Role of corticosterone in the murine enteric nervous system during fasting

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Lowette K, Tack J, Vanden Berghe P. Role of corticosterone in the murine enteric nervous system during fasting. Am J Physiol Gastrointest Liver Physiol 307: G905–G913, 2014. First published September 11, 2014; doi:10.1152/ajpgi.00233.2014.—Food intake depends on a tightly controlled interplay of appetite hormones and the enteric (ENS) and central nervous system. Corticosterone (CORT), levels, which are mainly studied with regard to stress, are also increased during fasting. However, the role of CORT in the ENS remains elusive. Therefore, we investigated whether CORT modulates activity of enteric neurons and whether its intracellular regulator, 11β-hydroxysteroid dehydrogenase (HSD) type 1, is present in the myenteric plexus, using immunohistochemistry and RT-qPCR. Effects of CORT on neuronal activity and expression of neuronal markers in the myenteric plexus were assessed via Ca2+ imaging and RT-qPCR, respectively, whereas modulations in mixing behavior were measured by video imaging. 11β-HSD-1 was present in enteric neurons along the gastrointestinal tract, and its expression increased after fasting (control: 0.58 ± 0.09 vs. fasted: 1.5 ± 0.23; P < 0.05). CORT incubation significantly reduced neuronal Ca2+ transients in tissues stimulated by electrical pulses (control: 1.31 ± 0.01 vs. CORT: 1.27 ± 0.01, P < 0.01) and in cultured neurons (control: 1.85 ± 0.03 vs. CORT: 1.76 ± 0.03, P < 0.05). CORT decreased small intestinal mixing (P < 0.05). Incubation of muscle myenteric plexus preparations with CORT induced an increase in cannabinoid receptor 1 (CB1, P < 0.05) and synaptobrevin (P < 0.05) but not in 11β-HSD-1 mRNA expression. In addition, fasting induced significant elevations in synaptobrevin (P < 0.05) and CB1 (P < 0.01) mRNA expression. In conclusion, we suggest CORT to be a downstream factor in maintaining state-related pathways that modulate important proteins in the fine tuning of enteric neurotransmission and gastrointestinal motility.

appetite; neuromodulation

Fasting and feeding cycles were shown to influence the activity of neurons in the enteric nervous system (ENS), a highly specialized network embedded in the intestinal wall along the entire length of the gastrointestinal tract (32). These nerves coordinate different gastrointestinal functions, including digestion of food, propulsion of gut content, and absorption of nutrients. The ENS circuits controlling intestinal function are built up by intrinsic primary afferent neurons, interneurons, and motor neurons, which form ganglioneuronal networks located in the submucous and myenteric nerve plexus. These neurons are activated by the presence of luminal contents and modulated by hormone levels that wax and wane depending on the feeding status (15). Satiety is induced by the rise of cholecystokinin, pancreatic polypeptide, and peptide YY, whereas hunger is associated with an increase in ghrelin (27).

In addition to these appetite hormones and their well-studied food intake-related effects in ENS and the central nervous system (CNS), corticosterone (CORT), one of the stress hormones, has also been shown to be involved in the regulation of appetite. Stress has been reported to affect food intake in a bidirectional way in humans, leading to increased eating in 70% and weight loss in 30% of individuals via a complex interplay with various appetite hormones (e.g., leptin, insulin, and neuropeptide Y) (1). These observations clearly demonstrate that stress is able to modulate hunger; however, interestingly, hunger itself has been shown to induce alterations in stress hormone (CORT) levels (25). Although it is clear that CORT levels rise during hunger to provide energy to fuel hepatic gluconeogenesis by mobilizing substrate, its role in the ENS during fasting remains unknown (9).

CORT has, upon crossing the blood-brain barrier, profound effects in the CNS as it binds to cytoplasmic or membrane-bound mineralocorticoid receptors (type I) or glucocorticoid (type II) receptors, which after translocation in the nucleus function as transcription factors (30). Derived from CNS studies, CORT has been recognized to target enzymes involved in neurotransmitter synthesis and ion channel and receptor expression (20). Plasma CORT levels depend on production in the adrenal cortex in response to the activation of the hypothalamic-pituitary-adrenal (HPA) axis by stressors. Activity of this axis is controlled by a tightly regulated feedback mechanism that is modulated by the endocannabinoid system. Endocannabinoids, via cannabinoid receptor 1 (CB1), mediate CORT-induced fast feedback inhibition of the HPA axis at the paraventricular nucleus (PVN) of the hypothalamus (14). Next to plasma CORT concentrations, intracellular CORT levels are regulated by 11β-hydroxysteroid dehydrogenase (HSD) type 1, an enzyme that is abundantly present in liver, adipose tissue, and the CNS (10). 11β-HSD-1 is a bidirectional enzyme that predominantly acts as a reductase to generate active CORT intracellularly from inert 11-dehydrocorticosterone. In the CNS, 11β-HSD-1 has been suggested to play an important role in HPA axis feedback regulation by modulating CORT levels (20). Moreover, the high expression of 11β-HSD-1 in hippocampus and cerebellum suggests a role for this enzyme, together with CORT, in behavior, cognition, neuronal development, and structure (18).

We hypothesize that CORT is an important neuromodulator of appetite-related fine tuning of ENS activity. Therefore, in this study, we investigate the role of elevated CORT levels during fasting in the ENS of mouse small intestine using Ca2+-imaging and intestinal motility studies. We start by assessing plasma CORT levels and 11β-HSD-1 expression in the ileum after fasting, and, considering the negative feedback role of endocannabinoids, we also measure the local effect of CORT on CB1 expression in the ileum. Interestingly, Burdyga et al. (7) have shown that CB1 expression in vagal afferent neurons increased after 24 h of fasting, demonstrating the involvement of endocannabinoids in the control of appetite.
Our data expand the understanding of the effects fasting and feeding have on the physiology of the ENS, which can be of therapeutic importance in view of food intake-related diseases such as obesity, bulimia, and anorexia nervosa.

METHODS

Animals

Male C57BL/6N mice (8–9 wk) were housed in the animal facility of the KU Leuven and randomly assigned to three groups each subjected to different food access conditions (control, fasted, and refed). Control mice were fed regular chow ad libitum, fasted mice were fasted for 20 h, and refed mice were fasted for 20 h and subsequently refed for 3 h.

Immunostaining

After the mice were killed by cervical dislocation (a euthanasia procedure approved by the Ethical Animal Committee of the KU Leuven, Belgium), the stomach and intestines were removed, cut along the mesentery, and pinned open with mucosa facing up. Tissues were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) containing Krebs solution for 30 min on ice. Subsequently, after being washed with PBS (1.54 mM KH2PO4, 155.17 mM NaCl, and 2.71 mM Na2HPO4, pH 7.4), the mucosal and circular muscle layers were removed by dissection. Next, longitudinal muscle myenteric plexus (LMPM) preparations were permeabilized in PBS with 0.5% Triton X-100 containing 4% donkey serum to block specific binding sites. Primary antibodies (Table 1) were diluted in donkey serum and incubated for 24 h at 4°C. After being washed with PBS (3 × 10 min), the cells were incubated for 2 h at 4°C with the appropriate secondary antibodies (Table 1). Immunoreactivity was visualized using a BX-41 fluorescence microscope (Cell Imaging Core). Six consecutive images (blue 458/470 – 495/505, green 505/510 – 550, and red 570 – 590/595/600 – 660) were captured using specific filter cubes (excitation/dichroic mirror/fluorescence microscope (Olympus, Aartselaar, Belgium) with a metal halide light source and specific filter cubes (excitation/dichroic mirror/fluorescence microscope (T500 SP2, Leica, Wetzlar, Germany) with a metal halide light source). Fluorescent signals were captured using the appropriate filter cubes (455–495 nm, 505–530 nm, and 570–615 nm).

Plasma CORT Measurements

Animals (n = 6 animals/group) were rapidly decapitated (a euthanasia procedure approved by the Ethical Animal Committee of the KU Leuven, Belgium) at 10:00 A.M., and trunk blood was collected. Blood was centrifuged at 2,000 g for 10 min at 20°C. Supernatant plasma was rapidly frozen using liquid nitrogen and stored at −80°C.

RNA was isolated from the isolated LMMP preparations using TRIzol reagent (Invitrogen, Merelbeke, Belgium) according to the manufacturer’s protocol. RNA samples of mouse small intestinal tissue were prepared using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed to cDNA with SuperScript II Reverse Transcriptase. The obtained cDNA served as a template for the PCR reaction, which was run on a LightCycler 480 system (Roche Diagnostics, Vilvoorde, Belgium) using LightCycler 480 SYBR Green I Master mix. Differences in 11β-HSD-1 (forward: CTCAGAGGTAGTTGTCCTCGC, reverse: CCTTGCACTAAATTGCTCCCGA), synaptotobrevin (forward: GAGGTGATCTGCGCCCATCAT, reverse: GGCAGACTCCTCAAGGGATT), CB1 (forward: CTGGTCTGTACCTGTTGTT, reverse: TTGCTAGCTGCTTGCTCCT), and mRNA expression in muscle myenteric plexus preparations were measured using specific primer sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: CCCCCATGTTGCGCCTGCGT, reverse: GCCTGTCTCCACCCCTTCT) was used as housekeeping gene, and for each gene a standard curve was created to obtain PCR efficiencies. Relative expression levels of all samples were calculated with the LightCycler 480 software and were expressed relative to GAPDH and corrected for interrun variability.

Table 1. Primary and secondary antibodies used in immunohistochemistry experiments

<table>
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<th>Primary/Secondary</th>
<th>Company</th>
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<th>Host</th>
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<td>HuC/D</td>
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</tr>
<tr>
<td>11β-HSD-1</td>
<td>Gift S. Webster (11)</td>
<td>1:1,000</td>
<td>Sheep</td>
</tr>
<tr>
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<td>Santa Cruz Biotechnologies</td>
<td>1:400</td>
<td>Rabbit</td>
</tr>
<tr>
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<td>Rabbit</td>
</tr>
<tr>
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<table>
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<td>Alexa Fluor 488</td>
<td>Molecular Probes, Invitrogen</td>
</tr>
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<td>Molecular Probes, Invitrogen</td>
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<td>Alexa Fluor 488</td>
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11β-HSD-1, 11β-hydroxysteroid dehydrogenase type 1.
supernatant was subsequently discarded. At the end of the third washing step, the pellet was resuspended in “complete” medium [DMEM F-12 (1:1) enriched with 10% FBS, 1% glutamine, 0.5% penicillin, and 0.5% streptomycin (Lonza Group)]. The cells were inoculated on round glass cover slips coated with poly-D-lysine hydrobromide (0.5 mg/ml in borate buffer) and laminin (20 μg/ml in PBS) (both from Sigma-Aldrich). The cultures were allowed to grow for 24 h incubated at 37°C, continuously gassed with 5% CO2. Next, the complete medium was replaced by a serum-free medium to which nerve growth factor (0.05%; Alomone Laboratories, Jerusalem, Israel) and neuron (0.2%)- and glia (0.2%)-specific (GIBCO Invitrogen) supplements were added (16).

**Ca2⁺ Imaging**

**Preparation.** The Ca2⁺-imaging experiments were performed on primary myenteric mouse cultures and mouse small intestine LMMP preparations (ileum). LMMP preparations were stretched over a small inox ring and immobilized by a matched rubber O ring (35). Subsequently, mouse cultures and LMMP preparations were incubated with CORT or vehicle (DMSO).

**Fluo 4 loading.** Primary myenteric mouse cultures/LMMP preparations were loaded with 5/1 μM fluo 4 acetoxyethyl (20 min) and washed in HEPES/Krebs solution (10 min, RT). Cultures were first transferred to a cover glass chamber before mounting on the micro-
scope stage. Changes in intracellular Ca$^{2+}$ concentrations were reflected in fluo 4 fluorescence intensity and recorded at 525/50 nm. Cultures/LMMP preparations were continuously perfused with HEPES/Krebs, and neurons were identified by depolarization (75 mM K$^+$, RT) for 5 s. After a recovery period of 3 min, cultures were either acutely exposed to CORT or stimulated in an electrical field ($\times$Hz).

**Imaging setup.** Neuronal activity was monitored using an inverted Zeiss Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany) with TILL Poly V light source (TILL Photonics, Gräfelfing, Germany), cooled CCD camera (PCO Sensicam-QE, Kelheim, Germany), and TILL-VisION (TILL Photonics) software.

**Analysis.** All image analysis was performed with custom-written routines in Igor Pro (Wavemetrics, Lake Oswego, OR). Regions of interest were drawn, after which the average Ca$^{2+}$ signal intensity was calculated, normalized to the initial fluo 4 values, and reported as F/F$_0$. The Ca$^{2+}$-response data (amplitude and peak duration) were compared between control and CORT groups.

**Video Imaging of Mixing Behavior**

Segments of ileum were suspended in an organ bath filled with Krebs solution kept at 37°C and continuously bubbled with 95% O$_2$-5% CO$_2$ (pH 7.4). Tetrodotoxin (TTX) (1 µM, 10 min), nifedipine (1 µM, 10 min), CORT (1 µM, 20 min), or vehicle containing Krebs solution (DMSO, 20 min) was used to investigate contractile behavior. After baseline was recorded (0 cm pH$_2$O), the intraluminal

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**Fig. 2.** Immunohistochemical staining for 11β-HSD-1 (red) in myenteric plexus of mouse ileum in combination with nNOS (green, $A$), calretinin (green, $B$), and neurofilament M (green, $C$). Scale bar: 50 µm.
pressure was subsequently elevated to 1.2, and 4 cm H2O at the oral side and filmed for 90 s (1 min interval between recordings). All movies (90 s, 10 Hz frame rate) were recorded with and sampled on a Sensory 611 PCI Frame Grabber. Images were read into Igor Pro (WaveMetrics) and analyzed using custom-written algorithms based on Henning et al. (19) to assess intestinal swelling and high-frequency mixing behavior (32).

Statistics

Unless stated otherwise, all data are presented as means ± SE. Distributions were tested for normality with a Kolmogorov Smirnov test. Depending on the distribution, data from different conditions were compared using parametric one-way ANOVA, nonparametric Mann Whitney U-test, or Kruskal Wallis ANOVA. Differences were considered significant if $P < 0.05$. Statistical analysis was performed with Statistica (StatSoft).

RESULTS

Sources of Fasting-Induced CORT Elevation

To address the effects elevated CORT levels as during fasting have on the ENS, we first investigated what possible sources of CORT were involved. The increased plasma CORT levels, as measured by a CORT enzyme immune assay, indicate the involvement of HPA axis activation during fasting (control: 6.4 ± 3.1 nM, fasted: 530 ± 200 nM, $P < 0.01$). This effect was partially restored after a 3-h refeeding period (refed: 47 ± 23 nM, $n = 6$) compared with the fasted ($P < 0.01$) and the control ($P < 0.05$) condition (Fig. 1A). Apart from an increase in circulating CORT levels, fasting also induced a significant increase in 11β-HSD-1 mRNA expression in muscle myenteric plexus preparations (control: 0.58 ± 0.09 vs. fasted: 1.5 ± 0.23, $P < 0.01$) that remained significantly elevated even after refeeding (refed: 1.08 ± 0.12, $P < 0.05$) (Fig. 1B). At present no information is available about the potential expression of 11β-HSD-1 in the ENS. Using specific antibodies against 11β-HSD-1 and the pan neuronal marker HuC/D we found that 11β-HSD-1 is expressed in myenteric neurons along the gastrointestinal tract from stomach to colon (Fig. 1C shows expression in small intestine). However, immunohistochemical stainings did not show significant differences in the number of 11β-HSD-1-positive myenteric neurons in the ileum during fasting and refeeding cycles (control: 36.91 ± 2.46% vs. fasted: 31.18 ± 3.63% vs. refed: 37.79 ± 2.64, $n = 4$). In addition, to identify whether 11β-HSD-1 expression was selective to specific subsets of neurons, we used three other neuronal markers (nNOS, calretinin, and neurofilament M). We found that 11β-HSD-1-positive cell bodies did not colocalize with nNOS and calretinin expression and that 11β-HSD-1-expressing fibers were mainly neurofilament M negative (Fig. 2, A–C).

CORT Reduces Ca2+ Responses in Enteric Neurons

To investigate whether CORT was able to acutely affect enteric neuronal and synaptic signaling, we performed Ca2+ imaging in primary myenteric mouse cultures using a single application of CORT at a high concentration (10 μM). In both neurons and varicose release sites (boutons), CORT induced slowly rising Ca2+ responses that were quite variable in terms of amplitude and shape, as seen from large standard deviations of the average responses (Fig. 3A). These acute responses to CORT were confirmed in tissue experiments where neurons also showed CORT-induced slowly rising Ca2+ responses that were significantly higher than those induced by the vehicle (details not shown). Because these Ca2+ transients were not like any of the responses elicited by neuronal depolarization (high K+) or neurotransmitter activation, we sought to investigate whether this slow rise could reflect long-term changes in enteric neurons (6, 34). Therefore, we recorded Ca2+ signaling in LMMP preparations that were exposed to CORT (20 min), at a concentration mimicking fasting plasma CORT levels (100 nM, $n = 4$). Electrical stimulation in CORT-incubated tissues induced significantly lower neuronal Ca2+ amplitudes compared with vehicle-treated control tissues (control: 1.31 ± 0.01 vs. CORT: 1.27 ± 0.01, $P < 0.01$) (Fig. 3, B and C). Because CORT levels, as seen by others in rats, already start rising after 12 h of fasting (12), longer-term effects of CORT were studied by incubating cultured myenteric neurons with the fasting CORT concentration (100 nM) for 20 h ($n = 4$). Resulting Ca2+ amplitudes confirm the inhibitory effect of CORT incubation on myenteric neurons compared with time-matched control cultures (control: 1.85 ± 0.03 vs. CORT: 1.76 ± 0.03, $P < 0.05$) (Fig. 3, D and E). In addition to the amplitude, 20 h of CORT incubation induced a significant decrease in Ca2+-response duration (at 50% of the maximum) after electrical stimulation of the neurons (control: 9.15 ± 0.19 s vs. CORT: 8.00 ± 0.14 s, $P < 0.001$) (Fig. 3, D and E).

CORT Modulates Small Intestinal Mixing Behavior

To study whether CORT has, in addition to its inhibitory effect on neuronal Ca2+ signaling, the ability to modulate small intestinal motility, we imaged the pressure-induced contractile patterns in the mouse ileum after exposure to CORT. Contrary to guinea pig small intestine, pressure stimuli do not elicit peristaltic waves in the mouse small intestine (23, 32). Therefore, two parameters (intestinal diameter and high-frequency contractions) were assessed (Fig. 4A). To validate these parameters, we made recordings either in the presence or absence of high K+ (100 mM), or nifedipine, an L-type Ca2+ channel blocker. TTX, to prevent neuronal contractions, or nifedipine, an L-type Ca2+ channel blocker. TTX significantly decreased the average diameter, whereas nifedipine caused dilation (Fig. 4C). The high-frequency mixing contractions were slightly decreased by TTX and abolished by nifedipine (Fig. 4, B and D). Incubation with CORT (20 min) did not have an effect on the diameter but induced a significant decrease in high-frequency contractions ($P < 0.05$) (Fig. 4).

Modulation of Gene Expression in CORT-Incubated Ileum

To elucidate the molecular changes underlying the CORT-induced modulations in Ca2+ buffering and intestinal motility, we investigated mRNA levels of two specific synaptic communication-related proteins (synaptobrevin and the CB1 receptor) in an in vitro setting by incubating ileal muscle myenteric plexus preparations with CORT (100 nM, 20 h). CORT incubation induced significant increases in the mRNA levels of both synaptobrevin (control: 1.87 ± 0.14 vs. CORT: 2.68 ± 0.4, $P < 0.05$) and CB1 (control: 0.59 ± 0.06 vs. CORT: 0.83 ± 0.11, $P < 0.05$) (Fig. 5, A and B). The 11β-HSD-1 mRNA expression after CORT incubation was not significantly altered, suggesting that an integrated system is required to
regulate levels of the local CORT-converting enzyme (control: 0.9 ± 0.18 vs. CORT: 1.35 ± 0.28). Next, to investigate if the specific feeding state-related changes in 11β-HSD-1 mRNA expression were associated with modulations in synaptobrevin and CB1 mRNA levels, we also measured mRNA levels after fasting and refeeding in muscle myenteric plexus preparations. Here again, synaptobrevin mRNA expression (control: 2.11 ± 0.13 vs. fasted: 3.02 ± 0.32 vs. refed: 2.24 ± 0.35, \( P < 0.05 \)) and CB1 (control: 0.56 ± 0.06 vs. fasted: 1.37 ± 0.25 vs. refed: 0.85 ± 0.16, \( P < 0.01 \)) mRNA expression were increased after fasting compared with the control mice, confirming the involvement of CORT in a feeding state-related pathway affecting the enteric nervous system (Fig. 5, C and D).

DISCUSSION

In this study, we investigated the effect of CORT on ENS activity, expression of neuronal markers in the ENS, and intestinal motility. Resulting data show for the first time that 11β-HSD-1, the intracellular regulator of CORT levels, is expressed in the myenteric plexus along the entire gastrointestinal tract and that its intestinal mRNA expression is modulated by feeding state. In our immunohistochemical studies on whole mount preparations, we found clear evidence for CORT-converting enzyme 11β-HSD-1 expression by subsets of enteric neurons that were neither nNOS nor calretinin positive. One earlier study by Ergang et al. (13) identified the presence of 11β-HSD-1 in the mucosa of rat colon where they showed
11β-HSD-1 mRNA and protein expression that increased in response to inflammation, however, without any indication that 11β-HSD-1 would be present in enteric nerves. At present, we have not been able to identify a known subclass of neurons that expresses 11β-HSD-1. In the CNS, 11β-HSD-1 expression had been found in hippocampal, PVN, and cerebellar neurons (17, 20). Roles for 11β-HSD-1 in neural pathways were discovered by using 11β-HSD-1 null mice (18) showing that, after deletion of the 11β-HSD-1 gene, mice expressed a longer CORT peak and increased plasma CORT and ACTH concentrations, suggesting attenuated negative HPA feedback. Apart from regulating HPA control, Densmore et al. (10) demonstrated that 11β-HSD-1 was able to modulate agouti-related peptide (AGRP) expression in the arcuate nucleus of the hypothalamus to regulate appetite. AGRP mRNA in 11β-HSD-1-deficient mice on a high-fat diet was elevated in the arcuate nucleus resulting in hyperphagia, suggesting a (diet-specific) role for 11β-HSD-1 in appetite control (10). The role of 11β-HSD-1 or even the effect of CORT itself on intestinal function has never been demonstrated; however, other stress-related hormones such as corticotropin-releasing factor (CRF) and urocortins have been recognized to delay gastric emptying and increase colonic motility in healthy volunteers (33). In addition, CRF has been recognized to induce activity in guinea pig myenteric neurons via a CRF-1 receptor-dependent mechanism (4).

In the present study, we could show for the first time that CORT, at concentrations similar as observed during fasting, reduces electrical stimulation-induced Ca²⁺-response amplitudes in myenteric neurons in tissue and in cultures. Moreover, durations of the Ca²⁺ responses were also decreased by CORT incubation in myenteric neuron cultures. In the CNS, the ability of CORT to modulate neuronal activity and synaptic transmission has long been recognized. Studies by Musazzi et al. (28) in the prefrontal and frontal cortex showed that acute stress

**Fig. 4. Video imaging of pressure-induced contractile patterns in the mouse ileum.**
A: color-coded spatiotemporal map (left) of diameter variations and contraction patterns in the mouse small intestine. A median filtered map highlights the slower changes in diameter over time (middle) that, after subtraction of the original map, generate a map (right) containing the high-frequency mixing contractions. B: spatiotemporal map of the high-frequency mixing after incubation with vehicle, tetrodotoxin (TTX), nifedipine, and CORT (1 μM) at 2 cm pH₂O pressure. C: change in intestinal diameter at 1, 2, and 4 cm pH₂O in control and in the presence of TTX, nifedipine, and CORT (1 μM). D: change in high-frequency mixing at 1, 2, and 4 cm pH₂O in control and in the presence of TTX, nifedipine, and CORT (1 μM). P < 0.05 (*) and 0.01 (**).
could increase the readily releasable pool of synaptic vesicles by increasing the amount of presynaptic SNARE protein complexes, of which synaptobrevin is a member. We demonstrated that CORT incubation also induces a local increase in synaptobrevin mRNA in myenteric neurons. However, apart from only a synaptic protein enhancement, we also demonstrated a CORT-induced increase in CB1 receptor expression. The increase in CB1 might be relevant regarding neuronal activity, since Boesmans et al. showed that Ca^{2+} buffering in myenteric neurons was inhibited by CB1 receptor activation (5). In addition, Hons et al. (22) demonstrated the inhibition of synaptic transmission in the myenteric plexus of the ileum by CB1, which was suggested to result from changes in Ca^{2+} influx into the presynaptic neuron. Furthermore, we were able to show that CORT decreased intestinal mixing without altering gut diameter. This CORT-induced reduction in mixing behavior is suggested to be associated with CB1 activity, since substantial evidence is available to demonstrate an inhibitory effect of cannabinoids on gastrointestinal motility (8, 29). Based on the Ca^{2+} and video imaging results, the elevated expression of CB1 seems to overrule any enhancing effect of CORT on synaptic transmission, which results in an overall negative effect on activity. Several mechanisms for CB1-induced inhibition of gut motility have been suggested, including the modulation of adenosine release, nonadrenergic noncholinergic excitatory transmission, and a decrease in acetylcholine release from enteric neurons in ileum and colon (2, 3, 24, 26). Local and direct CORT effects have also been shown in mouse colon, where, in contrast to our observations, CORT decreased CB1 protein expression in dorsal root ganglia innervating the colon (21). In addition, Reich et al. (31) showed significantly downregulated CB1 protein levels in the male rat hippocampus in response to chronic mild stress and upregulation in the hippocampus of female rats. These data show that the CORT-mediated modulation of CB1 expression differs not only between genders but also between central and peripheral neurons. Probably also the type of stressor is important.

In conclusion, the present study shows a neuromodulatory role for CORT during fasting in the gastrointestinal tract leading to reduced neuronal Ca^{2+} responses in myenteric neurons and mixing behavior in mouse ileum. Adding further support to a role for CORT in altering enteric neuronal control, we demonstrated expression of 11β-HSD-1 in the myenteric plexus. Because fasting induced higher CORT levels, increased 11β-HSD-1 mRNA and synaptobrevin and CB1, whereas CORT application only changes the levels of the latter two, we propose a model in which modulation of synaptic communication is downstream of CORT, which in turn is a consequence of the HPA axis and local (11β-HSD-1)-mediated CORT production during fasting. We conclude that CORT is an important modulator of proteins involved in the fine tuning of enteric neurotransmission and gastrointestinal mixing movements (Fig. 6). Although more research is needed to fully unravel the mechanisms underlying the CORT-induced effects, current results confirm a role for CORT in a feeding state-related pathway affecting enteric neurons, which adds to an improved understanding of appetite physiology. This is of great importance in view of the emerging role for the ENS in appetite-regulating function and for the development of therapeutic strategies in diseases such as obesity, bulimia, and anorexia nervosa.

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The authors disclose no conflicts of interest.

AUTHOR CONTRIBUTIONS
Author contributions: K.L. and P.V.B. conception and design of research; K.L. performed experiments; K.L. analyzed data; K.L. and P.V.B. interpreted results of experiments; K.L. prepared figures; K.L. drafted manuscript; J.T. and P.V.B. edited and revised manuscript; J.T. and P.V.B. approved final version of manuscript.

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