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SYMPATHETIC EXCITOTOXICITY IN SEPSIS: PRO-INFLAMMATORY PRIMING OF MACROPHAGES BY NOREPINEPHRINE

Michael Miksa [1], Rongqian Wu [1], Mian Zhou [1], and Ping Wang [1,2]

[1] Division of Surgical Research North Shore University Hospital and Long Island Jewish (LIJ) Medical Center, Center for Immunology and Inflammation, Institute for Medical Research, North Shore-LIJ Health System, Manhasset, NY 11030, [2] Department of Surgery, Albert Einstein College of Medicine, Bronx, NY 10461

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1. ABSTRACT

In the history of medicine, the interaction between mind and body has been repeatedly proposed. However, the influence of the nervous system on the immune regulation has, until now, drawn little attention. In this regard, the adrenergic system has been explored, and mainly catecholamine-mediated anti-inflammatory effects have been described. These inhibitory effects of epinephrine and norepinephrine were found to be mediated by $beta_2$-adrenoceptors expressed on mononuclear cells. Recently, the role of the parasympathetic nervous system in the local anti-inflammatory reflex has been investigated. Stimulation of the vagus nerve decreases the pro-inflammatory response of macrophages via $alpha_7$-cholinergic receptors. Thus, both the sympathetic and parasympathetic nervous systems are thought to work hand in hand in their anti-inflammatory responses. Here we discuss the deteriorating effects of the release of norepinephrine in sepsis. We have discovered that organ dysfunction in severe sepsis is mediated at least in part by an increase in pro-inflammatory cytokine release from Kupffer cells, which is caused by a priming via gut-derived norepinephrine. The sympathetic nervous system and gut-derived norepinephrine mediate the pro-inflammatory effects by activating $alpha_{2A}$-adrenoceptor on Kupffer cells. In this review, we will focus on the differential function of the noradrenergic system on local and systemic inflammatory responses and the possibilities of the modulation of sympathetic outflow by centrally active inhibitors such as the novel peptide ghrelin or NMDA-receptor blockers. Furthermore, we will introduce the new concept of "sympathetic excitotoxicity in sepsis" characterized by the neurogenic priming of the systemic pro-inflammatory response.

"The brain and the nerves are the true body of our Ego; the remainder is just the body of that body, the

nourishing and protecting bark of the delicate core." (Jean Paul, a German philosopher of the European Enlightenment period, 1763-1825)

2. INTRODUCTION

Over the past 20 years, the annual incidence of sepsis in the United States increased by 8.7%, resulting in 240 cases per 100,000 inhabitants (a total of 660,000 cases in the US alone). In turn, there have been increasing cases of Gram-positive (52% versus 38% Gram-negative and 5% polymicrobial) and fungal infections (3-fold increase). With the increased incidence of sepsis, morbidity from single and multiple organ failure has more than doubled over the last 20 years (1). Sepsis is generally defined as a systemic inflammatory response associated with a proven or suspected infection (2, 3), which eventually progresses to multiple organ dysfunction (severe sepsis) and intractable hypotension (septic shock). Recently, Wang *et al.* proposed to distinguish the two distinct entities "severe sepsis" and "septic shock" (4). Accordingly, severe sepsis is accompanied by a systemic inflammation that typically progresses over weeks until the patients finally succumb to organ dysfunction while showing few signs of inflammation or necrosis prior to death. Septic shock, on the other hand, is more acute and many patients die within 24-48 hours displaying widespread inflammation and necrosis. Although these are viewed as two distinct conditions, progression from severe sepsis to septic shock and vice versa is possible. Typically, those authors view the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-alpha, "early cytokine") and high mobility group box protein-1 (HMGB1, "late cytokine") as major mediators in septic shock and severe sepsis respectively (4). Although infection is a major component of sepsis, treatment of septic patients with antibiotics does not seem to be sufficient. Physicians and scientists have therefore focused on the modulation of the potentially detrimental pro-inflammatory mediators. In the past, various efforts have been made to inhibit cytokines such as TNF-alpha and interleukin (IL)-1beta in septic patients by utilizing antibodies and soluble receptors. However, because efforts targeting these pro-inflammatory mediators in clinical trials have mostly failed (5), there has been a shift in interest towards other pathologic mechanisms involved in sepsis.

The interaction between the central nervous system (CNS) and immune system under various inflammatory diseases has found considerable interest in the past few decades. Sympathetic influence on immune function was initially demonstrated by showing that epinephrine (E) and norepinephrine (NE) inhibited histamine secretion from mast cells (6). Along with the discovery of cytokines in the late 1970s, greater research efforts were undertaken to investigate neuro-immune interaction. Various studies have shown that catecholamines (CA) and glucocorticoids inhibit immune function of peripheral blood mononuclear cells (PBMC) and other immune cells. The adrenergic anti-inflammatory effects are mostly mediated by $beta_2$-adrenoceptors, which are expressed on lymphocytes and monocytes (7, 8). Borovikova *et al.* and Wang *et al.* recently described the novel concept of a cholinergic anti-inflammatory pathway mediated by the activation of the vagus nerve and nicotinic $alpha_7$-cholinergic receptor on macrophages (9, 10). It has also been shown that electrical stimulation of the vagus nerve during endotoxemia can inhibit synthesis of TNF-alpha in the liver, spleen and heart (10). Thus, sympathetic and parasympathetic nerves were viewed as anti-inflammatory systems that act synergistically.

In this review article, we will present evidence showing that sympathetic activation in sepsis leads to increased levels of enteric NE that enter the circulation, causing an override of the systemic anti-inflammatory effects of circulatory CA. NE, at concentrations observed in sepsis (~20 nM), mediates a pro-inflammatory response by the $alpha_{2A}$-adrenoceptor on hepatic macrophages (i.e., Kupffer cells) that is further boosted by endotoxins and pro-inflammatory cytokines. We will also discuss the causes responsible for the switch from a systemic, anti-inflammatory condition to a pro-inflammatory condition and introduce the new concept of "sympathetic excitotoxicity in sepsis". Contrasting the locally active "cholinergic anti-inflammatory pathway" recently described by Tracey *et al.* (11), this sympathoexcitation primes a neurogenic systemic inflammatory response, leading to organ toxicity. Finally, we will discuss the beneficial effects of the novel peptide ghrelin in sepsis that are mediated by increased vagotonic and decreased sympathetic nerve activities.

3. NEURO-IMMUNE INTERACTION

3.1. Pro-Inflammatory Cytokines in Sepsis

Two commonly used experimental models for studying sepsis are endotoxemia and cecal ligation and puncture (CLP) (12-14). CLP induces a polymicrobial sepsis, which provokes an early (up to 10 hrs) hyperdynamic phase with an increase in cardiac output (CO) and a decreased total peripheral vascular resistance (TPR), followed by a decrease of CO and an increase in TPR in the late phase (later than 12-15 hrs after CLP). Thus, CLP serves as an appropriate model for clinical sepsis by mimicking "warm shock" and "cold shock", respectively. Bacteremia as well as a concurrent increase of pro-inflammatory cytokines (TNF-alpha, IL-1beta and IL-6) occur very early after CLP, while late in sepsis, increased plasma levels of the cytokine HMGB1 can be found. The "classical" pro-inflammatory cytokines TNF-alpha and IL-1beta are mainly released by macrophages after recognizing bacterial structures with specific molecular patterns. Lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria, is such a molecular pattern that activates cells of the innate immune system, which express CD14 and toll-like receptor- (TLR-) 4 (15). The stimulated cells subsequently release pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-12), chemokines, prostaglandins, and leukotrienes (e.g., LTB_4), which lead to a local inflammatory response causing increased permeability, recruitment of other immune cells, and pain. Bacterial components other than LPS have been found to elicit a similar response. Peptidoglycans, lipoteichoic acid, bacterial DNA, polysaccharides and

fimbriae each activate specific TLRs. At a later stage of sepsis (12-32 hrs), the novel "late cytokine" HMGB1 is secreted by activated monocytes and macrophages and passively released from necrotic cells. HMGB1 acts as a potent mediator of inflammation (16-18). Uncontrolled release of pro-inflammatory cytokines induces the systemic inflammatory response, a hallmark of sepsis. This overwhelming inflammation leads to the activation of other immune cells and endothelial cells and increased cytokine production at sites distant from the primary inflammation. Although a contained, pro-inflammatory response is advantageous against localized infectious, such as pneumonia (19-21), an uncontrollable systemic inflammation has been shown to be detrimental (22), and the removal of early or late cytokines proved to be beneficial to septic animals (17, 23-25). We will show that the induction of a systemic pro-inflammatory response in sepsis is in part mediated by neuro-endocrine pathways.

3.2. Anatomy and Physiology of Neuro-Immune Interaction

There is a bi-directional cross talk between the CNS and the immune system; the brain influences the immune system by both the hypothalamo-pituitary-adrenal (HPA) axis (glucocorticoids) and by the sympathetic (E and NE) and parasympathetic (acetylcholine, ACh) pathways of the autonomic nervous system (ANS). The ANS is subdivided into two complimentary systems that usually produce opposing effects on various functions: the sympathetic (noradrenergic) and parasympathetic (cholinergic) nervous system. Homeostasis of these systems can be perturbed by internal and external challenges leading to the activation of the HPA and the sympathetic nervous system (SNS) (26, 27). The ANS is controlled by several nuclei within the hypothalamus and brain stem. The nucleus that has been best described is the paraventricular nucleus (PVN), a region close to the third ventricle consisting of magnocellular and parvocellular neurons. While magnocellular cells send efferent axons to the neurohypophysis, the parvocellular neurons communicate with regions in the brain stem and spinal cord and receive inputs from the brain stem, hippocampus, and other regions. Two principal neurotransmitter systems involved in the regulation of the SNS are the "CRHergic" system using corticotropin-releasing hormone (CRH) as a neurotransmitter in the PVN and the noradrenergic system of the locus coeruleus (LC). Both excite each other by sending signals through communicating fibers. The LC activates the ascending reticular activating system, whereby inducing alertness. It also increases peripheral sympathetic nerve activity. Neuronal axons from the PVN and brain stem communicate with preganglionic sympathetic neurons located in the intermediolateral column of the spinal cord. The neurotransmitter from these preganglionic fibers is ACh (the same as for the parasympathetic nervous system); they act through nicotinic receptors on the ganglionic neurons. Postganglionic fibers of the SNS are predominantly noradrenergic, and the effects on target organs depend upon the receptor specificities expressed on those cells. The ANS releases its neurotransmitters from varicosities in a paracrine fashion into their environment where the diffusion of neurotransmitters may be across several micrometers (as compared to 5-20 nm in synaptic transmissions) (7, 8). This type of connection is referred to as "nonsynaptic" and targets a larger cell population than synaptic connections. This nonsynaptic transmission bears the risk that in a hyperactive state such as sepsis, transmitters (in this case NE) may spill over into the circulation and reach distant organs, inducing undesired effects. How does the hyperactive state and spillover happen? We will try to answer this question by taking a closer look at the noradrenergic system under physiologic and pathologic conditions such as sepsis.

3.3. Communication between the Immune System and Central Nervous System

As the CNS modulates immune function, the immune system also influences the brain. Studies by Besedovsky *et al.* have shown that cytokines are able to alter neuronal excitability (28). Two distinct ways of communication between the immune system and neurons of the CNS have been proposed (28). One is the direct stimulation of sensible nerve terminals that project afferent fibers towards the spinal cord, brain stem and hypothalamus. These nerve terminals express cytokine receptors (TNFR and IL-1R) that are directly activated by inflammatory mediators (28). The other is an indirect peripheral activation by pain fibers via substance P (a nociceptive mediator of the dorsal horn of the spinal cord) and by endorphins, which also alter central nervous excitability (29). However, cytokines can also directly influence the nervous system bypassing the peripheral nervous system. Pro-inflammatory mediators can enter the brain through areas of a physiologically diminished blood-brain barrier and directly influence CNS activity. This pathway has been shown to be the major regulator of fever induction and anorexia during inflammatory reactions (30, 31).

4. SYMPATHETIC NERVOUS SYSTEM

4.1. Noradrenergic Pathway

The adrenergic effects of the SNS are mediated primarily by NE and partly by E through nine different adrenoceptors: three $alpha_1$ receptors ($alpha_{1A}$, $alpha_{1B}$, $alpha_{1C}$), three $alpha_2$ receptors ($alpha_{2A}$, $alpha_{2B}$, $alpha_{2C}$), and three beta receptors ($beta_1$, $beta_2$, $beta_3$). Other mediators such as neuropeptide Y (NPY) or opioids also seem to regulate sympathetic effects although details are not well understood (7, 32). It is known, however, that in times of low sympathetic activity (under physiologic conditions) only NE is released from the nerve terminals while high activity leads to a release of both NE and NPY (33). Upon binding of $beta_2$-adrenoceptors, E and high doses of NE increase intracellular cAMP levels through activation of the adenylate cyclase. cAMP by itself has been shown to induce anti-inflammatory pathways through activation of the cAMP response element binding protein (CREB) and inactivation of nuclear factor-kappa B (NF-kappa B) (34). $alpha_2$-Adrenoceptors are G_i- and G_0-protein coupled receptors that decrease intracellular cAMP, open K^+ channels, and inhibit voltage gated Ca^{2+} channels, all of which lead to hyperpolarization of neurons and activation of immune cells (34). In the CNS, $alpha_2$-adrenoceptors are predominantly presynaptic. They

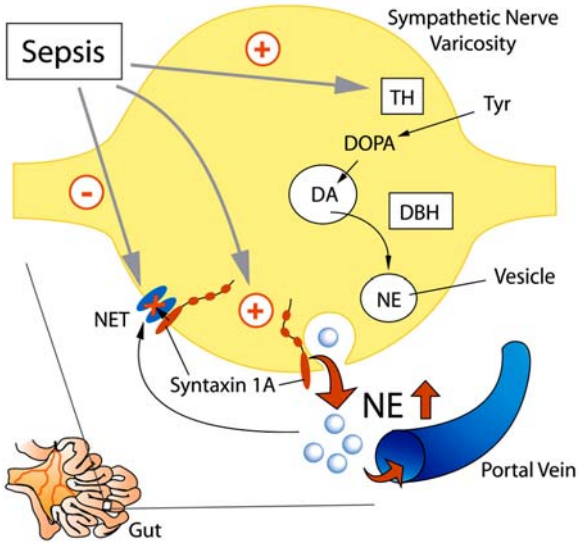

Figure 1. Altered activities of the sympathetic nerve varicosity in sepsis. In sepsis, TH is increased, which increases NE production. In addition, increased levels of syntaxin 1A mediate fusion of the presynaptic NE-vesicle with the varicosical membrane and promote NE release. The expression of NET, the major uptake and signal termination mechanism in the noradrenergic system, is decreased, leading to an extraneuronal accumulation of the previously released NE. Syntaxin 1A can also block the NET and further attenuate the already reduced NE uptake. Thus, the high levels of NE enter into the circulation via the portal vein where they reach the liver and the Kupffer cells. NE=norepinephrine, NET=NE transporter protein, Tyr=tyrosine, TH=tyrosine hydroxylase, DA=dopamine, DBH=dopamine beta-hydroxylase.

regulate the release of neurotransmitters through a negative feedback and modulate certain functions such as wakefulness, pain, and central blood pressure regulation. Functional studies of the genetic receptor subtypes have linked the $alpha_{2B}$-adrenoceptor to peripheral vasoconstriction and analgesic effects of N_2O (nitrous oxide). The $alpha_{2A}$-adrenoceptor, either alone or with $alpha_{2C}$-adrenoceptor co-activation, is involved in the central inhibition of sympathetic activities, modulation of neurotransmitter release, sedation, and anti-epileptic effects. In the periphery, the $alpha_{2C}$-receptor regulates the release of NE from sympathetic nerve fibers (34). As discussed below, the pro-inflammatory action of NE is mediated by the $alpha_{2A}$-receptor subtype expressed on hepatic macrophages (i.e., Kupffer cells).

4.2. Release of Gut-Derived NE into the Circulation

The enteric nervous system consists of neurons with a dense axonal network that is predominantly distributed along the myenteric (Auerbach) plexus and the submucosal (Meissner) plexus. These neurons sense local changes within the intestinal tract and act largely autonomously although they are fine-tuned by the sympathetic and parasympathetic nervous systems. Noradrenergic nerve fibers are distributed in clusters around the enteric neurons throughout the intestinal tract; a slightly higher density is found in the myenteric plexus than in the submucosal plexus (35). Hahn *et al.* demonstrated in 1995 that plasma levels of CA are elevated in sepsis (36). NE in particular, sustains elevated levels not only in the early stage, but also in the later stage of sepsis (36). In 2000, Yang *et al.* discovered that the gut is a major source of the sustained elevation of NE in sepsis (37). They compared the levels of portal to systemic NE, found 74% higher concentrations in the portal system and showed that systemic NE levels are reduced by 51% after enterectomy (37). They also found that the increased NE levels were associated with plasma increases of the pro-inflammatory cytokines, TNF-alpha, IL-1beta, and IL-6.

Why does this massive release of NE into the circulation occur in sepsis? Through several approaches we try to answer this question. Zhou *et al.* discovered a sepsis-induced elevation of the tyrosine hydroxylase (TH) in the gut, the rate-limiting enzymatic reaction in the synthesis of NE (35). The increased TH activity is predominantly found in the myenteric plexus. TH converts tyrosine into DOPA that is eventually converted to dopamine, a process that takes place in the cytosol of the nerve terminal. After uptake into a presynaptic vesicle, dopamine is converted to NE by the enzyme dopamine beta-hydroxylase (DBH). In addition to an increased production of NE, we found several mechanisms involved in the increased release that contribute to the massive spillover of NE into the circulation. Release of NE-vesicles from the sympathetic varicosities depends upon electrical stimulation that induces a cascade of cytoplasmic events, leading to fusion of the neurotransmitter vesicle with the plasma membrane. Members of the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) family have been shown to be involved in the intracellular vesicle transport in neurons and immune cells (38-42). Syntaxin 1A is a SNARE protein known to function as a presynaptic regulator of neurotransmitter release. Upon electrical stimulation, syntaxin 1A fuses NE vesicles with the presynaptic membrane and promotes NE release from the varicosities. Increased quantity or activity of syntaxin 1A thus leads to increased NE release (43-45). Additionally, syntaxin 1A can inhibit the NE uptake activity of the norepinephrine transporter (NET) that is also located on the presynaptic plasma membrane (46, 47). NE uptake into the presynaptic cytoplasm normally accounts for over 90% of the termination of NE signaling (48). Thus, both syntaxin 1A and NET play an important role in controlling NE release at the local level. NE can, after it has been released, bind to presynaptic $alpha_2$-receptors and through modulation of these two proteins, inhibit further NE release (46). However, this effect seems to be abrogated in sepsis. We have recently found that in early sepsis, enteric levels of NET decrease while syntaxin 1A levels increase (preliminary data). This effect leads to a net increase in NE release that eventually spills over into the portal system (Figure 1). Obviously, the typical feedback inhibition does not function anymore. This leads to the question whether or not these changes are due to local effects of pro-inflammatory cytokines found during sepsis. We tried to answer this question by *in vitro* experiments

and analyzed the effects of LPS and TNF-alpha on cultured neurons that express syntaxin 1A. Our preliminary data have shown that neither of those agents increased NE release from the cultured cells. Although it is not known whether the same is true in enteric varicosities, this indicates that the increase in NE release found in sepsis may not be a local inflammatory effect on nerve terminals. Rather, central mechanisms may control these changes, involving the activation of preganglionic neurons and autonomous nuclei in the hypothalamus and brain stem.

4.3. Pro-inflammatory Effects of NE in Sepsis

Our studies have indicated that intraportal injection of NE, at concentrations found under septic conditions (~20 nM), produced an increase of circulating levels of TNF-alpha, IL-1beta, and IL-6, similar to that found in sepsis (37, 49, 50). Hepatocellular dysfunction caused by these pro-inflammatory cytokines was determined by the increase of hepatic enzymes in circulation and by the reduction of indocyanine green clearance (37). Our previous studies have shown that gut-derived NE up-regulates TNF-alpha and IL-1beta production in Kupffer cells through an $alpha_2$-adrenergic pathway (49-51). This appears to be, in part, responsible for the increased pro-inflammatory cytokines in the circulation. At first glance, these findings seem to contradict previous findings of NE-mediated anti-inflammatory effects that are mediated by $beta_2$-receptor activation. However, the dose given in our study increased the pre-hepatic plasma NE concentration to ~20 nM. At this concentration, which also represents the level found under septic conditions, NE selectively activates $alpha_2$-adrenoceptors (52-54).

4.4. The Molecular Interface: $alpha_{2A}$-Adrenoceptor

Only supraphysioliogic levels of NE can inhibit cytokine release from monocytes/macrophages, which is an outcome different from the effects described here. Many studies focusing on the immunomodulation by NE used high concentrations of NE (i.e., 10^{-4} M) and thus were more likely to activate $beta_2$-receptors that override $alpha_2$-receptor-mediated pro-inflammatory responses (55-57). In previous studies, NE was also administered directly into the systemic circulation, and the first immune cells targeted were PBMCs that have a different receptor composition than tissue macrophages (7, 8). Intraportal infusion of NE at a concentration of 20 nM used in our study did not alter cardiac output (49), suggesting that the NE concentration in the circulation was low enough not to influence beta-receptor-mediated cardiac performance. This further implies that alpha-adrenoceptors on Kupffer cells become selectively activated (8). Through *in vitro* experiments with selective alpha-adrenergic inhibitors, we later found that the $alpha_{2A}$-receptor subtype on Kupffer cells is responsible for the increased cytokine release (58). Intraportal or systemic application of the $alpha_{2A}$-adrenoceptor inhibitor BRL-44408 maleate also showed a significant decrease in cytokine release *in vivo*, attenuation of organ injury, and improvement of survival in sepsis (59). Because previous reports of pro-inflammatory effects of the SNS were assigned to $alpha_{2B}$-adrenoceptors (7), this is a novel discovery. We also found that the receptor distribution pattern on Kupffer cells changes during the course of sepsis by increasing pro-inflammatory $alpha_{2A}$-adrenoceptor expression while showing no changes in the expression of the other two $alpha_2$-adrenoceptor subtypes $alpha_{2B}$ and $alpha_{2C}$ (58). Thus, these highly specialized macrophages express a different pattern of adrenoceptors and display a differential response to CA, which drives the noradrenergic effect towards a systemic pro-inflammatory response. In addition, the macrophages' state of activation is important, because NE does induce the production and release of pro-inflammatory cytokines but it does so more efficiently (and synergistically) after co-stimulation with bacterial endotoxins (51). This co-stimulatory effect seems to be mediated by activation of the NF-kappa B and MAP-kinase pathways. Furthermore, Roy *et al.* recently found that the macrophage response to NE differs depending on the dose and duration of stimulation (60). At high doses over a short period of time, responses are mediated by fast protein-kinase cascades, while at a low-dose stimulation for a prolonged period, responses are mediated by changes in gene expression eventually leading to opposite effects (60). These differential effects are not necessarily detrimental. In sepsis, the immune system tries to fight bacteria in the circulation. Thus, a hyper-inflammatory state, which seems at least transiently favorable, is eventually counteracted by a change in response through altered gene expression. Under non-septic conditions, an increase of CA-mediated effector functions makes sense from a teleological standpoint. The SNS activates the cardiovascular system while simultaneously suppressing a systemic inflammation through beta-adrenergic inhibition of cells not primarily involved in the local inflammation. This mechanism prevents the destruction of the host tissues located away from the site of inflammation while mature macrophages at the inflammatory site get ready to fight the bacterial intruders. Under septic conditions, however, the spillover of NE into the portal system activates cells that are not primarily involved in the inflammatory response, leading to the destruction of hepatic, downstream pulmonary, and other organ tissues through the subsequent release of pro-inflammatory cytokines.

5. IMPLICATIONS OF THE SYMPATHETIC INFLUENCE IN SEPSIS

5.1. Ghrelin: A Novel Modulator of the Autonomic Nervous System

In sepsis, modulating the sympathetic excitotoxic effect becomes an issue. Direct inhibition of the $alpha_{2A}$-adrenoceptor expressed on Kupffer cells is one promising approach. A possibility of modulating the SNS activity directly involves the use of a novel peptide, ghrelin. Ghrelin is a small peptide (28 amino acids) that was discovered in 1999 by Kojima *et al.* (61) and found to have somatotrope, lactotrope, and adrenocorticotrope properties by stimulating growth hormone (GH), prolactin (PRL), and adrenocorticotropin (ACTH), respectively (62, 63). Its orphan receptor however, has been known for over 20 years. Synthetic agonists, the growth hormone secretagogues (GHS), mediate their GH releasing effect through GHS receptors (GHSR) expressed in the pituitary gland. The GHSR is a G-protein coupled receptor with 7

transmembrane domains and exists in two variants, the GHS-R1a and the GHS-R1b, where the latter is thought to be inactive (64). Aside from being expressed in numerous tissues throughout the body (e.g., lungs, pancreas, stomach, kidneys and adipose tissue), the GHS-R1a can mainly be found in the pituitary gland (somatotropic cells), the hypothalamus (arcuate and ventromedial nuclei), and pontomedullary region. The receptors are thus logistically distributed in areas involved in neuroendocrine signaling. Since the detection of ghrelin as an endogenous ligand of the GHS-R1a, numerous publications on its orexigenic activity (i.e., the enhancing effect on hunger and food intake) have been found. It is now thought to be the most important mediator of food intake and a functional antagonist of leptin that is released by adipose tissues and mediates satiety in the hypothalamus (65, 66). Ghrelin is released during periods of starvation and hypoglycemia from the X/A-like cells, which comprise about 20% of the gastric endocrine tissue located in the oxyntic mucosa (67, 68). This peptide then binds to the GHSR in the hypothalamus and induces hunger and food intake (63, 69). Centrally administered ghrelin also inhibits lipolysis in adipose tissues, which is mediated by a decrease in sympathetic activity as recently shown by Matsumra *et al.* (70). Moreover, ghrelin has other effects such as anti-proliferative properties on neoplastic cells, involvement in the glucose metabolism, and on the cardiovascular system, such as vasodilation. Many of these latter effects have been attributed to activation of GHSRs expressed on peripheral tissues. With regard to the cardiovascular response, ghrelin induces vasodilation together with an increase in cardiac index without a change in heart rate (71). This has led to the notion that these effects are partly mediated by modulating the activity of the ANS. Indeed, IV and intracerebroventricular (ICV) injection of ghrelin has been shown to induce an increase in vagal efferent discharge (64, 72). Other publications have cited a decrease in the sympathetic nerve activity in brown adipose tissues and kidneys after IV or ICV injection of ghrelin (70, 73, 74).

Wu *et al.* recently discovered that plasma ghrelin levels dramatically decreased by 64-72% in CLP-induced sepsis (75), similar observations have been made in endotoxemic rats (76, 77). However, the mechanism responsible for the downregulation of ghrelin remains unknown. In the early, hyperdynamic stage of sepsis, hyperglycemia may be the reason for a strong inhibitory function on ghrelin release from gastric X/A-like cells (64). In the late, hypodynamic phase of sepsis, however, glucose levels decrease without inducing ghrelin release. Direct influence of endotoxin or pro-inflammatory cytokines on these cells is one possible mechanism that is currently under investigation. Wu *et al.* further reported that ghrelin has anti-inflammatory properties that are not mediated through direct receptor interaction on immune cells, but through the activation of the vagus nerve (78). In studies involving surgical dissection of the nerve (vagotomy) prior to CLP, we found that the anti-inflammatory effects induced by IV injection of ghrelin could be abrogated by vagotomy (78).

It is thought that, similar to cytokines, ghrelin enters the brain through the "loopholes" of the blood-brain barrier, which are closely related with the hypothalamic region: the circumventricular organs (CVO). The hypothalamus contains the control centers of food intake and temperature regulation, and the regulating centers of sympathetic and parasympathetic activity: the paraventricular (PVN) and dorsomedial (DMN) nuclei and the lateral hypothalamus (LH), respectively. After injection of ghrelin into the third ventricle, the hypothalamic ventromedial (VMN) and arcuate nuclei (ARC) become highly activated as shown by increased expression of the immediate early genes *c-fos* and the early growth response gene-1 (*egr-1*) in these areas (69). Unlike the ARC and VMN, the PVN and DMN do not display high levels of GHSR expression. Nevertheless, it has been shown that these hypothalamic nuclei are also activated, probably mediated by neuronal fibers received from the areas that express GHSRs. The majority of investigators in this field focus on the orexigenic pathway and have found that ARC neurons release the inhibitory γ-amino butyric acid (GABA), but also NPY and agouti related peptide (AgRP, a melanocortin receptor antagonist), all being linked to food intake (65, 79, 80). The NPY/AgRP neurons and the melanocortin releasing proopiomelanocortin (POMC) neurons in the ARC are involved in a complicated network with each other and with their afferent target neurons in the PVN and DMN exerting inhibitory and stimulatory effects, respectively. As the co-released AgRP inhibits the melanocorticotrope activity of POMC neurons, the net effect on the afferent target sites is inhibitory (65). This, and the co-release of GABA from both the NPY/AgRP and POMC neurons, could indeed explain the decreased sympathetic nerve activity that is mediated by the decreased activation of the DMN and PVN (Figure 2) (72, 81). It remains unknown, however, whether or not the same pathways also modulate the control centers of the ANS in sepsis. Other neurotransmitters released from GHSR-rich neurons include CRH, glutamate and NE and it requires further investigation to determine whether these mediators play a role in sympathoexcitation during sepsis. To support the hypothesis of ghrelin's role in the sympathoinhibition, our preliminary data have demonstrated that ghrelin can directly inhibit NE release from the SNS in septic animals and from the cultured neuronal CAD cell line. We also found that, in early sepsis (5 hours after CLP), GHS-R1a is upregulated at the transcriptional and translational level in different regions involved in the regulation of the SNS (the hypothalamus, pontomedullary region and limbic system). In the late phase of sepsis, however, this increase in receptor expression no longer prevails. Although the receptor levels seem to return to normal in the late stage of sepsis, the normal function cannot be resumed, due to the constant, decreased levels of ghrelin in the circulation (Table 1). This indicates that ghrelin has not only the ability to activate the cholinergic anti-inflammatory pathway but also can inhibit the sympathetic excitotoxicity observed in sepsis. Thus, ghrelin appears to be a potential modulator of the SNS in sepsis treatment.

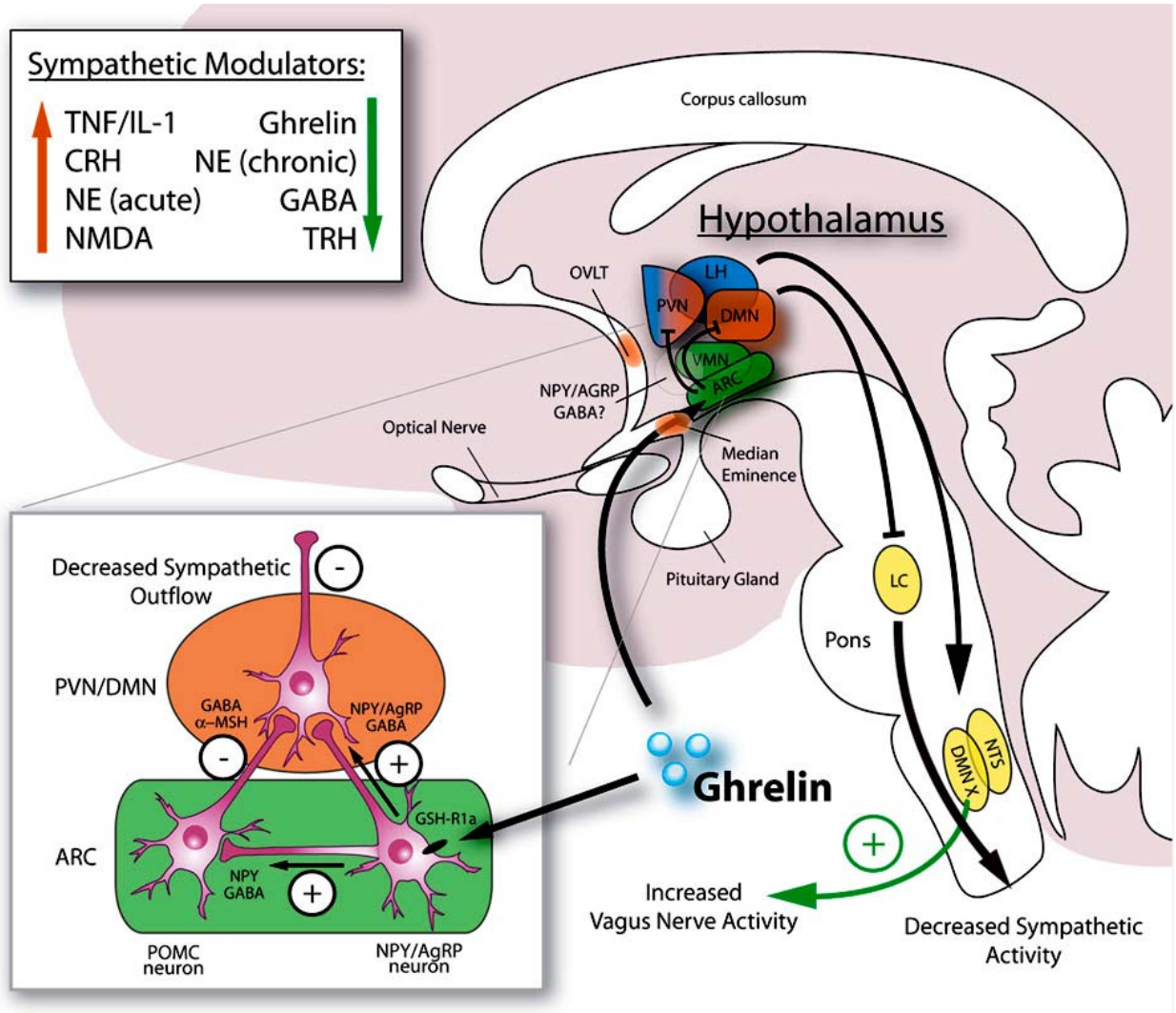

Figure 2. Central regulation of the sympathetic nerve activity (SNA). Several modulators activate or inhibit the sympathetic pathways in the hypothalamus and brain stem. Hypothalamic activation by NMDA or CRH increases sympathetic outflow. Pro-inflammatory cytokines also increase SNA after entering the hypothalamus through the circumventricular organs (CVO), including the OVLT and median eminence. The central NE pathways in the LC and hypothalamus show a biphasic effect on SNA. An initial increase in SNA followed by a sustained inhibition is possibly due to effects on central alpha$_1$- and alpha$_2$-adrenoceptors, respectively. TRH, GABA, and ghrelin inhibit sympathetic outflow and enhance parasympathetic activity. Ghrelin enters the hypothalamus and brain stem through the CVO, whereby binding to specific receptors (GHSR-1a) in the hypothalamic ARC and VMN, in the limbic system, and in the pontomedullary region. Ghrelin may directly enhance the DMN X activity and decrease LC activity, thus favoring a vagotonus and reducing SNA. Hypothalamic control of parasympathetic (LH, anterior PVN) and sympathetic (dorsal PVN and DMN) activity can be modulated by peptidergic (NPY/AgRP and CRH) and noradrenergic efferents from the ARC and VMN. Ghrelin also could influence the activity of the limbic system, which in turn regulates hypothalamic function. OVLT=organum vasculorum laminae terminalis, PVN, DMN, VMN, ARC=paraventricular, dorsomedial, ventromedial and arcuate nuclei, LH=lateral hypothalamus, LC=locus coeruleus, NTS=nucleus tractus solitarius, DMN X=dorsomedial (motoric) nucleus of vagus nerve, NMDA=N-methyl-D-aspartate, NE= norepinephrine, TNF=tumor necrosis factor, IL-1=interleukin-1, CRH=corticotropin- releasing hormone, GABA=gamma-amino butyric acid, TRH=thyroid releasing hormone, NPY= neuropeptide Y, AgRP=agouti related peptide, alpha-MSH=alpha-melanocorticotrope hormone, POMC=proopiomelanocorticotrope neuron.

5.2. Other Targets of Sympathetic Modulation: N-methyl-D-aspartate and GABA

N-methyl-D-aspartate (NMDA) receptors are voltage gated ion channels that open after ligation of the neurotransmitter, glutamate and mediate excitatory functions in the brain (82). Because glutamate has been shown to increase the sympathetic activity, the use of NMDA inhibitors to decrease sympathetic overexcitation in sepsis may be beneficial (81, 83). Studies indicate that NMDA and non-NMDA receptors mediate increased

Table 1. Changes of parameters in early and late sepsis induced by cecal ligation and puncture (CLP) in the rat

System	Parameter	Sepsis		Reference
		Early (2-5h)	Late (20h)	
Systemic	Ghrelin (peptide)	↓	↓	75, 78
CNS (Hypothalamus)	Ghrelin (peptide)	↓	↓	[1]
CNS (Hypothalamus)	GHSR-1a (mRNA, protein)	↑	-	[1]
Enteric	Tyrosine Hydroxylase (protein)	↑	↑	35
Enteric	Syntaxin 1A (mRNA, protein)	↑	-	[1]
Enteric	NET (mRNA, protein)	↓	-	[1]
Hepato-Portal	NE	↑	↑	37, 49, 95
Hepatic Kupffer Cells	$alpha_{2A}$-Adrenoceptor (mRNA)	↑	↑	49, 50, 58, 95
Systemic	TNF-alpha, IL-1beta, IL-6 (protein)	↑	↑	37, 49, 50

[1] our unpublished data

activation of the PVN of the hypothalamus and lead to enhanced the sympathetic outflow with consecutive cardiovascular and metabolic changes (81, 83, 84). However, neuronal activation of the PVN through NMDA receptors in sepsis may not be necessarily mediated by its natural ligand glutamate, because quinolinic acid (QA), which is produced by activated macrophages, can also do the same. QA binds to NMDA receptors and leads to glutamate-like excitatory activation (85). To our knowledge, NMDA-antagonists have not been investigated in septic animals. Other neurotransmitters may also play a role in the activity of the SNS. Studies have shown that the GABAergic tone in the forebrain of septic animals is enhanced (86-88), while others indicated that PVN activity can be inhibited by $GABA_A$ergic neuronal inputs from the suprachiasmatic nuclei – an effect that can be reversed by the application of GABA-antagonists (84). Both systems (i.e., the excitatory glutamatergic and inhibitory GABAergic) have been extensively investigated and are known to regulate learning and memory in other regions of the brain, but their regulation of the sympathetic nerve activity remains elusive. Hence, there remains an immense opportunity to explore their interaction in septic sympathicotonus.

6. SWITCH IN PARADIGM: NEUROGENIC PRIMING OF THE INFLAMMATORY RESPONSE

The immune system has elaborate mechanisms to sustain inflammation at the primary site of injury by releasing pro-inflammatory cytokines while inhibiting a systemic inflammatory response by anti-inflammatory cytokines. At the same time, cytokine or pain- and stress-induced activation of the HPA axis also counterbalances a systemic inflammatory response that is generally seen as detrimental. The fast anti-inflammatory reflex mediated by the cholinergic system supports this control of inflammation at the local level (11). Because most of the effects of CA on the immune system described in the past were anti-inflammatory, it has been assumed that the activation of the SNS produces anti-inflammatory responses, and that it works hand in hand with both the HPA-axis and parasympathetic nervous system (11). Although this may be the case in minor injuries or localized infections, a switch in paradigm occurs in sepsis, causing the previously anti-inflammatory SNS to become a pro-inflammatory pathway. We have clearly showed that an overexcitation of the SNS leads to a spillover of NE from the gastrointestinal tract into the portal and systemic circulation. This eventually primes Kupffer cells via activation of the alpha2A-adrenoceptor and causes a greater pro-inflammatory cytokine release. These cytokines (TNF-alpha, IL-1beta and eventually HMGB1) then contribute to the systemic inflammatory response seen in sepsis and produce toxic effects on various tissues (Figure 3A-B). This has lead us to term this effect "sympathetic excitotoxicity", which is analogous to the glutamate-mediated excitotoxic effects in the brain, as first named by Olney et al. (89). Although it remains unclear whether this sympathoexcitation is the initiating effect of sepsis, we believe that if the anti-inflammatory reflex system fails and the SNS enters a hyperactive state, sepsis can occur. In support of this hypothesis, Prass et al. recently described that cerebrovascular insults can prime spontaneous infections of the lungs through sympathetic excitation, which will eventually lead to sepsis (90). Most patients, who do develop sepsis, are already in a deteriorated condition with several co-morbidities or they are more prone to infection due to their decreased immune function (e.g., infants and the elderly). Normally, opportunistic microbes are well contained by the body's physical, chemical, and immune barriers. However, a morbid priming through severe trauma, burns, or chronic diseases may in fact be the initiator of sepsis. Such severe injuries could lead to an increased sympathicotonus with prolonged and elevated NE release. NE has also been shown to stimulate bacterial growth (91, 92). Due to the sympathicotonus-mediated disturbances of the intestinal microcirculation, bacteria may translocate into the peritoneum and circulation (93, 94), and potentiate the pro-inflammatory state by co-stimulation of the NE-primed Kupffer cells with bacterial endotoxins. Pro-inflammatory cytokines can enter the brain and then induce a vicious circle by further enhancing sympathoexcitation (Figure 3A).

7. CONCLUSION

Although advances are constantly made in the fields of immunology and neurophysiology, we still do not fully understand the complex interaction between the two. During sepsis, the sympathetic as well as

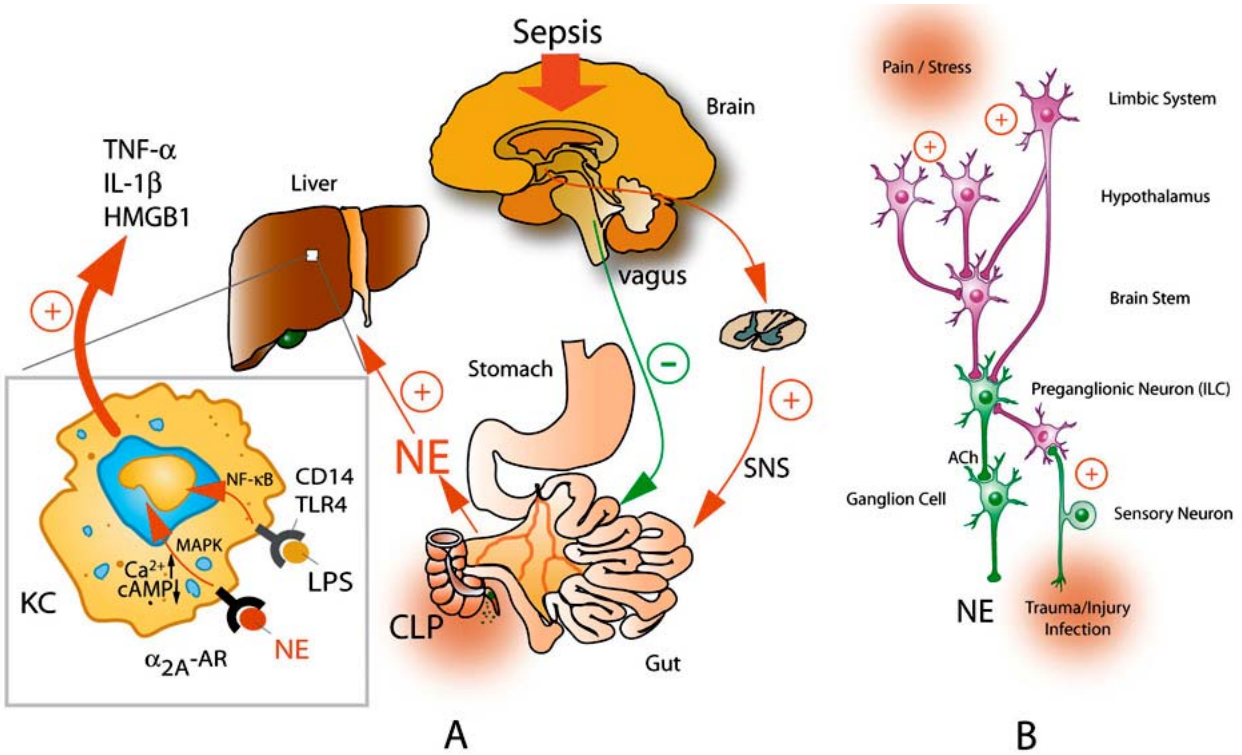

Figure 3. **A**. Sympathetic excitotoxicity inducing systemic inflammatory response in sepsis. Severe trauma and injury cause pain and stress, which activate the sympathetic nervous system. Sustained, increased levels of NE in the gut spill over into the portal circulation and reach Kupffer cells in the liver. NE and LPS along with Gram-negative bacteremia synergistically increase the release of pro-inflammatory cytokines TNF-alpha, IL-1beta and HMGB1 that enter the circulation and eventually lead to septic shock or severe sepsis with multiple organ failure. Inlet: After binding of both NE and LPS to their respective receptors, alpha$_{2A}$-AR and CD14/TLR4, intracellular cAMP decreases and Ca^{2+} increases. At the same time, NF-kappa B and MAP kinase pathways become activated and mediate a systemic pro-inflammatory response. **B**. Neuronal network of sympathetic activation. Activation takes place by two routes: in the periphery via afferent fibers and a reflectory increase of sympathotonus, sustained by stress/pain and cytokine-induced activation of the limbic/hypothalamic regions centrally controlling the activity of the autonomic nervous system. SNS=sympathetic nervous system, CLP= cecal ligation and puncture, NE= norepinephrine, CD14=cluster of differentiation protein 14, TLR4=toll-like receptor 4, LPS=lipopolysaccharides, alpha$_{2A}$-AR=alpha$_{2A}$-adrenoceptor, KC=Kupffer cells, cAMP=cyclic adenosine monophosphate, Ca^{2+}=calcium, MAPK=mitogen-activated protein kinase, NF-kappa B=nuclear factor kappa B, TNF-alpha=tumor necrosis factor-alpha, IL-1beta=interleukin 1beta, HMGB1=high mobility group box protein-1, ILC=intermediolateral column of gray matter in spinal cord.

parasympathetic nervous systems become activated. These two systems counterbalancing each other during the early stage of sepsis, causes the body to drive its reserves towards exhaustion. This hyperactivity might be controlled in the early phase pharmacologically by administration of prophylactic sympathoplegic drugs, such as ghrelin. Just as the physicians and philosophers of the European Enlightenment period began to recognize the close interaction of body and mind, we are now beginning to understand more and more about the aspects of the neuro-immune interactions. The tremendous accumulation of knowledge from a vague understanding of our brain to current advances in neurobiological science gives us a glimpse of hope that we will some day be able to grasp the communication between these two complex systems and develop tools that give us the chance to prevent sepsis in susceptible patients.

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Abbreviations: ACh: acetylcholine; ACTH: adrenocorticotropin; AgRP: agouti-related peptide; ANS: autonomous nervous system; ARC: arcuate nucleus (of hypothalamus); CA: catecholamines; *c-fos:* cellular transcription factor gene ("Finkel-[Biskis-Jinkins] osteosacroma"); CLP: cecal ligation and puncture; CNS: central nervous system; CO: cardiac output; CREB: cAMP response element binding protein; CRH: corticotropin releasing hormone; CVO: circumventricular organ; DBH: dopamine beta-hydroxylase; DMN: dorsomedial nucleus (of hypothalamus); E: epinephrine; *egr-1:* early growth response gene-1 ; GABA: gamma-amino butyric acid; GHS: growth hormone secretagogue; GHSR: growth hormone secretagogue receptor; HMGB-1: high mobility group box protein-1; HPA: hypothalamic-pituitary-adrenal; ICV: intracerebroventricular; IL-1R: interleukin-1 receptor; IV: intravenous; LC: locus coeruleus; LH: lateral hypothalamus; LPS: lipopolysaccharides; LTB_4: leukotriene B4; NE: norepinephrine; NET: norepinephrine transporter protein; NF-kappa B: nuclear factor-kappa B; NMDA: N-methyl-D-aspartate; NPY: neuropeptide Y; PBMC: peripheral bood mononuclear cells; POMC: proopiomelanocorticotrope neuron; PRL: prolactin; PVN: paraventricular nucleus (of hypothalamus); QA: quinolinic acid; SNARE: soluble N-ethylmaleimide sensitive factor attachment protein receptor; SNS: sympathetic nervous system; TH: tyrosine hydroxylase; TLR: toll-like receptor; TNFR: tumor necrosis factor receptor; TPR: total (vascular) peripheral resistance; VMN: ventromedial nucleus (of hypothalamus).

Key Words: Sepsis, Sympathetic Nervous System, Norepinephrine, Inflammation, Macrophages, Kupffer cells, $alpha_{2A}$-Adrenoceptor, Ghrelin, Review

Send correspondence to: Ping Wang, M.D., Division of Surgical Research, Institute for Medical Research, North Shore-LIJ Health System, 350 Community Drive, Manhasset, NY 11030, Fax: 516-562-2396, E-mail: pwang@nshs.edu