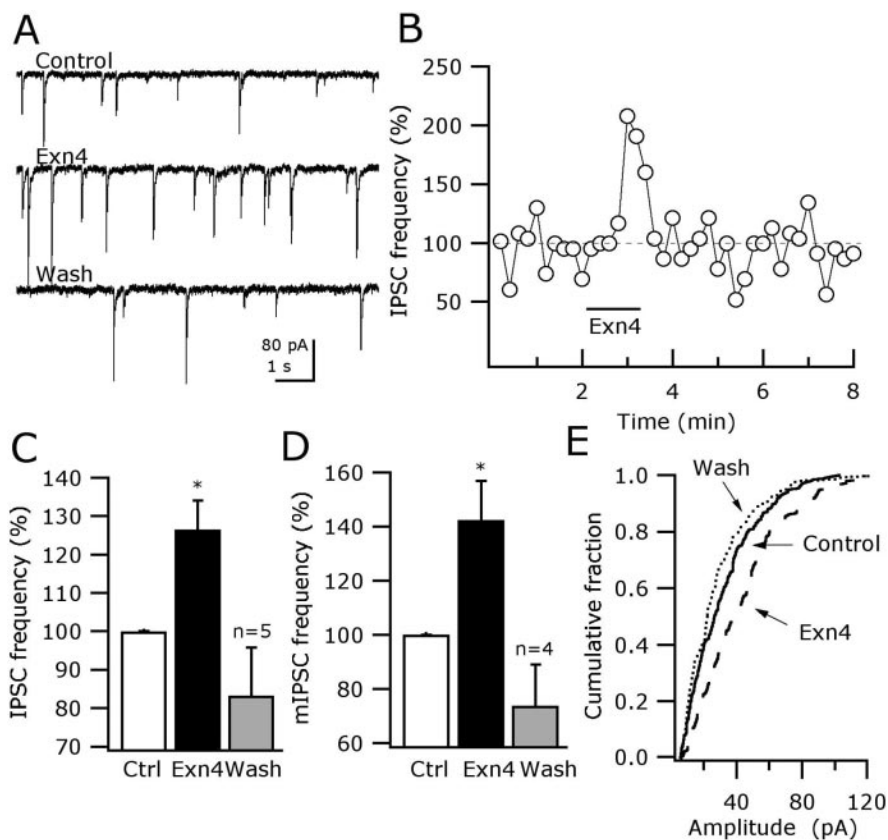


Figure 6. GLP-1 agonist enhances inhibitory, GABA-mediated, synaptic transmission in hypocretin neurons. *A*, Exn4 enhanced the frequency of IPSCs. In these experiments, KCl was used in the recording pipette and IPSCs were detected as fast inward currents. *B*, Time course of the Exn4 actions on IPSC frequency. *C*, Exn4 increased the frequency of IPSCs in five cells tested. *D*, *E*, Exn4 actions on the frequency and the amplitude of mIPSCs in hypocretin cells. The GLP-1 agonist increased the frequency of mIPSCs by $42.5 \pm 14.3\%$ (*D*). The cumulative distributions of the mIPSCs were also significantly altered in all of the cells tested (*E*). *Statistically significant. Ctrl, control; Wash, washout.

(Fig. 7F1–F3). In 12 cells (57.2%), the application of $1 \mu\text{M}$ Exn4 resulted in a robust, long-lasting depolarization of the membrane potential, followed by an increase in the frequency of action potentials (Fig. 7F1). In these neurons, Exn4 enhanced the firing rate by $35 \pm 7.8\%$ compared with control (data not shown; $p < 0.05$; ANOVA). In three neurons (representing 14.3%), Exn4 depressed the spike frequency of PVN neurons by $43.5 \pm 9.5\%$ compared with the control pretreatment levels ($p < 0.05$; ANOVA). Little or no alteration in the spike frequency or membrane potential after Exn4 was detected in the remaining six PVN recorded cells (28.1%). To determine whether these actions were attributable to direct Exn4 activation of postsynaptic receptors, we conducted additional experiments in the presence of TTX ($0.5 \mu\text{M}$) in the bath solution. Under these conditions, stable recordings were obtained from 11 neurons located around the magnocellular portion of the PVN. In five neurons (45%), depolarizing shifts of the membrane potential were detected after the addition of Exn4 to the bath (Fig. 7G, top trace). The Exn4 application depolarized PVN cells by $7.4 \pm 2.1 \text{ mV}$ compared with the resting pretreatment conditions. These effects were reversible, because the membrane potential returned to the control value after peptide washout. ANOVA analysis showed that these Exn4 actions on the membrane potential were statistically significant ($p < 0.05$). In the six remaining cells, no significant change in the membrane potential was detected (Fig. 7G, bottom trace). No hyperpolarizing effects of Exn4 on the membrane potential of PVN neurons were observed in the presence of TTX ($0.5 \mu\text{M}$),

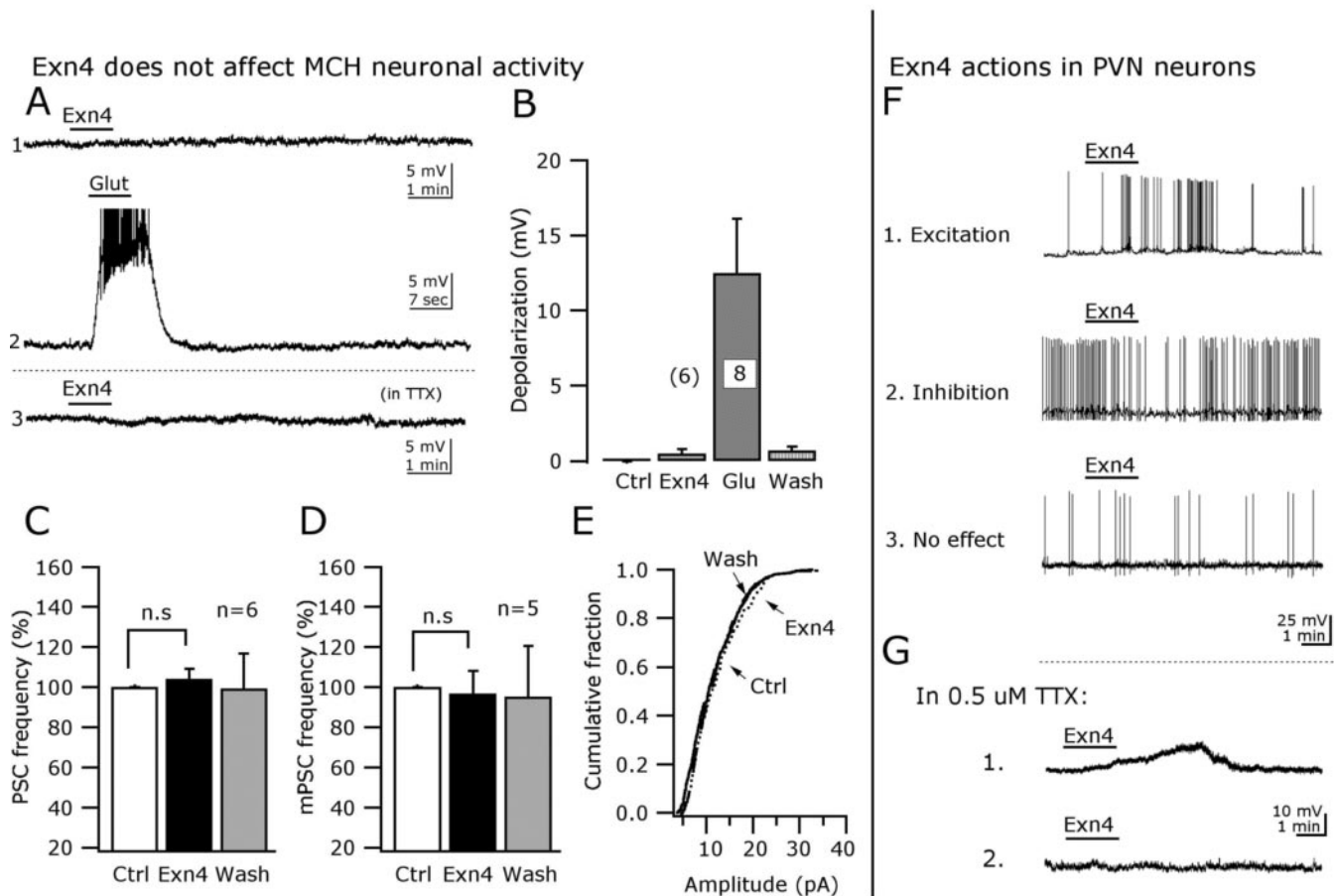


Figure 7. Effects of GLP-1 agonist on MCH and paraventricular hypothalamic neurons. *A*, Trace 1, Exn4 ($1 \mu\text{M}$) did not evoke any detectable change in the firing rate or the membrane potential in an MCH neuron. Trace 2, $50 \mu\text{M}$ glutamate (Glu) induced a robust depolarization followed by a burst of action potentials in an MCH neuron. The action potentials were truncated in this figure. Trace 3 shows the lack of Exn4 effects on the membrane potential in the presence of the TTX ($0.5 \mu\text{M}$). *B*, Bar graph showing the effects of Exn4 and glutamate on the membrane potential of MCH neurons in the presence of $0.5 \mu\text{M}$ TTX in the bath. *C*, Exn4 showed no effect on the synaptic inputs to MCH neurons, because it did not increase the frequency of PSCs in these cells. *D*, *E*, In the presence of TTX ($0.5 \mu\text{M}$) in the bath, Exn4 did not evoke any significant change in the frequency (*D*) or the amplitude (*E*) of mEPSCs recorded in MCH neurons. *F*, *G*, Diverse Exn4 responses were detected in PVN cells. An excitatory (increase in spike frequency) response (*F1*) was detected in 57% of the recorded neurons, whereas an inhibitory (depressing in the firing rate) response (*F2*) was observed in 14% of the recorded cells. We did not detect any change in the spike frequency or membrane potential in the remaining 28% of the recorded cells (*F3*). *G*, In the presence of $0.5 \mu\text{M}$ TTX in the bath, only depolarizing responses were detected in 45% of the cells (top trace). The remaining cells (bottom trace) did not show a significant change in their membrane potential with Exn4 application. Ctrl, control; n.s., not significant; Wash, washout.

suggesting that the depressing effect on the spike frequency described above might be attributable to an enhancement of the inhibitory synaptic inputs to these PVN neurons.

Discussion

In this study, we used whole-cell voltage clamp and current clamp to examine the cellular actions of GLP-1 agonists on the activity of hypocretin neurons identified by selective GFP expression in mouse hypothalamic slices. GLP-1 agonists depolarized the membrane potential of hypocretin neurons and increased spike frequency. These excitatory actions on the membrane potential were attributable to direct postsynaptic activation of GLP-1 receptors, dependent on extracellular Na^+ , mediated by the activation of G-proteins, and blockable by a GLP-1 receptor antagonist, exendin (9–39). GLP-1 also enhanced glutamate release at axon terminals synapsing onto hypocretin cells, in part by activation of presynaptic GLP-1 receptors. PVN neurons were also excited by GLP-1 agonists, consistent with the high levels of GLP-1-containing fibers and receptors found here. In contrast, GLP-1 agonists had no effect on identified MCH neurons in the LH.

GLP-1 presynaptic and postsynaptic excitation of hypocretin neurons

To the best of our knowledge, GLP-1 actions have not been studied previously at the cellular level in the CNS. We show here that GLP-1 excites LH hypocretin neurons by both postsynaptic and presynaptic mechanisms. Direct depolarizing effects of GLP-1 were evident in the presence of TTX, suggesting the activation of postsynaptic GLP-1 receptors expressed by hypocretin neurons. Our experiment using a nonhydrolyzable GTP ($\text{GDP}\beta\text{S}$) analog in the recording pipette confirmed that the GLP-1 depolarizing action in hypocretin cells were G-protein dependent (Hoosein and Gurd, 1984), and that the receptor–G-protein complex is located on the somatodendritic region of the recorded hypocretin cell.

GLP-1 caused an increase in spike frequency and an associated inward current. The replacement of extracellular sodium with choline strongly depressed or eliminated the direct depolarizing actions of GLP-1. The reversal potential for the inward current was near -28 mV , a potential between the negative K^+ and the positive Na^+ reversal potentials. Furthermore, the current was

not substantially altered by low extracellular calcium, both findings consistent with activation of a nonselective cationic current (Formenti et al., 2001; Liu et al., 2002; Yang et al., 2004). Although nonselective cationic conductances do not appear to have been reported previously in hypocretin neurons, they have been observed in other neurons expressing G-protein-coupled peptide receptors (Kirkpatrick and Bourque, 1995; Liu et al., 2002), including the G_s subtype (Yang et al., 2004). One mechanism of GLP-1 actions in non-neuronal cells is the depression of an inward rectifier K^+ conductance (Light et al., 2002). In hypocretin neurons, we found no evidence for GLP-1 modulation of whole-cell K^+ conductances activated either by voltage steps or by voltage-ramp commands, using protocols typically used to study K^+ currents, including the inward rectifier subtypes (Sodickson and Bean, 1996; Yang et al., 2004). This suggests that these K^+ currents were not the primary mechanism of excitation involved in the modulatory actions of GLP-1 in hypocretin neurons. Another mechanism by which transmitter may regulate neuronal excitability is the modulation of the AHP current (Sah, 1996; Stocker et al., 1999). GLP-1 attenuated the AHP current in hypocretin cells, suggesting that modulation of the K^+ currents involved in hyperpolarization after an action potential may be one mechanism by which GLP-1 increased spike frequency.

GLP-1 depresses Ca^{2+} currents in hypocretin neurons. GLP-1 receptors have been suggested to couple to the G_s type of G-protein. Activation of G_s -coupled receptors have been reported to enhance Ca^{2+} currents in many cells (Dolphin, 1998); in addition, several reports have shown that, in some cell types, activation of G_s may depress Ca^{2+} currents (Shapiro and Hille, 1993; Zhu and Yakel, 1997), a finding consistent with the data here. A recent report showed that GLP-1 receptors may also couple to $G_{i/o}$ subtypes of G-proteins (Hallbrink et al., 2001). Previous studies have shown depression of calcium currents by activation of $G_{i/o}$ proteins (Gao and van den Pol, 2001). The critical factor for the inhibitory modulation of voltage-dependent calcium channels may be the presence of the G-protein $\beta\gamma$ subunits (Herlitz et al., 1996; Ikeda, 1996). The depression of the calcium current by GLP-1 may be one mechanism by which the amplitude of the calcium-dependent potassium AHP current is attenuated.

In addition to the direct activation of postsynaptic receptors, GLP-1 agonists indirectly activated hypocretin neurons by increasing the frequency of spontaneous EPSCs. These currents were abolished by AMPA and NMDA receptor antagonists, suggesting that GLP-1 enhances the release of glutamate from axon terminals synapsing onto hypocretin cells. GLP-1 agonists increased the frequency of miniature EPSCs with little change in the event amplitude, suggesting that GLP-1 agonists enhance glutamate release by activation of GLP-1 receptors on the presynaptic terminals innervating hypocretin cells. Axons that release glutamate provide the primary excitatory input to hypocretin neurons, and some of these arise locally from excitatory neurons within the LH that might play a role in orchestrating the output of the dispersed hypocretin neurons (Li et al., 2002; Acuna-Goycolea et al., 2004); thus, GLP-1 would potentially enhance hypocretin cell activity by enhancing glutamate release.

Whereas hypocretin neurons showed only excitatory responses, PVN neurons showed more heterogeneous responses, consistent with the presence of different types of neurons within the PVN. The primary GLP-1 response of PVN neurons was excitatory, as shown by an increase in spike frequency. A small number of PVN neurons showed a decrease in spike frequency; hypothetically, this inhibition could be attributable to GLP-1-mediated excitation of inhibitory interneurons, resulting in a sec-

ondary indirect inhibition of other PVN cells. This view is consistent with the finding that, when spike-dependent synaptic activity was blocked with TTX, only depolarizing actions of GLP-1 were found in PVN neurons. Because both hypocretin and paraventricular neurons showed similar excitatory responses, it is probable that other neurons that express the GLP-1 receptor will respond similarly.

MCH neurons are intermixed, but constitute a group of cells different from the hypocretin neurons within the LH (Elias et al., 1998; Peyron et al., 1998). Mice lacking MCH peptide or its receptors are hypophagic and lean; intracranial MCH injections increase food intake (Qu et al., 1996; Rossi et al., 1999), suggesting MCH neurons may participate in energy homeostasis. We found little or no effect of GLP-1 agonists on the activity of identified MCH neurons, supporting the view that GLP-1 actions in the LH are highly selective, affecting hypocretin but not the adjacent MCH neurons.

Functional considerations

GLP-1-containing neuronal cell bodies are primarily located in the caudal NTS (Han et al., 1986; Jin et al., 1988), and axons from these cells innervate both the lateral hypothalamus and PVN. The NTS receives a broad spectrum of sensory signals from the gut, carried in part by the subdiaphragmatic vagus nerve; signals include sensory input from liver, stomach, and intestinal receptors (Powley, 2000; Saper et al., 2002; Vrang et al., 2003). These signals may be associated with the homeostatic control of feeding, and possibly involved in initiation or termination of meals (Saper et al., 2002). In addition to release from neurons of the NTS, GLP-1 may also be released from the gut into the vascular system. Although it is possible that LH neurons might be activated by circulating GLP-1, this appears less likely because the large peptide would not pass freely through the blood–brain barrier into the LH; circulating GLP-1 could, however, activate the NTS via axons from the area postrema, which may respond to circulating GLP-1 via a weak blood–brain barrier (Yamamoto et al., 2003). CNS administration of GLP-1 causes a profound inhibition of food intake, suggesting that GLP-1 may be critical for meal cessation (Tang-Christensen et al., 1996; Turton et al., 1996). However, mice with a targeted disruption of the GLP-1 receptor gene have normal body weight and feeding behavior (Scrocchi et al., 1996; Scrocchi and Drucker, 1998), suggesting that GLP-1 may not be critical for the long-term regulation of energy balance. GLP-1 neurons that project to the hypothalamus are activated by interoceptive stress (Rinaman, 1999), and GLP-1 neurons may mediate hypothalamic endocrine and anxiety responses generated by this stress (Seeley et al., 2000; Kinzing et al., 2003). Association of food with GLP-1 administration can lead to conditioned food avoidance, suggesting that GLP-1, at least with high levels, may provide negative cues or an unpleasant sensation that can be associated with food. Negative cues from the viscera may not be related solely to energy homeostasis; cardiac stress signaling to the NTS may also initiate arousal through the hypocretin neurons. Finally, hypocretin neurons project to the area of the GLP-1 cells in the NTS (Peyron et al., 1998) and hypocretin enhances activity of unidentified NTS neurons (Smith et al., 2002), providing potential direct feedback.

GLP-1 neurons in the NTS that project to the PVN increase expression of *c-fos* (a marker of neuronal activation) after interoceptive stress stimuli that included lithium chloride, bacterial lipopolysaccharides, and cholecystokinin; the excitatory response of PVN neurons to GLP-1 may activate the endocrine response to interoceptive stress (Rinaman, 1999; Seeley et al.,

2000; Kinzing et al., 2003). In addition to responding to GLP-1, PVN neurons also respond to a number of other neuropeptides considered to regulate energy homeostasis (Cowley et al., 1999).

Recent experiments have suggested that hypocretin neurons may regulate arousal that is dependent on the energy state (Yamanaka et al., 2003). In this report, we show that GLP-1 agonists consistently excite hypocretin neurons, suggesting that GLP-1 cells may enhance hypothalamic arousal. Because GLP-1 neurons appear to receive a variety of gut signals involved in the regulation of food intake, GLP-1 effects on hypocretin neurons might be an important substrate for the integration of gut signals relating energy homeostasis with hypothalamic arousal, or for enhancing arousal signaled by visceral or cardiac stress.

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