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CCK₂ receptor nullification attenuates lipopolysaccharide-induced sickness behavior

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Weiland, Tracey J., Nicholas J. Voudouris, and Stephen Kent. CCK₂ receptor nullification attenuates lipopolysaccharide-induced sickness behavior. Am J Physiol Regul Integr Comp Physiol 292: R112–R123, 2007. First published July 20, 2006; doi:10.1152/ajpregu.00156.2006.—Systemic infection produces a highly regulated set of responses such as fever, anorexia, adipsia, inactivity, and cachexia, collectively referred to as sickness behavior. Although the expression of sickness behavior requires immune-brain communication, the mechanisms by which peripheral cytokines signal the brain are unclear. Several mechanisms have been proposed for neuroimmune communication, including the interaction of cytokines with peripheral nerves. A critical role has been ascribed to the vagus nerve in mediating sickness behavior after intraperitoneally delivered immune activation, and converging evidence suggests that this communication may involve neurochemical intermediaries afferent and/or efferent to this nerve. Mice lacking functional CCK₂ receptors (CCK₂KO) and wild-type (WT) controls were administered LPS (50, 500, or 2,500 μg/kg; serotype 0111:B4; ip). Results indicate a role for CCK₂ receptor activation in the initiation and maintenance of LPS-induced sickness behavior. Compared with WT controls, CCK₂KO mice were significantly less affected by LPS on measures of body temperature, activity, body weight, and food intake, with the magnitude of effects increasing with increasing LPS dose. Although activation of CCK₂ receptors at the level of the vagus nerve cannot be excluded, a possible role for these receptors in nonvagal routes of immune-brain communication is suggested.

cholecystokinin; lipopolysaccharide; infection; vagus; fever

The GUT-BRAIN PEPTIDE, CCK, exhibits great potential as a neurochemical intermediary of vagus-to-brain signaling. CCK binds to CCK₁ and CCK₂ receptors; the former are primarily localized in peripheral organs and some discrete areas of the brain, whereas CCK₂ receptors are the predominant subtype in the brain (55). Both subtypes are present on the vagus nerve and vagal afferent connections, including the brainstem and hypothalamus (46, 51). Thus the peripheral and central positioning of CCK receptors provides an anatomical basis for CCK to play a role in vagally mediated neuroimmune communication.

A constellation of findings points to a role for CCK in immune regulation, including a protective function in septic shock induced by LPS (41, 42), a compound of the outer cell wall of Gram-negative bacteria (60). CCK induces monocyte chemotaxis both in vitro and in vivo (16, 65) and stimulates production of proinflammatory cytokines, TNF, IL-1β, IL-6, and IL-8 (16). Peripheral administration of CCK induces a pattern of c-fos expression that is similar to that seen after administration of the immune activators, LPS, or IL-1β (19). Increases in plasma CCK have been reported in the rat after intraperitoneal IL-1α (18) or intravenous IL-1β (40). However, limitations in assay sensitivity and specificity render these findings questionable, and contradictory evidence has recently been presented (84).

Studies examining the involvement of CCK receptor activation in sickness behavior (anorexia, inactivity, cachexia) have focused primarily on the role of the CCK₁ receptor. Antagonism of the CCK₁ receptor blocked the decrease in feeding after intraperitoneal IL-1α in rats (18), an effect that could not be reproduced for food-motivated behavior in mice with low doses of LPS or IL-1β administered ip (8). The role of CCK receptor activation in fever genesis and maintenance is also equivocal. The febrile effects of LPS and IL-1β administered to rats in low doses were not attenuated by CCK₁ receptor antagonism (34, 44), although naturally occurring mutant rats deficient in CCK₁ receptors exhibited an exaggerated febrile response, a result attributed to a secondary trait of the strain (34).

Although several studies have examined the role of the CCK₁ receptor in sickness behavior, relatively little attention has been paid to CCK₂ receptors. Blockade of CCK₂ receptors, either peripherally (subcutaneously) or centrally (icv), attenuated fever in rats administered a low dose of LPS intraperitoneally (77). The role for CCK₂ receptors in fever, however, has yet to be confirmed when immune activators are administered intraperitoneally or with other pyrogens and species.

Because the effectiveness of vagotomy (VGX) in attenuating behavioral depression (17, 71) and fever (25, 63) induced by LPS or IL-1 is typically dose-dependent, studies of the role of CCK in sickness behavior have used low doses of the immune activators. However, given the diverse peptide and receptor distribution of CCK and its possible involvement in thermoregulatory effector mechanisms (24, 86), it is important to acknowledge that CCK may have a role in sickness behavior that is independent of vagal activation, thus underscoring the importance of examining a broad range of doses of immune activators.

The purpose of this study was to examine the role of CCK₂ receptors in the expression of LPS-induced sickness behavior. Changes in body temperature (Tb), locomotor activity, body weight, and food and water intake were monitored in response to a 50-fold range of LPS doses. The responses of mice that...
lack functional CCK\(_2\) receptors (CCK\(_2\)KO; 53) were compared with responses of the background strain, or wild-type (WT). Investigations of the mechanisms underlying LPS-induced fever and anorexia are typically restricted to observations of a few hours (e.g., 70, 79). However, recent evidence suggests that the sickness response follows a protracted time course (20). Given this and the large range of doses used in the present study, all parameters were monitored for 4 days postinjection.

MATERIALS AND METHODS

Animals. Thirty-two male homozygous CCK\(_2\)KO mice (La Trobe University, Melbourne, Australia), and 32 male C57BL/6J mice (Mus musculus) as WT mice (La Trobe University) were used for the present study. All CCK\(_2\)KO mice were homozygous for the disrupted CCK\(_2\) receptor gene and were developed through gene targeting, as described previously (53, 58). Briefly, the CCK\(_2\) gene was isolated from a 129sv genomic library, and the clone inserted in a vector. Clones exhibiting homologous recombination on the disrupted CCK\(_2\) gene were microinjected into blastocysts of C57BL/6J females. Chimerics were bred with C57BL/6J mice to enable germ line transmission. The first generation of heterozygous animals were then bred for 10 generations, selecting for the mutant CCK\(_2\) gene at each generation. Fifth-generation heterozygous mice were then bred to generate CCK\(_2\)KO mice. When backcrossed with C57BL/6J for several generations, the WT strain or genetic background for such transgenic strains becomes the C57BL/6J (54). Confirmation that the CCK\(_2\)KO strain is, in fact, deficient in a functional CCK\(_2\) receptor has been provided previously (53). Every effort was made to age-match the animals. At the commencement of the recording period, CCK\(_2\)KO mice were aged between 84 and 120 days (means expressed as 98 ± 2 SE, median = 96), and their average body weight was 29.5 ± 0.4 and 26.8 ± 0.2 g (median = 28.2 g; range: 24.0 to 35.1 g).

Drugs. An immune response was triggered through the intraperitoneal administration of LPS (serotype: 011:B4; Sigma, Sydney) in doses of 50 μg/kg, 500 μg/kg, and 2,500 μg/kg. Lyophilized LPS was dissolved in sterile, pyrogen-free, isotonic saline solution (Astra Pharmaceuticals, North Ryde, Australia), aliquotted, and stored at −20°C in Eppendorf tubes until required. Before injection, aliquots were thawed at 4°C, and an appropriate volume of saline was added to bring the diluent to the correct concentration for injection. Solutions were then raised to room temperature. All LPS injections were administered 1 h after the onset of the photophase.

Procedure. Throughout experimentation, mice were housed individually under a 12:12-h light-dark cycle with food and water available ad libitum, and ambient temperature set at 30 ± 1°C, which is within the thermoneutral zone for this species. Sawdust and tissue were provided as bedding. Following a 2-wk acclimation period, a bioteflometry device (22 mm × 8 mm; 1.83 g; E-4000, MiniMitter, Bend, OR) was surgically implanted into the peritoneal cavity of each animal, as described previously (83).

A 7–10-day recovery period followed surgery, after which time, mice were housed singly in polypropylene enclosures. This apparatus, which enables the determination of Tb and the quantification of general locomotor activity and food and water intake, has been described in detail previously (83). Data for Tb, locomotor activity, and food intake were decoded by VitalView software (MiniMitter) at 1-min intervals and stored electronically.

Uneaten pellets were removed from the enclosure each morning and the number subtracted from the total number delivered each day for the final data set. Body weight and water consumption were manually recorded each morning. Body weight was measured to 10\(^{-2}\) g using top-loading scales. Water was dispensed following contact with a water nipple fed from a 25-ml vertical, polypropylene tube with 0.2-ml graduated markings.

All dependent variables (Tb, activity, body weight, food hopper entries, and water intake) were monitored during an acclimation period of at least 1 wk. Mice were considered to be adapted to the housing environment when 1) clear and consistent circadian rhythms in Tb, activity, and food hopper entries were present for at least 3 consecutive days; 2) daily water intake and body weight varied by less than 1 ml and 1.5 g, respectively, during this period; and 3) body weight was greater than presurgical weight. After successful acclimation, a 4-day baseline period commenced, during which time, all dependent measures were monitored.

After the baseline period, each animal was administered one of three treatments: 50, 500, or 2,500 μg/kg LPS, or the LPS vehicle, saline, 1 h after the onset of the photophase. Allocation of treatments was random, and treatments were administered intraperitoneally in a maximum volume of 100 μl/mouse. Dependent measures were then monitored for a further 4 days postinjection.

All procedures were conducted in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals and were approved by the La Trobe University Animal Ethics Committee.

Data analyses. Data were averaged, and means ± SE were calculated for each group and all variables for the 8-day testing period. Data for Tb were converted to 15 min, and 2-, and 12-hourly means. Activity data and food hopper entries were converted to 12-hourly means and totals, respectively, and expressed as percentage difference from the 4-day average baseline. Raw data for body weight and water consumption were collated and daily means for these variables were derived.

Data for each dose were analyzed separately, using univariate and two-way (time by strain or time by pretreatment) repeated-measures ANOVAs or ANCOVAs. This permitted the levels of time in the analyses performed to vary as a function of dose, an important consideration given that the duration of the effects of LPS is dose-dependent.

Identification of potential covariates was made using partial correlation. Missing data were replaced with the group mean when using food or water intake as covariates. To overcome violations of sphericity in the Tb data, the Greenhouse-Geisser statistic is reported for within-subjects effects. Where required, simple main effects were analyzed using Fisher’s least significant difference test. This statistic is suitable when the number of comparisons is small (32).

Basal strain differences in Tb, activity, body weight, and water intake from this data set have been reported previously (83). To control for preexisting individual and group differences, results for LPS-induced differences in Tb were analyzed on change from 4-day average baseline. LPS-induced group differences for all other dependent variables were tested on a percentage difference from average baseline and are presented as such. For Tb, activity, and food intake, data obtained for photophase and scotophase were analyzed separately.

For 15-min Tb expressed as a percentage difference from baseline, analyses were conducted on the initial 4-h period postinjection regardless of treatment. For 2-h Tb, analyses were conducted on the initial 24 h postinjection. For 12-hourly Tb, the first 24 h postinjection were analyzed for mice treated with saline or 50 μg/kg LPS, and the first 48 h were analyzed for mice administered either 500 or 2,500 μg/kg LPS. For activity data (percentage difference from baseline), the number of postinjection days analyzed was 1 for mice administered saline, 2 for mice administered 50 μg/kg LPS, and 3 for mice treated with 500 or 2,500 μg/kg LPS. Regardless of LPS dose, body weight analyses were conducted on data for the entire postinjection period since bodyweight typically failed to return to baseline. For saline-treated mice, however, body weight data were examined using data collected during the first day postinjection. Food pellet consumption, expressed as a percentage difference from baseline, was analyzed using separate two-way repeated-measures ANOVAs on data for the first 48 h for mice administered saline or 50 μg/kg LPS, and 96 h for
mice administered 500 or 2,500 µg/kg LPS. Water consumption analyses were conducted on data obtained during the first day postinjection, for all treatments except 2,500 µg/kg LPS, which was tested for a 48 h period.

RESULTS

Body temperature. The febrile response to 50 µg/kg LPS was less prominent in CCK2KO mice than WT mice, with the latter exhibiting a more rapid onset. ANOVA conducted on 15-min mean difference from average baseline Tb revealed significant effects for time, strain, and their interaction. The strain difference became significant from 90 to 105 min postinjection \([F(1, 14) = 5.26, P = 0.038 \eta^2 = 0.19]\) when a change from baseline of 0.48 ± 0.18 and −0.06 ± 0.08°C occurred for WT and CCK2KO mice, respectively (Fig. 1A). For 15 min of data, this difference remained significant for the remainder of the first 4 h period after injection. The strain difference was greatest 2–3 h postinjection (difference = 0.65°C), and for both strains, fever peaked at 5 h postinjection when an increase from baseline of 1.32 ± 0.26 and 0.65 ± 0.17°C occurred for WT and CCK2KO mice, respectively (Fig. 1B). ANOVA conducted on 2-hourly means indicated a significant strain difference throughout the photophase \([F(4, 56) = 3.34, P = 0.016 \eta^2 = 0.19]\). The mean 12-hourly increase from baseline during the initial photophase was significantly greater in WT circadian patterns of Tb were reestablished by injection. The febrile response compared with CCK2KO mice; this difference began to emerge 105–120 min post-LPS injection (Fig. 1). For 2 h, the Tb of CCK2KO mice remained 0.5°C or more greater than at baseline. The strain differences throughout the initial photophase and scotophase were confirmed by ANOVA on 2-hourly means \([\text{photophase: } F(1, 14) = 5.39, P = 0.036 \eta^2 = 0.28; \text{scotophase: } F(1, 14) = 23.54, P < 0.001 \eta^2 = 0.40]\). Inspection of 12-hourly means indicated substantial strain differences in Tb during the first 35 h after injection (Fig. 1F). ANOVA on scotophase data revealed significant effects for time, strain, and their interaction; the Tb of CCK2KO mice was significantly higher than that of WT mice during the initial postinjection scotophase \([F(1, 14) = 22.84, P < 0.001 \eta^2 = 0.62]\).

In response to saline, the Tb of WT mice initially decreased in the photophase by 0.39 ± 0.12°C (Fig. 1A) but increased by up to 0.55 ± 0.12°C by the end of this period (Fig. 1A and B). The Tb of CCK2KO mice remained similar to baseline during the start of the photophase but increased by 0.72 ± 0.21°C at the end of the first photophase. Although CCK2KO mice did not differ greatly from baseline during the initial scotophase period following injection, a decrease from baseline of up to 0.80 ± 0.17°C was found for WT mice (Fig. 1B). Examination of 15-min mean difference from baseline data for the first 4 h postinjection revealed a significant main effect for strain \([F(1, 14) = 6.05, P = 0.027 \eta^2 = 0.30]\) but not time or their interaction. The mean Tb of saline-treated WT mice was lower than that of CCK2KO mice 5–9 h postinjection (Fig. 1B). For 2 hourly photophase data, ANOVA indicated significant effects for strain \([F(1, 14) = 5.89, P = 0.029 \eta^2 = 0.30]\) and time, but not their interaction. Analyses of scotophase data indicated a significant interaction between time and strain. Simple main effects analyses revealed a significantly higher mean Tb for WT mice compared with CCK2KO mice from 20 to 23 h postsaline injection \([F(1, 14) = 12.94, P = 0.003, \eta^2 = 0.48; F(1, 14) = 4.95, P = 0.043, \eta^2 = 0.26]\). ANOVA indicated a significant difference between strains in 12-hourly mean Tb following saline during the first postinjection photophase \([F(1, 14) = 5.92, P = 0.029 \eta^2 = 0.30]\) and scotophase \([F(1, 14) = 5.68, P = 0.032 \eta^2 = 0.29]\) (Fig. 1C).

Locomotor activity. Both strains exhibited a decrease in locomotor activity during the first 2 days after 50 µg/kg LPS (Fig. 2A). These were most prominent during, but not limited to, the scotophase periods, and were greatest during the postinjection scotophase. Compared with WT mice, the activity levels of CCK2KO mice appeared less affected by LPS (Fig. 2A); however, the difference failed to reach significance.

After 500 µg/kg LPS, locomotor activity levels of both strains decreased substantially (Fig. 2B). A clear strain difference occurred in response to LPS, with CCK2KO mice being less affected than WT mice for the entire 4-day period (Fig.
This difference was most pronounced during the second scotophase when WT mice were 33 ± 11% less active and CCK2KO mice were 12 ± 28% more active, than at baseline. For scotophase data, ANOVA indicated significant effects for time, and strain [F(1, 11) = 5.94, P = 0.033, \( \eta^2 = 0.35 \)] but not their interaction.

Both strains exhibited a profound reduction in activity after 2,500 \( \mu \)g/kg LPS (Fig. 2B). Although activity during the
photophase periods remained less than baseline until day 4 postinjection, the decrease in activity was greatest for both strains during the initial scotophase period after LPS. During this period, WT and CCK2KO mice were 73% and 59% less active than at baseline, with activity falling below photophase levels (Fig. 2B). Although slight strain differences were present for this dose, they were not as distinct as those seen in mice treated with 500 μg/kg LPS (Fig. 2B), and they failed to reach significance.

Following saline intraperitoneal injection, both WT and CCK2KO mice produced a slight decrease from baseline activity during the initial photophase (Fig. 2A). In the ensuing scotophase, WT mice approached baseline whereas the activity of CCK2KO mice decreased to 26% less than baseline. A strain difference in activity was evident in the first scotophase and second photophase postinjection. However, only the scotophase difference reached significance \[F(1, 13) = 9.79, P = 0.008, \eta^2 = 0.43\].

Body weight. The cachexic effects of 50 μg/kg LPS were greatest for both strains on day 1 postinjection; WT and CCK2KO mice lost 4.7 ± 0.9 and 2.0 ± 0.6% body weight, respectively (Fig. 3A). The percentage weight loss of WT mice exceeded that of CCK2KO mice throughout the postinjection period with the strain difference peaking on day 2 postinjection (WT, −2.6 ± 0.9%; CCK2KO, 0.5 ± 0.4%, relative to baseline). ANOVA indicated significant main effects for strain \[F(1, 14) = 13.08, P = 0.003, \eta^2 = 0.48\] and time, but not their interaction.

When challenged with 500 μg/kg LPS WT and CCK2KO mice lost a maximum of 9.4 ± 0.2 and 3.5 ± 0.5%, respectively, with cachexia greatest on day 1 (Fig. 3B). CCK2KO mice were less affected by LPS, with the strain difference being greatest on day 2 postinjection. At this point, WT mice had lost only 8.3 ± 0.8%, whereas CCK2KO mice had lost 2.7 ± 0.7% of their baseline weights. By the end of the testing period, CCK2KO mice had returned to within 0.3 ± 0.6% of baseline body weight. In contrast, WT mice had returned to within 3.7 ± 1.2% of their initial weights. ANOVA indicated that the difference between strains was significant across the entire testing period \[F(1, 14) = 25.02, P < 0.001, \eta^2 = 0.64\].

When treated with the largest dose of LPS, WT, and CCK2KO mice lost 12.1 ± 0.6 and 6.4 ± 1.4% of body weight, respectively. In contrast to lower doses of LPS, the cachexic effects were greatest on day 2 postinjection, but weight loss was once again greatest in WT mice (Fig. 3B). The strain difference was largest on day 3 postinjection when CCK2KO mice lost 2.3 ± 1.1% and WT mice lost 9.6 ± 0.9% body weight (Fig. 3B). ANOVA indicated significant main effects.
CCK2KO mice consumed significantly more than their WT counterparts on the first 2 days postinjection only (Fig. 4A). During this period, the number of pellets consumed by WT and CCK2KO mice during the first scotophase period after injection. The decrease in consumption was greatest for both strains. Compared with WT mice, the food intake of CCK2KO mice was slightly less affected, and by the second scotophase, CCK2KO mice they had returned to within 6 ± 7% of baseline, whereas WT mice consumed 29 ± 6% less than baseline. Analyses of scotophase data revealed food consumption to be significantly less during the first postinjection scotophase regardless of strain. The main effect for strain was significant [F(1, 14) = 4.88, P = 0.040, η² = 0.26], but the time by strain interaction was not.

In response to 500 μg/kg LPS, both WT and CCK2KO mice decreased food consumption during the 4-day postinjection period (Fig. 4B). The decrease in consumption was greatest for both strains during the first scotophase period after injection. During this period, the number of pellets consumed by WT and CCK2KO mice decreased by 39.3 ± 2.6 and 28.9 ± 4.2, respectively. Strain differences were observed in mice treated with 500 μg/kg LPS; most notably during the first scotophase after LPS administration, when WT mice consumed 94 ± 1% and CCK2KO mice consumed 58 ± 10% less than baseline. ANOVA indicated that scotophase food intake varied significantly as a function of time, strain [F(1, 12) = 8.01, P = 0.015, η² = 0.40], and their interaction [F(1, 10) = 5.90, P = 0.002, η² = 0.33]. Simple main effects analyses revealed that CCK2KO mice consumed significantly more than their WT counterparts on the first 2 days postinjection only [F(1, 12) = 9.98, P = 0.008, η² = 0.45; F(1, 12) = 10.17, P = 0.008, η² = 0.46].

Consistent with other doses of LPS, the anorexic effects of 2,500 μg/kg LPS were most pronounced during the postinjection scotophase periods (Fig. 4B). For WT mice, the effects persisted for the entire postinjection period, whereas CCK2KO mice had recovered to baseline by the third postinjection photophase. Again, for both strains, the effects of LPS on food consumption were maximal during the first scotophase postinjection when food intake by WT and CCK2KO mice decreased by 36.2 ± 1.8 and 42.6 ± 4.5 pellets, respectively. Although strain differences were observed during each scotophase, the greatest differences between strains occurred during the third photophase, at which time CCK2KO mice had returned to baseline and WT mice ate 51 ± 13% less compared with the preinjection period (Fig. 4B). ANOVA on photophase data revealed significant effects for time, strain [F(1, 14) = 10.71, P = 0.006, η² = 0.43], and their interaction [F(3, 42) = 6.08, P = 0.002, η² = 0.30]. Simple main effects analyses indicated significant differences between strains on the first 3 days post-LPS administration with WT mice consuming more than CCK2KO mice during the initial photophase [F(1, 14) = 5.82, P = 0.030, η² = 0.29], and CCK2KO mice consuming more than controls during photophases 2 and 3 [F(1, 14) = 4.69, P = 0.048, η² = 0.25; F(1, 14) = 7.56, P = 0.016, η² = 0.35]. ANCOVA performed on photophase data revealed significant main effects for time, strain [F(1, 13) = 16.47, P < 0.001, η² = 0.56], and their interaction [F(3, 42) = 5.33, P = 0.003, η² = 0.28]. Simple main effects analyses revealed that during the scotophase, CCK2KO mice consumed significantly more than WT mice on days 1–3 postinjection [F(1, 14) = 5.28, P = 0.038, η² = 0.27; F(1, 14) = 14.31, P = 0.002, η² = 0.51; F(1, 14) = 10.51, P = 0.006, η² = 0.43].

Following saline intraperitoneal injection, food pellet consumption by WT mice increased by 2.2 ± 1.3 and 5.6 ± 1.3 (6 ± 3% and 14 ± 2%) during the first and second scotophases (Fig. 4A). In contrast, consumption by CCK2KO mice decreased 8 ± 1% during the first scotophase, but no difference was found during the second scotophase. A minimal but statistically significant strain difference was observed during the first 2 scotophase periods postinjection (Fig. 4A). For scotophase data, ANOVA revealed a significant main effect for time and strain [F(1, 11) = 9.85, P = 0.012, η² = 0.45] but not their interaction.

Water consumption. The decrease in water intake for mice administered 50 μg/kg LPS was restricted to the first 24 h postinjection (Fig. 5A). For WT and CCK2KO mice, the...
differences from baseline were 37 ± 9 and 30 ± 4%, respectively, a difference that failed to reach significance.

When treated with 500 μg/kg LPS, both strains exhibited a decrease in water consumption that persisted for 48 h postinjection but was most pronounced in the first 24 h after LPS (Fig. 5B). At this time, CCK$_2$KO mice consumed 48 ± 11% less than baseline, and WT mice consumed 84 ± 6% less than at baseline. A clear strain difference was apparent, with CCK$_2$KO mice consuming more than WT mice (relative to baseline) on the first day postinjection, but not on subsequent days [$F(1, 14) = 8.62, P = 0.011, \eta^2 = 0.38$] (Fig. 5B).

Similar to the 500 μg/kg dose of LPS, water consumption by mice treated with 2,500 μg/kg LPS was affected for the first 48 h after injection, with both strains decreasing consumption by ~85% (Fig. 5B). CCK$_2$KO mice, however, recovered more quickly, and a strain difference was apparent on day 2 of testing. ANOVA revealed a significant time by strain interaction [$F(1, 13) = 10.62, P = 0.006, \eta^2 = 0.45$], and simple main effect analyses revealed a significant increase in water consumption within each strain [WT, $F(1, 13) = 41.91, P < 0.001, \eta^2 = 0.76$; CCK$_2$KO, $F(1, 13) = 110.63, P < 0.001, \eta^2 = 0.90$] during postinjection day 2 compared with day 1.

In response to saline, water consumption by CCK$_2$KO mice decreased from baseline by 8 ± 2% (Fig. 5A). In contrast, the difference from baseline by WT mice was minimal, though somewhat variable (~1 ± 5%). After accounting for variance due to bodyweight (44%), ANCOVA indicated a no significant effects of strain.

**DISCUSSION**

Compared to controls, CCK$_2$KO mice were significantly less affected by LPS on measures of Tb, activity, body weight, and food intake. That the effects observed were apparent for a wide range of doses of LPS and dependent variables has important implications for the involvement of CCK$_2$ receptors in pathways of immune-brain signaling following intraperitoneal injection of LPS. Studies concerned with elucidating pathways of neuroimmune communication have ascribed differing levels of importance to vagal activation required for LPS or cytokine-induced fever compared with behavioral depression (2, 25). A decrease in social interaction has been shown to depend upon the integrity of vagal afferents regardless of dose (10, 11, 36, 43). Subdiaphragmatic VGX attenuated the fever and anorexia induced by a low, but not a high, dose of LPS (2, 71, 12, 43, 63). Thus the mechanisms of communication from the periphery to the brain, and their dose-dependence, differ for different components of the immune response. Consequently, the following discussion will consider the dependent measures of sickness separately.

There are several reasons to consider CCK as a neurochemical regulator of Tb under pathological conditions. In addition to the activation of gastric vagal afferents (6), CCK is capable of activating the common hepatic branch of the vagus (15), thought to be critical in the genesis of LPS-induced fever (73). The expression of Fos protein in central autonomic nuclei by CCK and LPS exhibits a similar pattern (23, 49). Both activate the central nucleus of the amygdala and hypothalamic structures, including the paraventricular nucleus (26, 80, 82), which has a role in temperature regulation (1, 26). Furthermore, in the rat, CCK is found abundantly in the preoptic anterior hypothalamus (47), an area long known for thermoregulatory integration (5), which has connections with the nucleus of the solitary tract (NTS; 68).

CCK$_2$KO mice were less affected than WT mice across a 50-fold range of LPS doses ranging from modest (50 μg/kg) to high (2,500 μg/kg). This is interesting with respect to a possible mechanism of action, as subdiaphragmatic VGX attenuates or abolishes LPS- and IL-1β-induced fever when they are administered in low doses only (25, 63). After high doses, VGX failed to prevent the induction of fever (2, 12, 43, 63). This raises the possibility that, in response to high doses of LPS, CCK may act on CCK$_2$ receptors to induce the expression of fever via a central, nonvagal route. This does not, however, preclude an involvement of CCK$_2$ receptors at the level of the vagus nerve. Rather, it points to the possibility that activation of CCK$_2$ receptors at this level may be sufficient, but not necessary, for immune-brain communication required for fever when doses of LPS are high. As highlighted by recent studies (36, 37), vagal activation may be less important for fever genesis and hypothalamo-pituitary-adrenal activation than for behavioral depression.
Our findings are in agreement with the reported inhibition of LPS-induced (sc, iv, 10 μg/kg) fever by a CCK2 receptor antagonist capable of crossing the blood-brain barrier (BBB; 79). Because fever suppression persisted for longer when the antagonist was administered intracerebroventricularly as opposed to subcutaneously, the LPS-induced febrile response may occur primarily via the activation of “central” CCK2 receptors rather than “peripherally” located CCK2 receptors. This hypothesis is consistent with the largely central distribution of CCK2 receptors, including areas implicated in thermoregulation (67).

In contrast to pharmacological studies examining the role of CCK2 receptors in fever (77, 79), we monitored Tb for several days following LPS. CCK2 receptor nullification attenuated both the early and late changes in Tb induced by LPS, with fever suppression persisting longer than 24 h with larger doses. This indicates a role for CCK2 receptor activation in both the induction and maintenance of thermoregulatory changes that occur in response to LPS.

The eicosanoid, PGE, is well accepted as one of the final steps in the fever process (69, 76, 81) but cannot fully account for all of the thermoregulatory changes of the fever course (79). PGE has been shown to act independently of CCK since the “feverlike” effects of intracerebroventricular administered PGE2 could not be attenuated by CCK2 receptor antagonism (77). The reverse is also true; the feverlike hyperthermic state induced by intracerebroventricular CCK was attenuated by CCK2 receptor antagonism, but not the PG synthesis inhibitor, indomethacin (77). Thus an exclusive role for either CCK or PGE in the mediation of fever is unlikely.

Cholecystokinin interacts with a variety of neurohormones and peptides, including endogenous opioids (48). This interplay is especially interesting with respect to the present study, since opioids have been implicated in thermoregulation associated with systemic inflammation. When administered to guinea pigs intravenously, the opioid antagonist, naloxone, decreased the first and suppressed the second febrile phase elicited by LPS and abolished the febrile changes evoked by interferon-α, TNF-α, and IL-6 (9). Although centrally administered μ-opioid receptor antagonist attenuated LPS-induced fever in rats (4) but not guinea pigs (62), peripheral administration attenuated fever in both species, suggesting that circulating opioids may play a role in fever production (9, 62). Given that the opioid system is upregulated in CCK2KO mice (58), one may have hypothesized an increased febrile response to LPS in this strain. This clearly did not occur except in response to the largest dose of LPS. Pharmacological modulation of the opioid and CCK systems may provide further insight regarding the coupling of these systems in thermoregulation.

The decrease in scotophase Tb compared with baseline may reflect a “true hypothermic” reaction to LPS, that is, involving a decrease in set point. The adaptive value of hypothermia has been discussed previously (64). More recently, data were presented suggesting that hypothermia and fever may be two different thermoregulatory strategies of the APR with differing underlying mechanisms (21). Data from the present study indicate that, after 2,500 μg/kg ip LPS, WT mice produced a substantial decrease in scotophase Tb compared with baseline, whereas no such decrease was observed for CCK2KO mice. CCK has been implicated in the regulation of sympathetic vasomotor function (24). Further, CCK-8 modulates the firing rate of sympathetic nerves innervating interscapular brown adipose tissue (85), induces skin vasodilation and a shock-like state, and subsequently inhibits heat production and increases heat loss (35). Interestingly, however, the hypothermia induced by CCK receptor activation has been demonstrated to depend on a CCK1, not a CCK2, receptor mechanism (59, 77).

Prolonged hypothermia accompanied by a fall in mean arterial blood pressure is a hallmark of septic shock (61). Although a brief decrease from baseline was present for WT mice soon after injection with 2,500 μg/kg ip LPS, this was rapidly followed by a prolonged fever, a pattern that contrasts with previous studies using this dose (39). Given the lack of prolonged decreases in Tb during the photophase, it is unlikely that any of the doses used in the present study were sufficient to induce septic shock. Interestingly, CCK has been demonstrated to reverse septic shock induced by large doses of LPS (8 mg/kg iv, serotype 0111:B4; 41, 42). It is unlikely that this is due to the activation of CCK2 receptors, since KO mice of the present study did not have an exacerbated shock response to the largest dose of LPS. Moreover, it is unlikely that the present findings resulted from strain differences in circulating CCK as a result of genetic compensation or an altered feedback loop. No differences were observed between CCK2KO and their background strain in basal concentrations of plasma, brainstem, or hypothalamic CCK (T. J. Weiland, unpublished thesis). Furthermore, we have recently presented evidence of a decrease in murine plasma CCK following low and high doses of LPS (84).

Evidence regarding the respective roles of CCK receptors in satiety is mixed. Because vagal activation has been demonstrated to be critical for satiety (74) and because CCK1 receptors are present on the vagus nerve and vagal afferent terminals, including the NTS, the CCK1 receptor has been favored for the regulation of food intake. However, satiety induced by endogenous CCK has been demonstrated to require activation of the CCK2 receptor, and although these receptors are located on the vagus nerve, they are also strongly represented in the hypothalamus. Indeed, it has been suggested that CCK may act not only at peripheral sites but also centrally to regulate food intake (22).

Results of the present study indicated that the anorexic effects of LPS were more profound in WT compared with CCK2KO mice. In contrast, Bret-Dibat and Dantzer (8) found that antagonism of the CCK2 receptor using a compound capable of crossing the BBB failed to attenuate the suppression of food-motivated behavior in mice induced by a low dose of LPS (serotype 0127:B8; 100 μg/kg ip) or IL-1β (20 μg/kg ip). The interpretation of studies involving pharmacological manipulations is complicated by issues such as incomplete antagonism, limited half-life, and bioavailability. While CCK2 receptor nullification through genetic means is permanent, pharmacological receptor antagonism is not. However, because the receptor antagonist used by Bret-Dibat and Dantzer (8) is reasonably long-lasting, the reason for the observed discrepancy is not clear. It should be noted, however, that they did not verify the biological activity of the CCK2 antagonist used. In addition, a variety of procedural differences may account for these discrepant findings. The present study differed from that of Bret-Dibat and Dantzer (8) in terms of ambient temperature (23 ± 2°C,

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CCK2 receptors are likely to mediate the anorexic effects of LPS following a high dose. With this in mind, central nervous system (CNS) processes associated with LPS administration and whether it impacts on thermoregulation and metabolic rate. Circulating CCK levels are suggestive of an exclusively central role for CCK2 receptors in LPS-induced changes in body temperature, activity, and timing of administration, and methods of quantifying changes in behavior.

Studies involving subdiaphragmatic vagal efferent fibers in moderating the response to illness. The use of a CCK2 antagonist incapable of crossing the BBB or intracerebroventricular injections may clarify this issue. Furthermore, since CCK exerts its effect on food intake via both afferent and efferent vagal fibers (50), future studies may seek to determine the contribution of CCK from vagal afferent fibers in moderating the response to illness.

The cachexic effects of LPS were more pronounced in WT mice compared with CCK2KO mice across all doses of LPS. CCK acts synergistically with leptin (45), an important circulating signal important for body weight regulation (31), and a recently recognized mediator of LPS-induced anorexia and fever (66). Leptin receptors are also critical in the recovery from LPS-induced hypothermia (75). In contrast to CCK, however, leptin is thought to be devoid of a true thermoregulatory action; it acts via metabolic rate but does not directly affect heat loss mechanisms (78). This synergy between CCK and leptin is particularly interesting with respect to our results for Tb and body weight, as circulating leptin is decreased in CCK2KO mice compared with WTs (H. Chen, S. Kent, and M. Morris, unpublished observations). It remains to be determined whether this strain difference remains following LPS administration and whether it impacts on thermoregulation and metabolism.

The close relationship of water consumption to food intake makes it plausible that the expression of LPS-induced adiposity and anorexia depends on a common pathway. Although CCK2KO mice were significantly less anorexic compared with WT mice in response to all three LPS doses, a strain difference in adiposity was observed only in response to the 500 µg/kg dose. Although this may be attributable to the larger variance in water consumption, it may also indicate that the role, if any, of CCK2 receptors in mediating LPS-induced adiposity is indirect or not robust.

In addition to food-motivated behavior and social exploration, decreased general locomotor activity has also been demonstrated to be vagally mediated (11, 43). Despite this, relatively little is known regarding the mechanisms that support illness-associated changes in locomotor activity. Under non-pathological conditions, CCK decreases locomotion in the rat via a vagally mediated pathway; conversely, CCK2 receptor antagonism increased the activity of mice (33). LPS typically results in decreased locomotor activity, but this was minimal in the CCK2KO mice. Consequently, it is possible that CCK may participate in LPS-induced hypoadiposity by acting on CCK2 receptors of the vagus nerve.

Further “downstream,” the locomotor effects observed may be due to the interaction of CCK with opioid or dopaminergic systems. In several areas of the CNS, CCK is colocalized with opioid peptides and/or dopamine (29, 57), and it antagonizes opioid and dopamine-mediated behavior (52, 56). Interestingly, opioid agonists increase locomotor activity in rodents, and it is well established that this involves dopamine (3).

The LPS-induced strain differences observed in the present study may be also be related to the role of CCK2 receptors in depression. There is considerable overlap between the APR and depressive episodes (85), and many features of the APR are prevented or attenuated by chronic antidepressants. For example, fluoxetine attenuates LPS-induced anorexia and cachexia and abolishes its hypothalamic effect (85). Similarly, tricyclic antidepressants prevent LPS-induced anorexia, cachexia, adiposity, and decreased locomotor activity in rats (72), and an atypical antidepressant attenuates the decreased social exploration and locomotor effects of peripheral but not central LPS and IL-1β (13). Interestingly, mice treated with CCK2 antagonists exhibit antidepressant-like responses in tests of reactivity to stress (27, 28). This antidepressant effect is prevented by a selective dopamine D1 receptor agonist (27) and by a δ-opioid antagonist (28). Thus it is conceivable that CCK2 receptor invalidation may attenuate the sickness syndrome by exerting an antidepressant effect involving stimulation of δ-opioid and/or D1 receptor stimulation. This hypothesis is in agreement with the enhanced opioid tone in CCK2KO mice.

Summary and future directions. Elucidating the role of CCK receptors in LPS-induced sickness behavior is far from straightforward. Interpretation of the literature is complicated by species differences, differences in immune activators, routes and timing of administration, and methods of quantifying changes in behavior.

Using a genetic approach, this study has explored the roles of CCK2 receptors in LPS-induced changes in Tb, activity,
body weight, food intake, and water consumption. Our findings strongly indicate an involvement of CCK2 receptors in LPS-induced sickness behavior. Given the doses used and the fact that most CCK2 receptors are located centrally, this has important implications regarding the possible mechanisms of action of immune-to-brain communication. Although these data cannot exclude activation of CCK2 receptors at the level of the vagus nerve, a possible role for these receptors in nonvagal routes of communication is suggested.

CCK is known to alter the firing rate of sympathetic nerves innervating interscapular brown adipose tissue and has a role in the action of immune-to-brain communication. Although these receptors mediate sickness behavior. Given the doses used and the fact that most CCK2 receptors are located centrally, this has important implications regarding the possible mechanisms of action of immune-to-brain communication. Although these data cannot exclude activation of CCK2 receptors at the level of the vagus nerve, a possible role for these receptors in nonvagal routes of communication is suggested.

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