Plasma hormone levels and central c-Fos expression in ferrets after systemic administration of cholecystokinin

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Billig, I., B. J. Yates, and L. Rinaman. Plasma hormone levels and central c-Fos expression in ferrets after systemic administration of cholecystokinin. Am J Physiol Regulatory Integrative Comp Physiol 281: R1243–R1255, 2001.—Posterior pituitary hormone secretion and central neural expression of the immediate-early gene product c-Fos was examined in adult ferrets after intravenous administration of CCK octapeptide. Pharmacological doses of CCK (1, 5, 10, or 50 μg/kg) did not induce emesis, but elicited behavioral signs of nausea and dose-related increases in plasma vasopressin (AVP) levels without significant increases in plasma oxytocin (OT) levels. CCK activated neuronal c-Fos expression in several brain stem visceral sensory regions, including a dose-related activation of neurons in the dorsal vagal complex (DVC). Activated brain stem neurons included catecholaminergic and glucagon-like peptide-1-positive cells in the DVC and ventrolateral medulla. In the forebrain, activated neurons were prevalent in the paraventricular and supraoptic nuclei of the hypothalamus and also were observed in the central nucleus of the amygdala and bed nucleus of the stria terminalis. Activated hypothalamic neurons included cells that were immunoreactive for AVP, OT, and corticotropin-releasing factor. Comparable patterns of brain stem and forebrain c-Fos activation were observed in ferrets after intraperitoneal injection of lithium chloride (LiCl; 86 mg/kg), a classic emetic agent. LiCl activated more neurons in the area postrema and fewer neurons in the nucleus of the solitary tract compared with CCK. Together with results from previous studies in rodents, our findings support the view that nauseogenic treatments activate similar central neural circuits in emetic and nonemetic species, despite differences in treatment-induced emesis and pituitary hormone secretion.

emesis; lithium chloride; catecholamines; glucagon-like peptide-1; oxytocin; vasopressin; corticotropin-releasing factor

Transient increases in plasma concentrations of vasopressin (AVP) and ACTH represent a physiological correlate of nausea in emetic species such as ferrets (7), sheep (5), pigs (22), monkeys (37), and humans (16). The similar neuroendocrine responses to nauseogenic stimuli across emetic species presumably are due to functional similarities in central neural circuits involved in nausea and/or vomiting in these species. The anatomic and neurochemical features of relevant neural circuits have been elucidated in part by using the immediate-early gene product c-Fos as a marker of stimulus-induced neural activation. Studies in emetic species include examination of brain stem c-Fos expression in macaque monkeys after systemic administration of high doses of CCK octapeptide (30), brain stem c-Fos expression in cats and ferrets after systemic administration of metacaine drugs (3, 17, 24), and brain stem c-Fos expression in ferrets after intraduodenal injection of hypertonic saline or after electrical stimulation of the supradiaphragmatic vagus nerve (3). However, only one study in an emetic species has reported the neurochemical phenotypes of activated brain stem neurons (30), and none have examined stimulus-induced c-Fos expression in the hypothalamus or other forebrain regions.

We now report plasma hormone concentrations and describe c-Fos activation in chemically identified brain stem and forebrain neurons in ferrets after systemic administration of synthetic CCK. Endogenous CCK released from the intestine after a meal contributes to normal digestive processes (see Ref. 13). However, pharmacological doses of CCK can produce gastric malaise and nausea accompanied by dose-dependent increases in pituitary hormone secretion (14, 16, 22, 35–37). Threshold nauseogenic and emetic doses of synthetic CCK range from 0.05 μg/kg in humans (16) to ~10 μg/kg in macaque monkeys (37). The anorexigenic, emetic, and neuroendocrine effects of exogenous CCK involve CCKA receptor-mediated stimulation of gastrointestinal vagal sensory inputs to the caudal dorsomedial medulla (18, 32). The behavioral and hormonal effects of CCK peak within 2–5 min and last only 10–15 min after acute intravenous administration (35–37). Thus systemically administered CCK provides a well-defined experimental model with which to examine the central neural mechanisms and neuroendocrine features of acute nausea. For comparative purposes, we also report brain stem and forebrain c-Fos expression in ferrets after systemic administration of a classic emetic agent, LiCl.

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EXPERIMENTAL PROCEDURES

Experiments were carried out in eight male ferrets (weight range of 1.5–2.0 kg) obtained from Marshall Farms (North Rose, NY). Animals were housed singly in a light- and temperature-controlled room. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Experimental Animals [DHHS Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and were approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

Surgical preparation. After at least 1 wk in the animal housing facility and acclimatization to daily handling, ferrets were implanted with a chronic indwelling cannula inserted into the left external jugular vein. This surgical procedure was performed in animals anesthetized by intramuscular injection of a mixture of ketamine (25 mg/kg) and xylazine (2.5 mg/kg) using aseptic procedures in a dedicated operating suite. Cannulas were made of silicone tubing (Esco Rubber; OD 1.5 mm, ID 0.5 mm) and were attached to an injection port that was sutured to the dorsal nuchal muscles and overlying skin. Animals were allowed to recover for at least 2 days before initiation of drug injection and blood sampling procedures.

Injection of drugs and blood sampling. The effects of systemically administered CCK on plasma levels of AVP and OT were determined in six animals. For this purpose, animals were gently restrained in the arms of one investigator while a second investigator withdrew a 2.0-ml baseline blood sample through the injection port followed immediately by intravenous infusion of 2.0 ml of 0.15 M NaCl containing 1, 5, 10, or 50 μg/kg CCK (Sigma, St. Louis). These doses were selected based on results in other emetic and nonemetic species, in which doses of CCK ≥1 μg/kg induce pituitary hormone secretion and brain stem and forebrain c-Fos expression 
(5, 18, 20–22, 30, 36, 37, 41). Blood samples were drawn over 15–30 s; drug infusions were made over ~15–20 s. Each animal was immediately returned to its home cage, and its behavior was observed for 8 min. Animals then were removed from their home cage to obtain a second (postinfusion) 2.0-ml blood sample, the volume of which was replaced immediately by infusion of an equal volume of 0.15 M NaCl. Each animal received at least two different doses of CCK accompanied by pre- and postinfusion blood sampling, with procedures in each animal separated by at least 2 days.

Plasma radioimmunoassay. Blood samples were collected into 3-ml heparinized borosilicate tubes (Vacutainer, Becton-Dickinson, Rutherford, NJ), immediately immersed into ice, and centrifuged at 4°C within 30 min of collection (3,000 g for 20 min). Plasma samples were removed and frozen at −80°C. Before assay, plasma samples were thawed on ice and extracted using C18 Sep-Pak Vac cartridges (1 ml, 50 mg; Waters, Milford, MA), as described previously (29). Each extract was frozen, dried using a Speed Vac (Savant Instruments), and then dissolved in 700 μl of buffer (50 mM NaPO4, 25 mM EDTA, 0.9% NaCl, 0.5% bovine serum albumin, 0.1% sodium azide). AVP and OT concentrations were measured by radioimmunoassay in separate 200-μl aliquots of each plasma extract. The initial incubation, performed at 4°C for 48 h, contained ~4,500 cpm of 125I-labeled OT or AVP (NEN-DuPont, Boston, MA) and a rabbit polyclonal antibody to OT or AVP (Dr. J. Fernstrom, Univ. of Pittsburgh, PA) in a volume of 400 μl. After incubation, antibodies were precipitated using a second antibody procedure: after overnight incubation with normal serum (1:600, Jackson Immunoresearch, West Grove, PA) and goat anti-rabbit IgG (1:120, Linco, St. Charles, MO), tubes were centrifuged (3,000 g, 30 min), the supernatant fluid aspirated, and the remaining pellets counted in a gamma counter (Packard-Auto Gamma Scintillation Spectrometer). Values of OT and AVP were calculated from standard curves generated with known amounts of synthetic peptide (Bachem, Torrance, CA) that were run through the same extraction procedure. All plasma samples were extracted at the same time and run together in the same assays for AVP and OT. Assay sensitivities for AVP and OT were 2.5 and 6.8 pg/ml, respectively.

Statistical analysis of plasma radioimmunoassay data. Plasma AVP and OT values were combined by treatment group (i.e., by CCK dose administered) and are expressed as means ± SE. Treatment-related differences in plasma AVP or OT compared with baseline (preinfusion) levels were tested for statistical significance by using one-way ANOVA. When f values indicated significant overall main treatment effects, the ANOVA was followed up with planned post hoc t-tests using Dunn’s (Bonferroni) correction for repeated-measures analysis. Differences were considered significant when P < 0.05.

c-Fos expression study. For analysis of stimulus-induced c-Fos expression, ferrets received an intravenous infusion of 2.0 ml 0.15 M NaCl containing 0 (n = 2), 10 (n = 2), or 50 (n = 2) μg/kg CCK. Two animals whose intravenous catheters were no longer patent instead received an intraperitoneal injection of 0.15 M LiCl (86 mg/kg; Sigma). Similar and lower doses of LiCl have been reported to induce emesis in ferrets (23). Animals were returned to their home cages for 60–75 min after CCK or vehicle administration or for 120 min after LiCl injection and then were anesthetized for perfusion fixation (see below). These postinjection survival times are similar to those used in previous studies of central c-Fos expression after CCK or LiCl treatment (20, 25, 28, 30, 33, 38, 40, 41).

Perfusion fixation and tissue preparation. After the appropriate time period, animals were deeply anesthetized by intravenous or intramuscular injection of a mixture of ketamine (35 mg/kg) and xylazine (2 mg/kg). Animals were perfused transcardially with 1.0 liter of 0.15 M NaCl, followed by 1.0 liter of a solution of 4% buffered paraformaldehyde and 2% acrolein (EM grade, Polysciences, Warrington, PA), followed by 500 ml of 4% paraformaldehyde. Fixed brains were removed from the skull, bisected coronally at the level of the superior colliculus, postfixed for 4–5 h in 4% paraformaldehyde, and then cryoprotected for 2 days at 4°C in 25% aqueous sucrose.

Coronal sections (30 μm thick) were cut with a freezing microtome from the caudal extent of the dorsal vagal complex (DVC; ~2.0 mm caudal to obex) to the rostral extent of the medial septum (~20.0 mm rostral to obex). Sections were collected sequentially in five sets so that each set contained a rostrocaudal series of brain stem or forebrain sections spaced 150 μm apart. Tissue sections were stored at ~20°C in cryopreservant solution (39) until further processing.

Immunocytochemical procedures. PBS (0.1 M, pH 7.2) was used for all incubation solutions and rinses, unless otherwise noted. Tissue sections were removed from cryopreservant, rinsed for 1 h, fixed in 2% acrolein for 10 min, rinsed for 1 h, treated for 20 min in 0.5% sodium borohydride (Sigma), rinsed for 30 min, treated in 0.3% H2O2 for 10 min, rinsed for 30 min, and then incubated for 1 h in blocking solution containing 1% BSA (Sigma), 1% donkey serum (Jackson ImmunoResearch), and 0.3% Triton X-100 (Sigma).

Primary and secondary antisera were diluted in PBS containing 1% BSA or donkey serum and 0.3% Triton X-100. The anti-c-Fos serum was generated against the NH2-terminal...
region of synthetic human c-Fos protein (provided by Drs. Philip J. Larsen and Jens D. Mikkelsen, Panum Institute, Denmark). The specificity of this antiserum for c-Fos protein has been verified (30). Tissue sections were incubated for 48 h at 4°C in rabbit anti-c-Fos (1:50,000) and then processed using the Vectastain Elite avidin-biotin immunoperoxidase method (Vector Laboratories). A solution of diaminobenzidine (DAB), nickel sulfate, and H2O2 was used to generate blue-black nuclear c-Fos immunolabeling.

Separate sets of c-Fos-labeled brain stem sections from each ferret were subsequently processed for immunoperoxidase localization of tyrosine hydroxylase (TH) or glucagon-like peptide-1 (GLP-1). c-Fos-labeled forebrain sections were processed for immunoperoxidase localization of OT, AVP, or corticotropin-releasing factor (CRF). For this purpose, tissue sections were incubated for 48 h at 4°C in monoclonal mouse anti-TH (Chemicon, Temecula, CA; 1:100,000), rabbit anti-GLP-1 (Peninsula Laboratories, San Carlos, CA; 1:10,000), rabbit anti-OT (Peninsula; 1:20,000), rabbit-anti-AVP (Peninsula; 1:20,000), or rabbit anti-CRF (Peninsula; 1:5,000).

After being rinsed, sections were incubated for 1 h in biotinylated donkey anti-mouse or donkey anti-rabbit IgG (Jackson ImmunoResearch; 1:600), rinsed and then processed using Elite Vectastain reagents and a solution of DAB and H2O2 to generate brown cytoplasmic immunolabeling.

AVP and OT neurons are not spatially segregated in the ferret hypothalamus as they are in rats, and so a blocking study was performed to test the specificity of the rabbit polyclonal AVP and OT antisera used in this study (Peninsula). Preincubation of the OT antiserum (1:20,000) with a 10-fold excess of synthetic OT peptide (Bachem) completely blocked immunocytochemical labeling in the ferret hypothalamus, whereas preincubation of the OT antiserum with excess AVP peptide (Bachem) did not noticeably alter OT immunostaining. Thus the OT antiserum used in this study is specific for OT. Conversely, a low level of perikaryal and axonal immunostaining persisted in the ferret hypothalamus after the AVP antiserum (1:20,000) was preincubated with excess AVP peptide. Peninsula reports a 1% cross-reactivity of this AVP antiserum with OT; correspondingly, immunostaining was completely eliminated by preincubating the AVP antiserum with both AVP and OT peptides. Consequently, preincubation of the AVP antiserum with excess OT resulted in immunostaining that was specific for AVP. This approach was used to verify the AVP phenotype of activated neurons in the ferret PVN and SON.

Immunoreacted sections were mounted onto Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and allowed to dry overnight. To determine the boundaries of neuronal structures in each animal, one series of slide-mounted sections immunoreacted only for c-Fos was counterstained for Nissl substance and fiber tracts using a modified Kluver-Barrera procedure (9). Slides were dehydrated in graded baths of alcohol, defatted in xylene, and placed under a coverslip with Cytoseal 60 (VWR Scientific, West Chester, PA).

Immunoreacted tissue sections were examined under bright-field illumination using a Zeiss Axiosplan microscope. Immunolabeling was assessed qualitatively using a ×20 objective. Electronic images from regions of interest were obtained using a Dage MTI 3CCD camera (Mutech, Billerica, MA) and the Simple 32 Image Analysis System (Compix, Lake Oswego, OR). Digital images were assembled into plates and prepared for publication using Adobe Systems (San Jose, CA) Photoshop software. Individual images were adjusted for size, brightness, and contrast, but color balance was not altered.
GLP-1-immunopositive neurons. Closely adjacent double-immunolabeled sections through the three selected levels of the PVN were used to examine c-Fos expression by AVP-, OT-, and CRF-immunopositive neurons.

RESULTS

Behavioral observations. All ferrets displayed increased licking and salivation within 5 min after intravenous injection of CCK at doses of 1, 5, 10, or 50 µg/kg. These effects persisted for ~10 min, during which time the animals assumed either a crouched or supine position in their home cage. Neither retching nor emesis was observed after any dose of CCK. The incidence and magnitude of CCK-induced licking and salivation were lowest in animals that received the lowest dose (1 µg/kg) and greatest in animals that received the highest dose (50 µg/kg). All animals appeared to recover completely within 10–15 min after CCK infusion, as evidenced by loss of licking behavior and increased mobility and exploration accompanied by rearing and nosepoking through the cage door (a normal and frequent behavior when investigators are present in the room).

Both LiCl-treated ferrets displayed increased licking and salivation within 17–20 min after intraperitoneal injection, accompanied by two or three episodes of emetic retching that were complete within 30 min. After emesis, both animals assumed supine and relatively immobile positions in their home cage, during which time licking and salivation gradually diminished. Mobility, exploration, rearing, and nosepoking behavior indicated that both animals recovered fully within 60–90 min after LiCl injection.

Effects of CCK administration on plasma levels of AVP and OT. Baseline plasma levels of AVP and OT were low and exhibited little variability (n = 15 preinjection samples; Fig. 1). Subsequent intravenous infusion of 2.0 ml of 0.15 M NaCl containing 1 (n = 5), 5 (n = 3), 10 (n = 4), or 50 µg/kg CCK (n = 3) caused

Fig. 2. Camera lucida drawings of transverse brain stem sections indicating c-Fos immunoreactivity in the dorsal medulla after intravenous injection of 0.15 M NaCl (control), 2 doses of CCK (intravenous), and LiCl (intraperitoneal). The numbers on the left side of the diagrams represent the approximate distance from obex. 5SL, laminar spinal trigeminal nucleus; 5SP, alaminar spinal trigeminal nucleus, parvocellular division A1, caudal ventral noradrenergic cell group region; AP, area postrema; Ce, central canal; d/dln, dorso/dorsolateral subnucleus of the nucleus of the solitary tract (NTS); IO, inferior olive; mn, medial subnucleus of the NTS; ncom, commissural subdivision of the NTS; ni, interstitial subnucleus of the NTS; Rob, nucleus obscurus of raphe; S, tractus solitarius; sg, subnucleus gelatinosus of the NTS; V4, fourth ventricle; v/vln, ventro/ventrolateral subnucleus of the NTS; X, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus. Scale bar 1 mm.
dose-related increases in plasma concentrations of AVP (Fig. 1, top). Plasma AVP levels were significantly higher than baseline after infusion of CCK at doses of $\geq$5 $\mu$g/kg ($P < 0.05$). Conversely, OT levels in plasma samples from the same animals did not increase significantly after any dose of CCK (Fig. 1, bottom; note the different y-axis scales for AVP and OT levels).

**Brain stem distribution of c-Fos labeling after CCK or LiCl treatment.** Figure 2 depicts the distribution of c-Fos labeling in the caudal medulla in ferrets after intravenous infusion of 0.15 M NaCl or CCK (10 or 50 $\mu$g/kg) or after intraperitoneal injection of LiCl. After CCK treatment, c-Fos-immunoreactive cells were present bilaterally in the DVC, including the nucleus of the solitary tract (NTS), area postrema (AP), and dorsal motor nucleus of the vagus (X). The number of c-Fos-immunoreactive cells in each DVC subregion is indicated in Table 1. Within the NTS, c-Fos expression was most prominent in the commissural and medial subnuclei (for nomenclature of NTS subnuclei in ferret, see Ref. 2). Less extensive labeling was present in other NTS subdivisions, including the dorsal/dorsolateral, ventro/ventrolateral, intermedial, and interstitial subnuclei (Fig. 2). Statistical comparisons of these data could not be made due to the small number of animals in each treatment group ($n = 2$). However, it appeared that more NTS neurons were activated after the high dose of CCK (50 $\mu$g/kg) compared with the lower dose (10 $\mu$g/kg), and both CCK doses appeared to activate more NTS neurons compared with the number activated after injection of 0.15 M NaCl vehicle (Fig. 2, Table 1). Intraperitoneal injection of LiCl appeared to activate fewer NTS neurons but more AP neurons compared with either dose of CCK (Fig. 2, Table 1). Neurons throughout the lateral and medial AP expressed c-Fos in animals treated with LiCl, whereas c-Fos labeling was mainly confined to the lateral AP in animals treated with CCK. Both CCK and LiCl activated c-Fos expression in neurons lying within the cytoarchitectural boundaries of X, particularly in its lateral margins. It was unclear whether these activated cells included vagal motor neurons.

Other medullary regions that consistently displayed stimulus-induced c-Fos expression after CCK or LiCl treatment included the caudal and rostral ventrolateral medulla (Fig. 3), dorsolateral portions of the lateral tegmental field, nucleus raphe obscurus, lateral parabrachial nucleus (Fig. 4), mesencephalic nucleus of the trigeminal nerve, magnocellular medullary reticular formation, inferior vestibular nucleus, locus ceruleus, periaqueductal gray, and Barrington’s nucleus.

**CCK- and LiCl-induced activation of brain stem cat- echolaminergic neurons.** Dual-label immunocytochemistry revealed that both CCK and LiCl treatment activated c-Fos expression in catecholaminergic (TH positive) neurons in the NTS, lateral AP, lateral X, caudal and rostral ventrolateral medulla, and lateral tegmental field (Fig. 5). The proportion of TH-positive cells in the NTS that expressed c-Fos appeared to be greater after injection of the 50 $\mu$g/kg dose of CCK compared with the 10 $\mu$g/kg dose, and both doses of CCK appeared to activate more TH-positive neurons than were activated after LiCl treatment. However, because each treatment group comprised only two animals, statistical comparisons of these data were not performed.

**Activation of GLP-1 neurons in the caudal brain stem.** CCK and LiCl treatment consistently activated c-Fos expression in GLP-1-positive neurons in the caudal medulla (Fig. 6). The perikarya of GLP-1-immunopositive cells were characterized by brown, fibrous-like immunoreactivity. GLP-1-positive cells typically were found in clusters in the NTS and adjacent dorsal reticular formation from 1.0 mm caudal to 0.5 mm rostral to obex. GLP-1-positive cells expressing c-Fos after CCK or LiCl treatment were present in the dorsal, lateral, and medial portions of the commissural subdivision of the NTS.

**Distribution of c-Fos labeling in the PVN.** PVN c-Fos labeling was sparse in vehicle-injected control animals (Fig. 7, Table 1). Conversely, a large number of c-Fos-immunoreactive cells were consistently present within the PVN in animals that received CCK or LiCl (Fig. 7, Table 1). The lower dose of CCK (10 $\mu$g/kg) appeared to activate fewer PVN neurons than did the higher dose of CCK (50 $\mu$g/kg), but the distribution of labeling was similar (Fig. 7). Activated neurons were concentrated in the medial parvocellular PVN, lateral and medial

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**Table 1. Number of c-Fos-activated cells per section per side in 5 selected areas of the dorsal vagal complex (from 1.5 mm caudal to 0.5 mm rostral to the obex) and in 3 selected areas of the paraventricular nucleus (between 16.5 mm and 17.3 mm rostral to the obex)**

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Values represent Fos-activated cells counted in each animal; 2 animals were included in each treatment group, and thus 2 values are provided for each region studied (1 from each animal). NTS, nucleus of the solitary tract; AP, area postrema; X, vagus.
magnocellular PVN, the dorsomedial cap, and the posterior PVN (Fig. 7).

Activation of phenotypically identified PVN neurons. Very few AVP- or OT-positive neurons were activated to express c-Fos in vehicle-injected control animals (Fig. 8). Conversely, LiCl and the 50 µg/kg dose of CCK activated substantial numbers of AVP- and OT-positive neurons in the PVN, particularly in the lateral and medial magnocellular subdivisions and the dorsal part of the posterior PVN (Fig. 8). Antibody blocking procedures (see EXPERIMENTAL PROCEDURES) confirmed the OT and AVP phenotypes of activated neurons in the PVN and supraoptic nucleus (SON) (described below). Less activation of AVP and OT neurons was observed in animals that received the 10 µg/kg dose of CCK. CRF-positive neurons in the medial parvocellular PVN rarely expressed c-Fos in vehicle-injected controls, whereas many CRF neurons were activated in animals treated with CCK or LiCl (Fig. 8).

Activation of neurons in the SON. Many c-Fos-immunopositive cells were present within the SON in animals that received 50 µg/kg CCK or LiCl, but very few cells were activated after injection of vehicle or 10 µg/kg CCK. Both AVP- and OT-positive neurons in the SON expressed c-Fos after CCK or LiCl treatment (Fig. 9), as confirmed by antibody blocking procedures (see EXPERIMENTAL PROCEDURES).

Distribution of c-Fos labeling in the limbic system. CCK and LiCl treatment activated c-Fos expression in the central nucleus of the amygdala (CeA) and in the medial bed nucleus of the stria terminalis (mBNST). Only moderate c-Fos labeling was observed in these regions after ferrets received infusions of vehicle or 10 µg/kg CCK, whereas the number of labeled cells was notably increased after 50 µg/kg CCK or LiCl (Fig. 10).

**DISCUSSION**

The locations of hindbrain and forebrain neurons activated in rats after systemic administration of pharmacological doses of CCK are similar to the locations of neurons activated after systemic administration of...
LiCl (20, 25, 28, 40), consistent with the comparable behavioral, autonomic, and neuroendocrine effects of these treatments in rats. In the present study, a largely similar distribution of neurons was activated in ferrets after systemic administration of CCK or LiCl, despite differences in the emetic effects of these agents. In ferrets, as in rats, brain stem activation was most prominent within the DVC, ventrolateral medulla, and parabrachial nuclei, and forebrain activation was most prominent within the PVN and SON, the CeA, and the mBNST.

Brain stem activation. Studies performed primarily in rats indicate that the behavioral and neuroendocrine effects of exogenous CCK result from activation of gastrointestinal vagal afferent inputs to the NTS (18, 32), whereas LiCl acts through vagal afferents and directly on AP chemoreceptor cells (1, 4, 40). Correspondingly, in the present study, CCK treatment induced more c-Fos activation in the ferret NTS compared with LiCl, whereas LiCl was more effective than CCK in activating neurons within the AP. Although these observations are tempered by the small number of animals used in this study, a similar differential distribution of c-Fos within the AP and NTS was reported in ferrets after treatment with cisplatin (a cytotoxic agent; Ref. 24) or after electrical stimulation of the subdiaphragmatic vagus (3). There were no other appreciable differences in the patterns of brain activation produced by CCK or LiCl despite our observations that LiCl treatment provoked emetic retching and CCK did not (even at doses as high as 50 μg/kg). Not all neurons express immunocytochemically detectable levels of c-Fos after receiving stimuli that increase their firing rate (6, 8). Thus it is likely that brain regions in addition to those revealed in this study were activated in ferrets after CCK and/or LiCl treatment. In particular, the emetic retching associated with LiCl treatment presumably was accompanied by activation of additional visceral and somatic sensory and motor pathways in these animals (12); however, corresponding treatment-related differences in central c-Fos expression were not evident. Previous reports of brain stem c-Fos expression in ferrets and cats treated with emetic stimuli also have failed to document significant activation of additional brain stem pathways (3, 17, 24).

The lack of emesis in ferrets that received even the highest dose of CCK in this study was unexpected, because much lower doses of CCK provoke emesis in other species (16, 37). Others have noted that the ferret is not an optimal model for all forms of emesis (10).

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The lack of emesis in ferrets that received even the highest dose of CCK in this study was unexpected, because much lower doses of CCK provoke emesis in other species (16, 37). Others have noted that the ferret is not an optimal model for all forms of emesis (10).
Nevertheless, the observed salivation, licking, prone posture, and dose-related increases in plasma AVP levels in the present study are evidence that exogenous CCK did provoke nausea. Others also have reported that nausea (as measured by taste-aversion learning) can occur in the absence of overt emesis in ferrets (23).

Both CCK and LiCl activated c-Fos expression in the DVC and other regions of the ferret caudal medulla that receive and process vagal sensory information, including the ventrolateral medulla and lateral parabrachial nuclei. Similar to findings in rats (25, 28), hindbrain neurons activated after CCK or LiCl administration in ferrets included catecholaminergic neurons in the dorsomedial and ventrolateral medulla and GLP-1-immunopositive neurons in the caudal NTS and adjacent reticular formation. In rats, subsets of activated catecholaminergic and GLP-1-positive neurons project to the hypothalamus (25, 27) to participate in stimulating pituitary hormone release after CCK and LiCl treatment. Hindbrain catecholaminergic and GLP-1-positive neurons presumably play a similar role in emetic species, although direct evidence for this is not yet available.
Our findings regarding CCK- and LiCl-induced activation of GLP-1-positive neurons in the ferret hindbrain are of particular interest. Recent studies indicate that central GLP-1 neural systems participate in treatment-induced anorexia and nausea in rats (25, 26, 31, 33, 34), but the presence of GLP-1 neurons and their functional role(s) in emetic species have not previously been reported. GLP-1 is a major splicing product of the preproglucagon peptide precursor. In the rodent brain, GLP-1 is expressed by a discrete group of neurons located in and around the DVC (11, 15). We observed a similar central distribution of GLP-1-positive neurons in ferrets. Although GLP-1-receptor gene expression and binding sites have not been examined in ferrets, in rats, GLP-1 receptors are localized to specific nuclei in the hypothalamus and other forebrain and brain stem regions that receive inputs from hindbrain GLP-1-positive neurons (15). The present observations in ferrets provide further support for the hypothesis that this small population of peptidergic neurons plays an important functional role in behavioral and endocrine responses to CCK, LiCl, and other interoceptive stressors in both emetic and nonemetic species.

**Hypothalamic activation.** The present study revealed a notable species-related difference between rats and ferrets in the neurochemical phenotypes of hypothalamic neurons that express c-Fos after nauseogenic treatments. CRF-, AVP-, and OT-positive hypothalamic neurons were activated in ferrets after CCK or LiCl administration. Conversely, in rats, CCK and

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**Fig. 7.** Camera lucida drawings of transverse sections through the paraventricular nucleus (PVN) illustrating the locations of c-Fos-activated cells in ferrets after intravenous injection of 0.15 M NaCl (control), 2 doses of CCK (intravenous), and LiCl (intraperitoneal). Top drawings show the rostral PVN, whereas middle and bottom illustrate more caudal levels; the distance between each depicted section is ~0.4 mm. 3V, third ventricle; f, fornix; pvdc, dorsomedial cap of the paraventricular nucleus; pvl, lateral magnocellular paraventricular nucleus; pvm, medial paraventricular nucleus; pvpa, parvocellular portion of the paraventricular nucleus; pvpo, posterior portion of the paraventricular nucleus. Scale bar 1 mm.
LiCl treatment activates CRH- and OT-positive neurons, with little or no activation of AVP-positive neurons (21, 38, and personal observations). These c-Fos expression data are consistent with evidence that experimental models of nausea and gastric malaise in rats are associated with increased plasma levels of OT, with no significant change in plasma levels of AVP (36). Conversely, in ferrets and several other emetic species studied to date, nauseogenic treatments are associated with increased plasma levels of AVP, with no significant change in plasma levels of OT (present study and Refs. 5, 7, 16, 22, 37). On the basis of these findings, we predicted that CCK and LiCl treatment in ferrets would activate AVP neurons but would not activate OT neurons. The former prediction was accurate, but the latter was not. Immunocytochemical blocking procedures confirmed that both AVP and OT neurons in the PVN and SON expressed c-Fos in ferrets after CCK and LiCl.

It is possible that some proportion of the activated OT-positive PVN neurons in ferrets includes parvocellular neurons with central axonal projections. In rats, central OT-containing neural pathways (including a descending projection from the PVN to the DVC) have been implicated in the autonomic and behavioral effects of CCK, LiCl, and other anorexigenic treatments (19, 21). However, presumably all of the activated OT neurons in the ferret SON are magnocellular neurons with axonal projections to the posterior pituitary gland. Insofar as c-Fos expression signals the treatment-induced activation of these neuroendocrine cells, it is unclear why plasma OT levels did not increase after CCK treatment in ferrets. Recent data obtained from one animal indicate that plasma OT levels also do not increase in ferrets after LiCl treatment, despite increases in plasma AVP (unpublished observation). An explanation for this apparent discrepancy in the data must await further study.

**Fig. 8.** Photomicrographs illustrating labeled cells in the PVN (−17.0 mm rostral to obex) in ferrets after injection of 2 doses of CCK (intravenous) or LiCl (intraperitoneal). Examples of dual-labeling for c-Fos and AVP (top), c-Fos and OT (middle), and c-Fos and corticotropin-releasing factor (CRF; bottom) are shown. The region depicted in the photomicrographs is indicated by a rectangle in the camera lucida drawings at the left. **A**: location of neurons containing AVP- and OT-like immunoreactivity combined with cFos expression; **B**: location of neurons with CRF-like immunoreactivity combined with c-Fos expression. Scale bar 250 μm.
In summary, the present report provides evidence that nauseogenic doses of CCK and an emetic dose of LiCl induce reproducible and specific patterns of brain stem and forebrain c-Fos expression in ferrets that are largely similar to c-Fos expression patterns in CCK- or LiCl-treated rats, despite species-related differences in posterior pituitary hormone release and despite the differential emetic effects of these agents. Thus rats appear to serve as adequate models for studying central neural responses to nauseogenic and emetic treatments in emetic species. The behavioral and neuroendocrine effects produced by CCK and LiCl appear to be at least partly mediated by very similar, if not the same, central neural pathways. The exact nature of these pathways remains to be determined, but catecholaminergic and GLP-1-containing projections from the caudal brain stem to the hypothalamus must be considered as strong candidates for mediating or modulating the neuroendocrine and behavioral effects of nauseogenic treatments in both emetic and nonemetic species.

**Fig. 9.** Photomicrographs illustrating labeled magnocellular neurons in the supraoptic nucleus, −17.0 mm rostral to obex, in a ferret that received 50 μg/kg CCK. Cells that were dually labeled for c-Fos and AVP (A) or c-Fos and OT (B) are shown. ot, optic tract. Scale bars 250 μm.

**Fig. 10.** Examples of c-Fos labeling in the central amygdaloid nucleus (CeA) and medial bed nucleus of stria terminalis (mBNST) after the injection of 50 μg/kg CCK. Right: enlargements of the areas circumscribed by a rectangle in the photomicrographs on the left. EN, entopeduncular nucleus; f, fornix; ic, internal capsule; lv, lateral ventricle. Scale bars 500 μm.
Perspectives

Together with results from previous studies, the present findings indicate that nauseogenic stimuli activate neurons in similar areas of the central visceral neuraxis in emetic and nonemetic species. These findings help justify the use of “lower” mammalian species, such as rats, as acceptable experimental models for studying the functional organization of central nausea circuits in “higher” species, perhaps including humans. Despite differences in pituitary hormone secretion and hypothalamic c-Fos expression, the similarities across species in nausea-induced brain stem and limbic forebrain activation patterns are striking. Phylogenetic conservation of these neural systems likely reflects the survival value of centrally mediated homeostatic responses to nausea and malaise and emphasizes the close interplay between the viscera and the limbic forebrain. In this regard, the present findings draw attention to hindbrain catecholaminergic and GLP-1 neurons as potentially important players in the behavioral, endocrine, and autonomic features of nausea. In rats, catecholaminergic and GLP-1 neurons relay visceral sensory signals to the hypothalamus and limbic forebrain and receive reciprocal descending projections that allow forebrain modulation of visceral sensory processing. Elucidating the functional organization of these central neural circuits in animal models should contribute to our understanding of how the functional state of the viscera influences subjective feelings, and vice versa, in humans.

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