ABSTRACT: Immune activation leads to production of mediators such as cytokines, which act to induce both brain-mediated and peripheral defense processes. We used intraperitoneal administration of the cytokine tumor necrosis factor-α (TNF-α) to investigate whether defense processes induced by this cytokine are mediated by vagal afferents and/or interleukin-1 (IL-1) receptors. Because some effects of TNF-α are mediated, at least in part, by the brain [plasma corticosterone (CORT) elevation] and some are mediated by peripheral organs [reduction of serum protein and corticosteroid binding globulin (CBG)], we also investigated whether the effects of vagotomy are specific to those defense processes mediated by the brain. Both vagotomy and IL-1 receptor antagonist attenuated serum CORT elevation, but had no effects on serum protein or CBG reduction. These results support the idea that vagal afferents provide a true immune-to-brain pathway that may include IL-1 receptors. © 1997 Elsevier Science Inc.

KEY WORDS: Cytokine, Vagus, TNF, Corticosterone, Vagotomy, CBG.

INTRODUCTION

Activation of a peripheral immune response by infection or inflammation induces a constellation of defense mechanisms mediated by both the central nervous system as well as peripheral organs. Peripheral injection of proinflammatory cytokines or agents that induce the release of endogenous cytokines from immune cells (e.g., lipopolysaccharide, LPS) causes changes in brain amines [10,21], induces sickness behavior [18,20], creates fever [8], activates an acute phase response [2], and increases hypothalamic–pituitary–adrenal (HPA) hormones [3]. Many of these effects of peripheral cytokines are mediated by the brain, and an important and unresolved issue is how peripheral cytokines signal the central nervous system to produce them.

Several possible mechanisms may mediate cytokine-to-brain communication. Cytokines may signal the brain directly either by entering at areas where the blood–brain barrier is weak or absent such as the organum vasculosum laminae terminalis [26], or entering via active transport [1]. Alternatively, peripheral cytokines may interact with receptors in blood vessel endothelial cells that respond by releasing a second factor (e.g., prostaglandins) into the brain parenchyma [27]. In addition, cytokines may communicate with the brain via interaction with peripheral nerves [28].

Research investigating the possibility of immune-to-brain communication via peripheral nerves has focused most closely on the vagus nerve. The vagus nerve contains sensory fibers that terminate in the nucleus tractus solitarius and area postrema of the brainstem. Subdiaphragmatic vagotomy blocks or reduces fever [28], sickness behaviors [4,7], hyperalgesia [29], ACTH [13], and corticosteroid increases [12], brain norepinephrine changes [12], and taste aversions [15] that follow the intraperitoneal administration of LPS and IL-1β ([22] for review). In addition, IL-1β binding sites have been localized on paraganglia, which make afferent synaptic contact with vagal fibers [16], thereby providing a potential mechanism for cytokine activation of vagal fibers. The literature documenting the role of the peripheral vagus in cytokine-to-brain communication has utilized primarily the administration of either IL-1β or LPS. LPS, however, induces the release of other proinflammatory cytokines in addition to IL-1β, such as IL-6 and TNF-α. The effects of LPS or other inflammatory agents can often be blocked by administration of the IL-1 receptor antagonist (IL-1ra) or antisera directed against IL-1 (e.g., [11,24]), suggesting that such effects may be mediated, at least in part, by IL-1 receptors. IL-1β, IL-1α, TNF-α, and IL-6, however, produce different patterns of neural activity [32] and so may well utilize somewhat different communication pathways. At present, the role of the vagus in mediating communication between cytokines other than IL-1β and the brain is largely unexplored.

Although reduction in responding to peripheral LPS or cytokines following vagotomy is thought to result from the interruption of vagal sensory fibers carrying immune-to-brain signals, there are a number of other possible mechanisms by which vagotomy may act. Vagotomy eliminates vagal parasympathetic (motor) fibers, as well as sympathetic fibers intermingled with the vagal branches, to organs such as the liver, which, are disturbed by the dissection of the hepatic and other branches of the vagus. Because many of the central nervous system-mediated responses to peripheral cytokines ultimately require activation of peripheral processes (e.g., brown fat metabolism, adrenocortical secretion, liver protein synthesis

[Received 17 February 1997; Accepted 24 June 1997]
shifts), the impact of vagotomy on these responses could be mediated by interruption of vagal motor rather than sensory function.

Vagotomy studies have been restricted to a study of cytokine responses that are themselves within the CNS (e.g., monoamine changes) or clearly require mediation by the CNS. For example, the peripheral changes that increase core body temperature during fever are organized in and require the activity of units in the preoptic region of the anterior hypothalamus [26]. However, the proinflammatory cytokines also produce other responses that do not require mediation by the CNS, and these should be unaffected by vagotomy. For example, LPS and cytokines produce shifts in liver metabolism away from the production of circulating serum proteins and carrier proteins such as corticosteroid binding globulin (CBG) and towards the production of acute phase proteins. Hepatocytes express cytokine receptors [25], and so peripheral cytokines can alter liver metabolism by direct action. If the impact of vagotomy is indeed mediated by interruption of cytokine-to-brain communication, then vagotomy should not influence such direct peripheral organ responses to cytokines.

In addition, it is also possible that either CNS systems or the peripheral structures required for illness responses are altered by vagotomy in such a way as to prevent the expression of the illness response, via a mechanism unrelated to the disruption of vagal afferents. For example, vagotomy may reduce the sensitivity of the hypothalamic paraventricular nucleus. This would result in either blunting or preventing the corticosterone response to any stimulus, including those independent of immune-to-brain pathways. Thus, to distinguish between the true consequences of loss of afferent pathways signalling immune activation and a loss of CNS sensitivity, vagotomized animals must be shown to be capable of responding normally to nonimmune stimuli, such as stressors.

Intraperitoneal administration of TNF-α produces both brain-mediated illness consequences, including elevation of plasma corticosterone, as well as peripheral organ responses such as reduction of serum proteins and CBG. Thus, we used intraperitoneal administration of TNF-α to address the following issues: (1) are the brain-mediated responses to TNF-α mediated by the vagus, and/or IL-1 receptors? and (2) Can the effects of vagotomy on brain-mediated illness consequences be explained by reduction in responsiveness of CNS integrative or motor neurocircuitry or peripheral organ insensitivity? To investigate these issues we administered TNF-α to vagotomized and sham-operated animals and measured plasma corticosterone, serum protein, and CBG. If vagal afferents function only to convey signals from TNF-α to the brain, then only the elevation of plasma corticosterone should be affected. Similarly, to determine whether vagotomized animals are capable of mounting normal neuroendocrine responses to nonimmune stimuli, we subjected vagotomized animals to inescapable shock, a stressor. Further, to determine whether the effects of TNF-α are mediated by IL-1 receptors, we pretreated intact animals with IL-1 receptor antagonist (IL-1ra) intraperitoneally prior to administration of TNF-α.

MATERIALS AND METHODS

Subjects

Adult male Sprague–Dawley viral free rats (Harlan & Sasco, 350–375 g) were used (n = 4–7 per group). All subjects were maintained on a 12:12 light/dark cycle (lights on 0600–1800 h) with standard rat chow and water freely available. Colony room temperature was maintained at 22–23°C. Care and use of animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgery

Rats were anesthetized with sodium pentobarbital (55 mg/kg IP) and received either sham surgery or complete subdiaphragmatic vagotomy. Testing was delayed 4–5 weeks after surgery. See Watkins et al. [28] for surgical and post operative care details.

Drugs

Recombinant human TNF-α (Amgen) was diluted in sterile PBS (Gibco) and injected IP at a dose of either 250 µg/kg/2 ml or 500 µg/kg/2 ml. Each dose of TNF-α was given in two injections separated by 30 min to yield a total dose of 500 µg/kg (250 µg/kg × 2) and 1.0 mg/kg (500 µg/kg × 2). This injection regimen was chosen because the level and duration of the subsequent corticosterone response closely matched that produced by the dose of rhIL-1β used in our previous vagotomy study [12]. In studies involving IL-1ra, rats were injected subcutaneously 30 min prior to the first injection of TNF-α (250 µg/kg × 2) with either rhIL-1β receptor antagonist (IL-1ra; Amgen) at a dose of 100 mg/kg in CSE buffer (10 mM citrate, 140 mM sodium chloride and 0.5 mM EDTA, pH 7) or an equal volume of CSE buffer (1 ml/kg).

Blood Sampling and Corticosterone Assessment

Samples were taken immediately prior to injection (baseline) in all experiments with the exception of the dose response experiment. The dose response study had samples taken at 30 min, 1 h, 2 h, and 4 h after initial TNF-α injection. All other TNF studies had samples taken 1 h, 2 h, and 4 h after initial TNF-α injection. In the final study, which assessed the effect of vagotomy on the corticosterone response to inescapable tailshock, blood samples were taken before exposure to inescapable shock (BL), after the 5th shock, after the 100th shock, 1 h after the end of the shock session, and 24 h later. Home cage controls were sampled at the same time of day, and corticosterone levels were measured at baseline, and at the timepoints equal to 1 h and 24 h after inescapable shock. Samples were not taken from home cage controls at the 5 and 100 shock time points because the time of day at these points was close enough to the baseline period so that controlling for circadian shifts was not necessary.

Blood samples (150 µl) were taken from the tail vein. Sampling was completed within 2 min of touching the cage to avoid elevated baseline corticosterone. Blood samples were allowed to clot, serum was removed, and frozen at −20°C until later analysis. Serum levels of total corticosterone were measured using a modification of the radioimmunoassay described by Sigma (intra- and inter- assay CVs <10%, corticosterone antibody Sigma Chemical Co. #C-8784).

Total Serum Protein and Corticosterone Binding Globulin Assessment

Total serum protein levels were measured using a version of the Coomassie blue protein assay adapted from Bradford [5]. The dye solution for the assay was prepared by first dissolving 100 mg Coomassie Brilliant Blue G-250 (Bio Rad, catalog #161-0406) into 50 ml of 95% ETOH, then adding 100 ml of 85% phosphoric acid. The solution was then diluted to a final volume of 1 liter with distilled water and filtered. A standard curve using Bovine Serum Albumin (BSA; Sigma, lot #24H0174) ranging from 1.0–15.0 mg protein (also in TEG buffer, as described above except that dithiothreitol was omitted) was generated and linear regression performed to obtain the formula for calculating unknowns. Serum samples were diluted 1:200 in TEG buffer, and 15 µl of the diluted sample was added to a microtiter plate (each sample executed in triplicate). The protein dye solution (250 µl) was then added to each well. Samples were then incubated for 15 min, following
which their absorbances were read at 595 nm on a microplate reader (Dynatech Laboratories, model MRX). All data are expressed in terms of mg protein/100 μl sample.

Serum CBG levels were assessed using a competitive binding assay adapted from Westphal [31]. The samples were initially diluted 1:200 in buffer consisting of 10 mM Trizma base, 1.0 mM EDTA, 10% Glycerol (v/v), and 1.0 mM dithiothreitol at pH = 8.0. The diluted sample was then mixed with 1H-corticosterone (15 nM) or unlabeled corticosterone (10 mM) at a final dilution of 1:600 and allowed to incubate over night at 4°C. Bound and unbound steroids were separated using activated charcoal (executed in duplicate). The bound fraction was mixed with scintillation cocktail and counted with a liquid scintillation counter (TriCarb 1600TR, Packard, Meriden, CT). Data are expressed as nmol specific 1H-corticosterone binding (CBG)/liter serum.

Inescapable Tailshock

Inescapable shocks were administered while the rats were restrained in Plexiglas tubes 17.5 cm in length and 7.0 cm in diameter. Each animal’s tail extended from the rear of the tube. One hundred shocks were applied through electrodes attached to each rat’s tail at an average intershock interval of 1.0 min. The shocks were 1.6 mA in intensity and 5.0 s duration.

Statistical Analysis

Baseline data and CBG data were analyzed using factorial ANOVAs. Repeated measures ANOVAs were performed on the corticosterone and protein data. Fischer Protected Least Significance Difference (F-PLSD) tests were performed post hoc.

RESULTS

TNF-α Dose Response

As shown in Fig. 1, TNF-α increased serum levels of corticosterone compared with saline injected controls, F(2, 11) = 8.3, p < 0.001. F-PLSD post hoc analyses revealed a reliable increase in serum corticosterone 1 h, 2 h, and 4 h after the initial TNF injection of either 250 μg/kg (×2) or 500 μg/kg (×2) (ps < 0.01) compared with saline injected controls. There was no reliable increase in serum corticosterone 30 min after injection compared with saline injected controls (p > 0.05). Both doses of TNF-α resulted in the same level of corticosterone increase (ps > 0.05).

Subdiaphragmatic Vagotomy Effect on the Serum Corticosterone Response to TNF-α

Based on the results of the dose–response experiment, the 250 μg/kg (×2) dose of TNF-α was used in this study. Baseline levels of serum corticosterone did not differ between the groups (Fig. 2; p > 0.05). Clearly, subdiaphragmatic vagotomy attenuated the TNF-α-induced increase in serum corticosterone. A 2 × 2 × 3 repeated measure ANOVA revealed a reliable drug × surgery interaction, F(1, 20) = 11.1, p < 0.01. Vagotomy resulted in a reliable decrease in serum corticosterone at 1 h, 2 h, and 4 h after TNF-α (ps < 0.01) compared with TNF-α injected sham controls. However, the corticosterone response was not totally eliminated.

IL-1ra Effect on the Corticosterone Response to TNF-α

As shown in Fig. 3, the baseline measure of serum corticosterone was not different between the groups (p > 0.05). IL-1ra reduced, but did not eliminate, the corticosterone response to TNF. An overall 3 × 3 repeated measure ANOVA revealed a reliable main effect of drug condition (TNF + IL-1ra, TNF + VEH, SAL + VEH) on serum corticosterone, F(2, 14) = 48.6, p < 0.0001. F-PLSD post hoc analyses revealed a reliable increase in serum corticosterone compared with saline/vehicle injected controls at 1 h, 2 h, 4 h after TNF-α injection (p < 0.05). TNF-α and IL-1ra-treated rats also had significantly elevated serum corticosterone levels compared with saline injected controls at 2 h and 4 h (p < 0.05) but not 1 h (> 0.05)) after TNF-α. IL-1ra did reliably reduce the TNF-α–induced corticosterone response compared with vehicle + TNF-α treated rats. F-PLSD post hoc analyses revealed a reliable reduction at 1 h and 4 h (ps < 0.05), but not 2 h (p > 0.05) after TNF-α.

Subdiaphragmatic Vagotomy Effect on Serum Total Protein and CBG Response to TNF-α

Baseline levels of total serum protein did not differ between the groups (Fig. 4a; p > 0.05). Clearly, TNF-α decreased total serum
protein, \((F(1, 19) = 903.3, p < 0.0001)\). Subdiaphragmatic vagotomy had no effect on the cytokine-induced reduction nor did vagotomy itself alter serum protein levels \((p > 0.05)\).

Baseline levels of CBG also did not differ between the groups (Fig. 4b, \(p > 0.05\)). There was a large decrease in serum levels of CBG, \((F(1, 18) = 130.6, p < 0.0001)\) 4 h after TNF-\(\alpha\). Subdiaphragmatic vagotomy had no effect on the TNF-\(\alpha\)–induced decrease in CBG nor did vagotomy itself alter CBG levels \((p > 0.05)\).

IL-1ra Effect on Serum Total Protein and CBG Reduction Produced by TNF-\(\alpha\)

Baseline levels of serum protein were the same for all groups prior to drug treatment (Fig. 5a, \(p > 0.05\)). TNF-\(\alpha\) resulted in a large reduction in serum total protein, \(F(2, 14) = 366.5, p < 0.0001\), and IL-1ra had no effect on this reduction \((ps > 0.05)\).

Baseline levels of CBG were equal across groups prior to drug treatment (Fig. 5b, \(p > 0.05\)), but not TNF-\(\alpha\) treatment reduced CBG, \(F(2, 14) = 120.3, p < 0.0001\). IL-1ra had no effect on this reduction \((p > 0.05)\).

Subdiaphragmatic Vagotomy Effect on Corticosterone Response to Inescapable Tailshock

Inescapable shock produced a large increase in serum corticosterone from basal levels of 4–5 \(\mu\)g/dl to above 40 \(\mu\)g/dl (Fig. 6). Vagotomy neither altered basal levels of corticosterone nor did it have even a slight effect in mitigating the corticosterone increase produced by the stressor. ANOVA yielded \(Fs < 1.0\) at all time points.

DISCUSSION

Cytokines can communicate with the brain by activating peripheral sensory nerves via paracrine action close to the local sites where they are released [22]. The vagus nerve is a logical candidate pathway because it innervates tissues known to participate in immune function (thymus, lung, liver, lymph nodes, etc.). The vagus becomes electrically active after hepatoporal vein injection of IL-1\(\beta\) [23] and projects to an area of the brainstem, the nucleus tractus solitarius, which is intensely activated following peripheral immune stimulation [6]. These considerations have led a number of investigators to examine the effect of transecting the vagus at the subdiaphragmatic level on immune-to-brain communication. This transection blocks afferent input of abdominal vagal fibers to the CNS. This work has revealed that a number of different CNS-mediated responses to either LPS or IL-1\(\beta\) are completely blocked or substantially reduced following vagotomy. In addition, specific binding sites for IL-1\(\beta\) have been found on vagal paraganglia, which are collections of cells thought to be chemoreceptive that are innervated by vagal afferents [16]. These findings suggest that the vagus provides an afferent pathway by which immune activation signals the brain.

The experiments presented here investigated whether vagotomy might exert its effects via mechanisms other than interruption of sensory communication of the cytokine signal to brain. In the
ever, had a selective effect on these changes. Vagotomy reduced
rum total protein and CBG. Subdiaphragmatic vagotomy, how-
taken 1.0, 2.0, and 4.0 h after the first TNF-
(100 mg/kg) was given 30 min prior to TNF-
a vehicle, TNF-
(a
vehicle (VEH), TNF-
a and IL-1ra vehicle, or TNF-
a and IL-1ra. IL-1ra
(100 mg/kg) was given 30 min prior to TNF-
a. TNF-
a injections were taken at 0 and 0.5 h, as indicated by the arrows. Baseline blood samples (BL) were taken prior to the first injection. Additional blood samples were taken 1.0, 2.0, and 4.0 h after the first TNF-
a injection. (B) Serum corticosteroid binding globulin following either saline (SAL) and IL-1ra vehicle (VEH), TNF-
a and IL-1ra vehicle, or TNF-
a and IL-1ra. IL-1ra
(100 mg/kg) was given 30 min prior to TNF-
a. TNF-
a injections were made at 0 and 0.5 h, as indicated by the arrows. Baseline blood samples (BL) were taken prior to the first injection. Additional blood samples were taken 1.0, 2.0, and 4.0 h after the first TNF-
a injection. CBG levels were assessed only for the 4.0 h blood sample.

FIG. 5. (A) Total serum protein following either saline (SAL) and IL-1ra vehicle, TNF-
a and IL-1ra vehicle (VEH), or TNF-
a and IL-1ra. IL-1ra
(100 mg/kg) was given 30 min prior to TNF-
a. TNF-
a injections were made at 0 and 0.5 h, as indicated by the arrows. Baseline blood samples (BL) were taken prior to the first injection. Additional blood samples were taken 1.0, 2.0, and 4.0 h after the first TNF-
a injection. (B) Serum corticosteroid binding globulin following either saline (SAL) and IL-1ra vehicle (VEH), TNF-
a and IL-1ra vehicle, or TNF-
a and IL-1ra. IL-1ra
(100 mg/kg) was given 30 min prior to TNF-
a. TNF-
a injections were made at 0 and 0.5 h, as indicated by the arrows. Baseline blood samples (BL) were taken prior to the first injection. Additional blood samples were taken 1.0, 2.0, and 4.0 h after the first TNF-
a injection. CBG levels were assessed only for the 4.0 h blood sample.

The present studies, increases in corticosterone produced by TNF-
a were substantially reduced by subdiaphragmatic vagotomy. However, this was not the result of a vagotomy-induced decrease in either adrenocortical or hypothalamic responsiveness because in-escapable shock, a stimulus effecting the CNS via mechanisms unrelated to vagal afferents, elicited an undiminished corticoste-
erone response in vagotomized subjects. Thus, the CNS neurocir-
cuity, as well as the peripheral endocrine structures supporting
elevated plasma corticosterone responses by TNF-
a, are unaffected by subdiaphragmatic vagotomy. It was also possible that interruption of vagal motor function might interfere with peripheral organ responses to cytokines. The intracerebroventricular administration of IL-1 leads to shifts in protein synthesis by the liver [17], for example, and so it is possible that communication proceeds from peripheral cytokines to the brain via blood-borne mechanisms, and then signaling to peripheral organs such as the liver involve vagal motor processes. In the present experiments intraperitoneal injec-
tion of TNF-
a increased serum corticosterone and decreased se-
rum total protein and CBG. Subdiaphragmatic vagotomy, how-
ever, had a selective effect on these changes. Vagotomy reduced
the corticosterone response, but had no effect on the reduction in
total protein and CBG. Thus, TNF-
a produces changes in liver
function using mechanisms that do not require intact vagal
communication. It should be noted, however, that given the size
(approx: 80%) and speed (within 1 h) of the reduction in total
serum protein and CBG produced by TNF-
a, there are potentially
additional mechanisms (e.g., plasma extravasation with egress of
proteins) that could be contributing to this effect. The failure of
vagotomy to blunt the impact of TNF-
a on serum protein levels is
also relevant to the possibility that vagotomy does not interrupt
signaling to the CNS, but instead renders the organism less able to
respond to cytokines. Clearly, serum protein responses were un-
affected. It was only the response (plasma corticosterone eleva-
tion) that is mediated, in part, by the CNS that was blunted. These
data support the argument that the impact of vagotomy on cytokine
responses is mediated by interruption of signaling to the brain.

The corticosterone response to TNF-
a was not completely
blocked by vagotomy. This could mean that TNF-
a signals the
CNS by an alternative pathway, in addition to the vagus. However,
vagotomy also produces only a partial blockade of the CORT
response to IL-1[12]. Because part of the corticosterone increase
produced by cytokines is mediated by direct action at the pituitary,
adrenal, and/or median eminence [24], the portion of the cortico-
sterone increase mediated in this fashion would not be expected
to be altered by vagotomy, rather only the portion mediated by
communication between the cytokine and the CNS.

Although the present data implicate the vagus in the commu-
nication pathways between TNF-
a and the CNS, the mechanism
may involve induction of IL-1, with IL-1 then acting as the
stimulus to the vagus. This scenario is supported by the IL-1 attenuation of the corticosterone response to TNF-
a. In a similar
fashion, intraperitoneal TNF-
a produces hyperalgesia as measured
by a decrease in the tail-flick reflex to radiant heat [30]. This
hyperalgesia is mediated by the CNS and is completely blocked by
both vagotomy and IL-1ra [30]. Interestingly, IL-1ra did not have
any effect whatsoever on the impact of TNF-
a on total protein and
CBG, the very same reactions that did not require vagal signalling.
This observation is consistent with the possibility that IL-1 recep-
tors are an obligatory part of vagal signalling. In contrast, hepa-
tocytes and other cells in the liver do express TNF-
a receptors
[14], and so responses mediated at this level do not require IL-1.
Thus, the effects of TNF-
a on peripheral structures and the CNS
may be mediated quite differently.

FIG. 6. Serum corticosterone levels following inescapable shock (IS) or
home cage control (HCC) treatment in subjects that had received either
subdiaphragmatic vagotomy (VAGOT) or sham surgery. A baseline (BL)
blood sample was taken prior to inescapable tailshock. Additional samples
were taken after 5 (5-IS) and 100 (100-IS) tailshocks.
In sum, these results demonstrate that elevated plasma corticosterone levels observed after peripheral TNF-α administration are dependent at least in part on the integrity of the vagus nerve. In contrast, decreased plasma protein and CBG levels, effects of TNF-α administration not initiated by the brain, are unaffected by vagotomy. Further, the corticosterone response to a stressor independent of immune stimulation (inescapable tailshock) was also unaffected by vagotomy. Thus, the vagus nerve appears to function selectively to signal the brain regarding peripheral immune activation, and the effects of vagotomy are unlikely to be the result of either CNS or peripheral organ insensitivity to cytokines. Because pretreatment with IL-1ra mimicked the pattern of vagotomy on TNF-α actions, it is possible that IL-1 receptors mediate the effects of other cytokines such as TNF-α that may signal the brain via vagal afferent fibers. Clearly, evidence exists for bidirectional communication between the brain and the immune system. The data presented here support immune-to-brain communication, which involves cytokine interactions with peripheral nerves. There is also evidence supporting brain-to-immune communication. For example, the brain (via peripheral nerves) can also regulate cytokine production [19]. Thus future work will continue to reveal the nature and importance of nervous system and immune system interactions.

ACKNOWLEDGEMENTS

This research was supported by Aagen and NIH grants MH 55283, and MH 45045.

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