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Messenger RNA for neuropeptide Y in the arcuate nucleus increases in parallel with plasma adrenocorticotropin during sepsis in the rat

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Abstract

Loss of appetite occurs in the cecal ligation and puncture (CLP) model of sepsis in conjunction with the activation of central neural stress pathways. Neuropeptide Y (NPY) in the arcuate nucleus of the hypothalamus is upregulated by several stressors and is stimulatory to feeding. To examine the response of NPY messenger RNA in the arcuate nucleus to sepsis, we used biotinylated RNA probes and a quantitative non-isotopic *in situ* hybridization approach in cryo-preserved sections from rats made septic by CLP. The mRNA in arcuate neurons was upregulated from the first day after CLP. By the afternoon of the third day through the morning of the fourth day, the average grey level of NPY mRNA clusters was 30% greater after CLP than after sham surgery ($P < 0.05$), and the integrated optical density based on both the grey level and the amount of area with detectable mRNA was 60% greater after CLP than after sham surgery ($P < 0.03$). Both the average grey level and area with detectable staining were positively correlated to plasma ACTH ($r = 0.953$ and $r = 0.917$, respectively, $n = 10$ and $P < 0.01$ in each case). Thus sepsis increases the expression of the mRNA for NPY in the arcuate nucleus in proportion to the magnitude of the stress response. However, the suppression of feeding behavior in the CLP model suggests that sepsis activates additional mechanisms that negate the orexigenic contribution of the neuronal increase in NPY mRNA.

Keywords

adrenocorticotropin; arcuate nucleus; cecal ligation and puncture; *in situ* hybridization; neuropeptide Y; sepsis

Sepsis as a result of bacterial or fungal infection is a potentially lethal complication in critically illness that leads to the suppression of appetite and negative nitrogen balance with a loss in body mass [7]. These metabolic effects result in part from the response of central stress

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pathways [21]. We recently mapped the central expression of the immediate gene product, Fos, in the cecal ligation and puncture (CLP) model of polymicrobial sepsis [9]. The most intense expression of Fos occurred during the first 28 h, but some responses were sustained for three or more days. In addition, certain brain areas showed a tonic increase in corticotropin releasing hormone (CRH) within neuronal somata consistent with a chronic state of stress [9].

Acute inflammatory stress induced by lipopolysaccharide suppresses food intake in parallel with an increase in the mRNA for proopiomelanocortin (POMC) and other anorexic peptides in the arcuate nucleus by 4 h but no change in the transcription of NPY suggesting a minor role for NPY-containing arcuate neurons in the early decrease in food intake during systemic inflammation [25]. Loss of body weight also occurs during chronic sleep deprivation [16,17], and during chronic dehydration induced by drinking 2.5% saline. The weight loss in these states parallels the upregulation of NPY neurons and the downregulation of POMC neurons in the arcuate nucleus [16,30]. In the absence of stress, NPY output from the arcuate nucleus potently stimulates feeding behavior [15,24]. However, a delayed feeding response occurs during sleep deprivation [17], and persistent anorexia occurs during the dehydration induced by hypertonic saline until water is reintroduced for drinking [29]. During such challenges the response of NPY in the arcuate nucleus may modulate inflammatory pain [18] and influence the resiliency to stress [33]. Because sepsis interrupts REM and slow-wave sleep [31] and may include dehydration, we hypothesized that CLP upregulates the mRNA for NPY in arcuate neurons. Our findings confirm this hypothesis and support further work to determine the extent to which this response affects host defense and ultimate outcome in sepsis.

Fifty-eight Sprague-Dawley male rats (Charles River) weighing 325–435 g were acclimated to a 12-hour light/dark cycle with lights on at 0600 h for at least five days. Rat chow and water were provided *ad libitum*. Experimental protocols were approved by the University of Maryland Institutional Animal Care and Use Committee in accord with the guidelines of the National Institutes of Health. As detailed previously [10], rats were prepared with indwelling arterial catheters and underwent CLP or sham surgery in randomized order through a midline abdominal incision under an intraperitoneal dose of ketamine (75 mg/kg) and xylazine (5 mg/kg). Individual caging allowed external access to the arterial lines and freedom of movement for each rat. During recovery from surgery, rats received ~3 ml/100g of normal saline as 2.5 ml injections at 20–30 min intervals. Rats were then returned to the animal facility. Blood samples (0.4–0.5 ml) were drawn daily in the AM (0800 h to 1000 h) and the PM (1600 h to 1800 h) and were replaced with equal volumes of heparinized (10 units/ml) saline. Rats were perfused with 2.5% acrolein in 4% paraformaldehyde in either the AM or the PM of day (D) 1 after surgery or in the PM of D3 or the AM of D4 as described previously [9]. Brains were removed and saved in 25% sucrose until thirty-five micron coronal sections were cut and stored in cyroprotectant antifreeze solution [28]. Cryo-preserved hypothalamic sections representing every 12th section through the arcuate nucleus were used for analysis of NPY mRNA.

The NPY cDNA construct originally prepared by Higuchi *et al* [14] was kindly provided by Dr. A. Sahu (University of Pittsburgh). It included a fragment of the cDNA for rat NPY mRNA (base pairs 1–511) ligated into the EcoR1 site of the Bluescribe M13 vector. The plasmid was linearized with FspI and transcribed with T3 RNA polymerase for the antisense NPY riboprobe or was linearized with SmaI and transcribed with T6 RNA polymerase for the sense-strand control. Biotinylated probes were synthesized using biotin-16-UTP (Roche Diagnostics Corp., Indianapolis, IN). All solutions were prepared with diethylpyrocarbonate-treated water. The previously described staining procedure [16] included multiple rinses of the sections in 0.05 M potassium phosphate buffered saline (KPBS) followed by 1% sodium borohydride in KPBS then rinses in KPBS to remove fixative before they were rinsed overnight in 0.5% Triton-X-100 at 4 °C. They were then rinsed twice in 0.1 M triethanolamine buffer (pH 8.0) and thrice in 2x SSC before being incubated overnight with heat-denatured biotinylated NPY riboprobe (600

ng/kbp/ml) with 0.25 mg/ml Yeast RNA (Ambion, Foster City, CA) in hybridization buffer [1.4 ml per well made with 750 µl deionized formamide, 300 µl 50% dextran sulfate, 90 µl 5 M NaCl, 12 µl 1 M Tris (pH 8.0), 2.4 µl 0.5M EDTA, 30 µl 50x Denhardt's solution, and 250 µl of H₂O] overnight at 50 °C.

Sections were rinsed in 4× SSC then in RNAase buffer [10 mM Tris (pH 8.0), 500 mM NaCl, 0.75 mM EDTA] before incubation in 20 mg/ml RNAase (Qiagen, Valencia, CA) in RNAase buffer for 30 min at 37 °C. After multiple rinses in 2× SSC and then in 1× SSC at room temperature, sections were incubated in 0.1 SSC for 1 h at 55 °C and then rinsed multiple times with KPBS before incubation in an antibody to biotin made in goat at 1:100,000 for 48 h at 4 °C.

Sections were rinsed 10x over 1 h in KPBS then incubated in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) to goat immunoglobulins diluted 1:600 for 1 h at room temperature. After rinsing 5x over 50 min in KPBS, sections were incubated in AB solution made with the ABC Elite Kit (Vector) using 45 µl of A and 45 µl of B per 10 ml of KPBS with 0.4% Triton X-100. After 3 × 5 min rinses with KPBS and 3 × 5 min rinses with 175 mM sodium acetate, sections were reacted for 20 min in nickel-enhanced 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution (1 mg DAB plus 125 mg nickel (II) sulfate with 4.2 µl 3% H₂O₂ per 10ml of 175 mM sodium acetate) to yield a black reaction product.

Neuronal counts and microdensitometry were done with an image analysis system and the Nova Prime software package (Bioquant Image Analysis Corporation, Nashville, TN). Staining of NPY mRNA was selected by digital thresholding in square fields of 10000 µ² in the heart of the arcuate nucleus. The average grey level of the selected pixels was corrected for the background grey level of an adjacent area with no detectable signal. Staining densities from six to twelve fields from three to six sections through the arcuate nucleus were averaged for each rat. We also recorded the number of positive cells in each analyzed field and the area fraction with detectable staining. Finally, we determined the integrated optical density over six to twelve fields in each rat that circumscribed the entire arcuate nucleus through its rostral-to-caudal extent. For each pixel within a field, the logarithm of the ratio of the measured grey level to background was computed so that pixels at the background level yielded a signal of zero. Results for all pixels were summed over each field and then normalized to its area before being averaged with results for the other fields from each rat. This measurement was sensitive to both the intensity of the staining and the area within the arcuate nucleus in which staining was detected.

Plasma ACTH was radioimmunoassayed based on [20]. Plasma corticosterone was determined by a radioimmunoassay kit (MP Biomedicals, Inc., Irvine, CA). Crossreactivities, sensitivities, and intra- and interassay coefficients of variation were reported previously [9].

Histological measurements were tested by Wilcoxon rank-sum test. Pearson correlation coefficients were used to assess the relationships between plasma ACTH and measurements of NPY expression. A P value of 0.05 was classed as significant.

Initially, we selected rats with the greatest central expression of Fos after CLP based on an earlier analysis [9] for a preliminary determination of the expression of mRNA for NPY in the arcuate nucleus. This sample included one rat perfused in the AM and one in the PM of D1, one in the PM of D3, and one in the AM of D4. Two rats with sham surgery and perfused on D1 were also screened. The expression of NPY increased after CLP and was most intense on D3 and D4 (Fig. 1). A larger sample from additional rats was then processed to confirm the response on D3 and D4. To ensure that the distribution of the stress responses in this sample was representative of the entire population, we ranked all rats challenged with CLP from the lowest (= 1) to the highest (= N) plasma concentration of ACTH. Of the five rats sampled from

an N of nine perfused in the PM of D3, the average rank was 5.4 compared to an average rank for all nine rats of 5.0. Of the five rats sampled from an N of ten perfused in the AM of D4, the average rank was 6.4 compared to an average rank for all ten rats of 5.5. After CLP, the mean concentrations of plasma ACTH and corticosterone did not differ significantly from the PM of D3 to the AM of D4 despite a return of the circadian PM-to-AM difference in plasma corticosterone after sham surgery [9].

Because no difference in the expression of NPY was detected between D3 and D4, the results from both days were combined. Increases in both the average grey level of staining (Fig. 2A, $P < 0.03$) and the total area with detectable NPY mRNA in the arcuate nucleus (not shown, $P < 0.03$) after CLP accounted for an increase in the integrated optical density of the staining (Fig. 2B, $P < 0.03$). Increased mRNA for NPY in the arcuate nucleus was also detected in the two brains sampled on D1 after CLP (Fig. 1B&E). Both the average grey level and the integrated optical density of mRNA staining in these rats were within the range of values measured on D3 and D4 after CLP and were greater than the values measured in the two sham rats from D1 (Fig. 1A&D). Thus, the expression of mRNA began to increase in the two rats examined on D1 after CLP and continued to increase in those rats shown previously [9] to have the most robust central expression of Fos (Fig. 1).

On D3 and D4 after CLP, the expression of mRNA for NPY in the arcuate nucleus was proportional to the circulating concentration of ACTH. Significant correlations were found between plasma ACTH and both the average grey level of staining ($r = 0.953$, $P < 0.01$, Fig. 3A) and the percent of the analyzed fields with detectable mRNA ($r = 0.917$, $P < 0.01$, Fig. 3B) after CLP but not after sham surgery. Plasma ACTH after CLP showed similar relationships to the number cells with detectable mRNA per field ($r = 0.785$, $P < 0.01$) and to the integrated optical density of staining ($r = 0.707$, $P < 0.05$). The expression of NPY was not correlated with plasma corticosterone.

The primary observation of this study was an increase in the mRNA for NPY in the arcuate nucleus in the well-established CLP model of sepsis. In a previous study [2], measurements of mRNA detected immunocytochemically as done in the present study showed a strong linear relationship ($r^2 = 0.854$) to autoradiographic grain counts of a radio-labeled probe in similarly treated sections supporting the validity of the present technique to determine the amount of transcription. The spatial resolution using a biotinylated probe is excellent and can reveal the appearance of mRNA for NPY in new areas within or beyond the boundaries of the arcuate nucleus in response to a stimulus. Increased activity in the arcuate surround was not found after CLP. However, both the density of staining within individual arcuate neurons and the absolute number of neurons in the nucleus with detectable message increased in proportion to the degree of stress.

Given the known effect of sepsis on feeding behavior, the upregulation of the mRNA for orexigenic peptide NPY in the arcuate nucleus appears paradoxical. In a more recent study [8] we found that more than 45% of rats do not resume noticeable consumption of chow until the third day after CLP. Similarly, in sepsis induced by the abdominal implantation of a fecal-agar pellet, food consumption is depressed initially after surgery and never exceeds control values for five days [6]. Although there may be a role for the observed neuronal response in the gradual recovery in eating after CLP, this response is insufficient to maintain body weight because we have found significant weight loss over seven days after CLP in similarly prepared rats [8].

Numerous mechanisms may account for the suppression of appetite by CLP including centrally acting cytokines and stress hormones [7,21]. CLP may increase the input of vagal afferents that suppress feeding [11] and contribute to the sustained response of central neural stress

pathways [9] while the plasma concentration of the potent and major orexigenic gut peptide, ghrelin [11], is greatly reduced [32]. Additional anorexic effects may arise from peptides such as cholecystokinin (CCK) that has been localized in areas of the brain [3,12] responsive to CLP [9]. The activation of CCK-1 receptors [19] in the medial hypothalamus inhibits feeding [4], and the anorexic peptides GLP-1 and oxyntomodulin [11] increase the expression of CRH in the hypothalamic paraventricular nucleus [22].

These neural and humoral signals are unlikely to account for the response of the NPY-containing arcuate neurons since gastric distension, the suppression of ghrelin, and the central actions of CCK constitute inhibitory signals for the expression of NPY [11]. A decrease in plasma leptin could increase the synthesis of NPY in the arcuate nucleus [15], but this hormone's response was not measured in the present study and was variable in earlier studies. Increased plasma leptin occurred during the initial phases of sepsis in humans [5] and mice [26]. In rats, an initial increase in leptin was found after CLP where caloric intake was augmented by total parenteral nutrition [13] whereas a decrease was seen in the fecal-agar pellet model of sepsis [6].

Normally the regulation of NPY neurons in the arcuate nucleus is tightly controlled so that the stimulatory effect of these cells on caloric intake is balanced with energy expenditure to maintain body mass [11]. However, the response of rats to sepsis resembles that during experimentally imposed sleep deprivation [17] and during dehydration [29] where the transcription of NPY in the arcuate nucleus increases while body weight decreases. Thus, these systemic challenges that interrupt metabolic homeostasis have a uniform effect on NPY-containing neurons despite differences in the mechanisms for the loss of body mass.

Significant weight loss with no change in feeding occurs during the first five days of sleep deprivation [17] consistent with a hypermetabolic state. Increased metabolism also develops early in sepsis [7] but is accompanied initially by anorexia [6,8]. Both CLP and sleep deprivation increase the content of CRH peptide in the hypothalamic paraventricular nucleus [9,16]. The correlation between plasma ACTH and the expression of NPY in the arcuate nucleus indicates a strong association between the degree of stress and the upregulation of NPY after CLP. Reciprocal connections between the arcuate and paraventricular nuclei [23] may underlie this relationship. The surgical interruption of the NPY pathway from the arcuate to the paraventricular nucleus inhibits the response of plasma ACTH to restraint [1], and the central administration of NPY stimulates the release of ACTH acutely [27].

On the other hand, our results do not prove a causal role for NPY in the response of ACTH. Dehydration-induced anorexia leads to reduced expression of CRH in the hypothalamic paraventricular neurons and no change in plasma ACTH [30]. Thus, the paraventricular expression of CRH and the arcuate expression of NPY do not always parallel one another in anorexic states.

In conclusion, CLP-induced sepsis elicits a sustained increase in the mRNA for NPY in the arcuate nucleus that is correlated with the response of ACTH. In a related study we found that rats showing the greatest increases in ACTH from one to five days after CLP were at greatest risk for death [8] suggesting that the upregulation of NPY parallels both the degree of stress and of lethal risk. In this previous study, non-survivors that had greatest increases in ACTH had the least loss of body mass (unpublished analysis of reference 8). Thus, the response of NPY may partially compensate for anorexia but with little benefit for survival. The absence of hyperphagia or weight gain in sepsis suggests that the myriad hormonal and neural controlling influences in this pathologic state either block the orexigenic signals from NPY neurons, downregulate the receptors for NPY in central areas that stimulate feeding [11], or provide an opposing anorexic input.

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References

1. Bell ME, Bhatnagar S, Akana SF, Choi S, Dallman MF. Disruption of arcuate/paraventricular nucleus connections changes body energy balance and response to acute stress. *J Neurosci* 2000;20:6707–6713. [PubMed: 10964976]
2. Berghorn KA, Le WW, Sherman TG, Hoffman GE. Suckling stimulus suppresses messenger RNA for tyrosine hydroxylase in arcuate neurons during lactation. *J Comp Neurol* 2001;438:423–432. [PubMed: 11559898]
3. Bhatnagar S, Viau V, Chu A, Soriano L, Meijer OC, Dallman MF. A cholecystokinin-mediated pathway to the paraventricular thalamus is recruited in chronically stressed rats and regulates hypothalamic-pituitary-adrenal function. *J Neurosci* 2000;20:5564–5573. [PubMed: 10884340]
4. Blevins JE, Stanley BG, Reidelberger RD. Brain regions where cholecystokinin suppresses feeding in rats. *Brain Res* 2000;860:1–10. [PubMed: 10727618]
5. Bornstein SR, Licinio J, Tauchnitz R, Engelmann L, Negrao AB, Gold P, Chrousos GP. Plasma leptin levels are increased in survivors of acute sepsis: associated loss of diurnal rhythm, in cortisol and leptin secretion. *J Clin Endocrinol Metab* 1998;83:280–283. [PubMed: 9435456]
6. Bryant P, Shumate M, Yumet G, Lang CH, Vary TC, Cooney RN. Capsaicin-sensitive nerves regulate the metabolic response to abdominal sepsis. *J Surg Res* 2003;112:152–161. [PubMed: 12888332]
7. Campbell IT. Limitations of nutrient intake. The effect of stressors: trauma, sepsis and multiple organ failure. *Eur J Clin Nutr* 1999;53(Suppl 1):S143–147. [PubMed: 10365992]
8. Carlson DE, Chiu WC. Absence of circadian cues during recovery from sepsis modifies pituitary-adrenocortical function and impairs survival. *Shock* 2008;29:127–132. [PubMed: 17693947]
9. Carlson DE, Chiu WC, Fiedler SM, Hoffman GE. Central neural distribution of immunoreactive Fos and CRH in relation to plasma ACTH and corticosterone during sepsis in the rat. *Exp Neurol* 2007;205:485–500. [PubMed: 17462630]
10. Carlson DE, Chiu WC, Scalea TM. Cecal ligation and puncture in rats interrupts the circadian rhythms of corticosterone and adrenocortical responsiveness to adrenocorticotrophic hormone. *Crit Care Med* 2006;34:1178–1184. [PubMed: 16484905]
11. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *J Clin Invest* 2007;117:13–23. [PubMed: 17200702]
12. Herbert H, Saper CB. Cholecystokinin-, galanin-, and corticotropin-releasing factor-like immunoreactive projections from the nucleus of the solitary tract to the parabrachial nucleus in the rat. *J Comp Neurol* 1990;293:581–598. [PubMed: 1691749]
13. Heuer JG, Bailey DL, Sharma GR, Zhang T, Ding C, Ford A, Stephens EJ, Holmes KC, Grubbs RL, Fynboe KA, Chen YF, Jakubowski JA. Cecal ligation and puncture with total parenteral nutrition: a clinically relevant model of the metabolic, hormonal, and inflammatory dysfunction associated with critical illness. *J Surg Res* 2004;121:178–186. [PubMed: 15501457]
14. Higuchi H, Yang HY, Sabol SL. Rat neuropeptide Y precursor gene expression. mRNA structure, tissue distribution, and regulation by glucocorticoids, cyclic AMP, and phorbol ester. *J Biol Chem* 1988;263:6288–6295. [PubMed: 2834371]
15. Hillebrand JJ, de Wied D, Adan RA. Neuropeptides, food intake and body weight regulation: a hypothalamic focus. *Peptides* 2002;23:2283–2306. [PubMed: 12535710]
16. Koban M, Le WW, Hoffman GE. Changes in hypothalamic corticotropin-releasing hormone, neuropeptide Y, and proopiomelanocortin gene expression during chronic rapid eye movement sleep deprivation of rats. *Endocrinology* 2006;147:421–431. [PubMed: 16210372]
17. Koban M, Sita LV, Le WW, Hoffman GE. Sleep deprivation of rats: the hyperphagic response is real. *Sleep* 2007;31:927–933. [PubMed: 18652088]
18. Li JJ, Zhou X, Yu LC. Involvement of neuropeptide Y and Y1 receptor in antinociception in the arcuate nucleus of hypothalamus, an immunohistochemical and pharmacological study in intact rats and rats with inflammation. *Pain* 2005;118:232–242. [PubMed: 16216414]

19. Moran TH, Bi S. Hyperphagia and obesity in OLETF rats lacking CCK-1 receptors. *Philos Trans R Soc Lond B Biol Sci* 2006;361:1211–1218. [PubMed: 16815799]
20. Nicholson WE, Davis DR, Sherrell BJ, Orth DN. Rapid radioimmunoassay for corticotropin in unextracted human plasma. *Clin Chem* 1984;30:259–265. [PubMed: 6319046]
21. Rothwell NJ. Central effects of CRF on metabolism and energy balance. *Neurosci Biobehav Rev* 1990;14:263–271. [PubMed: 2234606]
22. Sarkar S, Fekete C, Legradi G, Lechan RM. Glucagon like peptide-1 (7–36) amide (GLP-1) nerve terminals densely innervate corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. *Brain Res* 2003;985:163–168. [PubMed: 12967720]
23. Sawchenko PE, Swanson LW. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. *J Comp Neurol* 1983;218:121–144. [PubMed: 6886068]
24. Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;404:661–671. [PubMed: 10766253]
25. Sergeyev V, Broberger C, Hokfelt T. Effect of LPS administration on the expression of POMC, NPY, galanin, CART and MCH mRNAs in the rat hypothalamus. *Brain Res Mol Brain Res* 2001;90:93–100. [PubMed: 11406287]
26. Stewart D, Fulton WB, Wilson C, Monitto CL, Paidas CN, Reeves RH, De Maio A. Genetic contribution to the septic response in a mouse model. *Shock* 2002;18:342–347. [PubMed: 12392278]
27. Suda T, Tozawa F, Iwai I, Sato Y, Sumitomo T, Nakano Y, Yamada M, Demura H. Neuropeptide Y increases the corticotropin-releasing factor messenger ribonucleic acid level in the rat hypothalamus. *Brain Res Mol Brain Res* 1993;18:311–315. [PubMed: 8392133]
28. Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 1986;7:155–159. [PubMed: 3520509]
29. Watts AG, Salter DS, Neuner CM. Neural network interactions and ingestive behavior control during anorexia. *Physiol Behav* 2007;91:389–396. [PubMed: 17531275]
30. Watts AG, Sanchez-Watts G, Kelly AB. Distinct patterns of neuropeptide gene expression in the lateral hypothalamic area and arcuate nucleus are associated with dehydration-induced anorexia. *J Neurosci* 1999;19:6111–6121. [PubMed: 10407047]
31. Weinhouse GL, Schwab RJ. Sleep in the critically ill patient. *Sleep* 2006;29:707–716. [PubMed: 16774162]
32. Wu R, Zhou M, Cui X, Simms HH, Wang P. Upregulation of cardiovascular ghrelin receptor occurs in the hyperdynamic phase of sepsis. *Am J Physiol Heart Circ Physiol* 2004;287:H1296–1302. [PubMed: 15155262]
33. Zhou Z, Zhu G, Hariri AR, Enoch MA, Scott D, Sinha R, Virkkunen M, Mash DC, Lipsky RH, Hu XZ, Hodgkinson CA, Xu K, Buzas B, Yuan Q, Shen PH, Ferrell RE, Manuck SB, Brown SM, Hauger RL, Stohler CS, Zubieta JK, Goldman D. Genetic variation in human NPY expression affects stress response and emotion. *Nature* 2008;452:997–1001. [PubMed: 18385673]

Fig. 1. Low (A, B, C, scale bars = 100 μ) and high power (D, E, F, scale bars = 10 μ) images of NPY mRNA in the arcuate nucleus after CLP (B, C, E, F) or sham surgery (A, D) on day 1 in the AM (A, B) and PM (D, E), or day 3 in the PM (C, F).

Fig. 2. Staining intensity (average grey level, A) and integrated optical density (B) of NPY mRNA detected in the arcuate nucleus is increased three to four days after cecal ligation and puncture (CLP, $n = 10$). * $P < 0.05$ for difference from sham surgery ($n = 5$). Error bars are SEM. AU = arbitrary units.

Fig. 3. Staining intensity (average grey level) of NPY mRNA (A) and the area within the arcuate nucleus where the mRNA was detected (B) are both correlated to plasma ACTH three (D3) or four days (D4) after cecal ligation and puncture (CLP; $r^2 = 0.909$ in A and $r^2 = 0.840$ in B; $P < 0.01$ in both cases) but not after sham surgery (B). AU = arbitrary units.