New Insights to Neuroimmune Biology
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Istvan Berczi
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Foreword

Neuroimmune biology is a newly emerging multidisciplinary science that aims to collect, organize, and interpret the knowledge concerning the coordination and integration of bodily functions in higher animals and humans. After a long period of empiric observations, exact scientific methods were used for the first time in the mid-1950s, which initiated modest progress in the subject. With the advent of cellular and molecular biology, it became possible to take a second look at long-standing issues and problems in this field. This research started in the mid-1970s and this time it was possible to make major advances in this difficult and complex area of scientific inquiry. Today it is clear that, unlike originally believed, the immune system is not an autonomous, intelligent system capable of recognizing antigens and defending the host. Rather, the immune system has to coordinate with the homeostatic organization of the host. It receives regulatory signals from the neuroendocrine system via hormones, neurotransmitters, and neuropeptides. In turn, the immune system provides feedback signals about the status of immune functions, which are delivered by cytokines. These discoveries both established and proved the existence of immune–neuroendocrine circuitry [1].

The field is now progressing rapidly, and it is becoming clear that the brain is itself capable of recognizing antigens and functioning as an immunocompetent organ. Moreover, cytokines are involved in the physiological regulation of many organs and tissues, including the nervous system. During acute illness, the entire organism is actively involved in host defense, through a complex network that constitutes the neuroimmune supersystem. In this book, various aspects of neuroimmune biology are presented by world-renowned authors.

Istvan Berczi

Reference

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Preface

The chapters of this book present and discuss various mechanisms that are relevant to neuroimmune biology. The investigators selected as contributors are in the forefront of research in their areas and are internationally recognized. The papers cover molecular regulation of cytokines in brain astrocytes, immunoregulation by the sympathetic nervous system, circadian regulation of immune reactions, antigen recognition by the central nervous system, modulation of the immune response in cases of head injury, neurogenic inflammation, the role of tachykinins in asthma and allergic disease, defense and defeat reactions, cytokines, behavior and affective disorders, and increased activity of type 1 helper T cell functions after reward stimulation.

This book has relevance and utility for the entire scientific community in the areas of biology, medicine, and veterinary medicine, as it discusses molecular, cellular, organic, and systemic aspects of neuroimmune interactions, as well as the physiological, pathophysiological, and behavioral mechanisms involved in this new and important discipline of general biology.

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Section A

Introduction

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The Brave New World of Neuroimmune Biology

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1.1 Introduction

1.1.1 How Did It Begin?

Sporadic suggestions of neuroimmune interactions date back to the late nineteenth century. The students and followers of Pavlov in Russia thoroughly investigated the nature of immunoregulation by neuroimmune mechanisms and eventually concluded that the hypothalamus is a likely center of immunoregulation [1,2].

Using modern technology, Szentivanyi and colleagues observed that hypothalamic lesions inhibited the development of anaphylactic shock in immunized animals [3]. Tuber cinereum lesions (TBLs) in the hypothalamus inhibited anaphylaxis in preimmunized guinea pigs and (in later experiments) also in rabbits. Anaphylaxis was induced in immunized animals by intravenous (IV) injection of the immunizing antigen. TBLs also inhibited antibody production if the lesions were created prior to immunization. TBLs did not affect antigen–antibody reactions, nor did the release of tissue materials mediating anaphylaxis. Hypothalamic lesions temporarily increased histamine resistance and inhibited the anaphylactic reaction even when the animals were provided with passively transferred antibodies, which elicited lethal shock in control animals. The Schultz–Dale test, which was performed with small pieces of intestine in vitro, was also inhibited by TBLs. The Arthus reaction, turpentine-induced inflammation, and the Sanarelli–Shwartzman phenomenon were unaffected. Lesions of other areas of the hypothalamus or of the central nervous system (CNS) were ineffective in modulating immune phenomena. Further, electrical stimulation of the mammillary region of the hypothalamus had an inhibitory effect on the anaphylactic response and increased the resistance of animals to histamine [4–6]. In 1964, Korneva and Khai [7] confirmed that hypothalamic lesions in rabbits, guinea pigs, and rats inhibited the production of complement-fixing antibodies.
Hans Selye described the stress syndrome in 1936 [8]. He established that the hypothalamus–pituitary–adrenal (HPA) axis was involved in mediating the stress response, which could be induced by various “nocuous” agents. The adrenal gland was stimulated by stress and the thymus and lymph nodes were involuted by glucocorticoids (GCs). He also observed gastrointestinal lesions [9]. Subsequently, Selye concluded that the stress response was a host defense reaction, which he called the “general adaptation syndrome” [10]. These observations clearly implied that the pituitary gland plays an important role in host defense. While I was in Selye’s laboratory, I decided to learn more about the interaction of the pituitary gland and of the brain with immune host defense. We established that adaptive immune (ADIM) function is regulated by pituitary hormones: for example, prolactin (PRL) and growth hormone (GH) stimulated immune function, whereas the HPA axis was inhibitory and antagonized the stimulatory effect of PRL and GH. Human placental lactogen (HPL) was as efficient as PRL or GH in restoring ADIM function in this system. Initially we used young (100 g birth weight) hypophysectomized (Hypox) rats, and the antigen was always injected without adjuvant 7–10 days after operation. Treatment of intact animals with the dopamine-agonist drug bromocriptine was as effective as Hypox in suppressing ADIM function [11–13]. Others showed that immune-derived cytokines (CTKs) are capable of stimulating the HPA axis. This discovery revealed that ADIM function is regulated by the CNS and that the HPA axis, but also PRL and GH, receive feedback regulatory signals from immune-derived CTKs [14,15].

Ader and co-workers, and Gorczynski and Kennedy showed that immune function is subject to Pavlovian conditioning [16,17]. These observations imply that the host is able to activate immune defense when danger is anticipated. However, the mechanisms involved in immune conditioning are complex and poorly understood [18].

1.2 Recent Developments in Neuroimmune Biology

1.2.1 The Concept of Neuroimmune Biology

During the past three decades, the field of neuroimmune interactions grew steadily, and has now evolved into a sophisticated and credible area of biology. A number of terms have been proposed to name this multidisciplinary science. Because this science deals with the physiology and pathophysiology of higher organisms, in 2000 we coined the term neuroimmune biology (NIB) [19], which has been accepted by the scientific community. It is clear that the CNS, the endocrine system (ES), and the immune system (IS) form a regulatory circuit, which integrates, coordinates, and regulates all functions in higher organisms from conception till death.

By now a lot of information has been accumulated about the recognition systems and signaling in this neuroimmune supersystem (NISS) [20]. The mediators for signaling are hormones, neurotransmitters, neuropeptides, CTKs, and chemokines, which are shared within the NISS. Indeed, sharing occurs throughout the entire organism, which makes it possible to signal efficiently within the animal or human being.
Additional communication is mediated by innervation and by recirculating leukocytes (Figure 1.1). Forward and feedback (or stimulatory and inhibitory) signaling is the rule. Under physiological conditions (homeostasis), all vital activities and values are maintained at “normal” or physiological levels.

Pathophysiological conditions (allostasis) occur in response to various insults by pathogenic microbes and agents. Pathological events may also be caused by endogenous abnormalities, defects, and malfunctions. Acute febrile illness leads to the acute phase response (APR), which is initiated by CTKs (e.g., interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), IL-6) released from the innate immune (INIM) system. Under these conditions, hypothalamic corticotropin-releasing hormone (CRH) and vasopressin (VP) stimulate the HPA axis and also induce sympathetic outflow. GCs and catecholamines (CATs) stimulate suppressor/regulatory T lymphocytes (Tsrs) and amplify INIM. Tsrs in turn suppress ADIM function. The synthesis of acute phase proteins is rapidly amplified in the liver, as is the synthesis of natural antibodies in specialized CD4+ B lymphocytes. The CNS, bone marrow, liver, and leukocytes are activated, whereas the function of other organs is reduced and catabolism prevails. Fever is a constant symptom of APR. APR is analogous to Selye’s general adaptation syndrome. It is an emergency host defense reaction against diverse pathogenic agents, which leads to healing in the overwhelming majority of cases [21–28].

In the absence of the HPA axis, there is excessive CTK response after immune activation/inflammation, which may have lethal consequences for the host. Even immunization with complete Freund’s adjuvant, which contains mycobacterial antigens (acts on toll-like receptor-4 (TLR-4)), would kill Hypox animals (unpublished results). Further, the sensitivity to bacterial lipopolysaccharide (LPS, also acts on TLR-4) of adrenalectomized (ADRX) mice is elevated by 500–1,000 times when compared to normal animals. TNF levels, induced by the same dose of LPS, were 60 times higher in ADRX mice than in intact controls. Dexamethasone restored the resistance of ADRX mice to LPS [29]. These experiments indicate that stimulation of the INIM system in the absence of the HPA axis results in excessive CTK production, which could kill the host. Further, Korneva and Novikova showed that after immunization, the C-fos gene is expressed in the hypothalamus. C-fos expression indicated that the cells were activated in the hypothalamus after immunization, which was true for several different types of antigens [30]. These experiments demonstrate that there is mutual and continuous regulatory interaction between the hypothalamus and the IS during immunization.

VP is the dominant hypothalamic regulatory hormone during chronic inflammatory conditions. VP stimulates both PRL and the HPA axis, and thus it is capable of maintaining both the ADIM and INIM systems in homeostasis and harmony. Indeed, our experiments revealed that VP maintains adaptive immunocompetence. On this basis it has been suggested that recovery from disease is regulated by VP [28].

1.2.2 INIM–ADIM Interactions

It is very well established that monocytes and macrophages, which are related cells, play a fundamental regulatory role in the INIM system and also present antigen to
**Figure 1.1 The NISS.**

This figure shows the major systemic neuroimmune regulatory pathways during homeostasis. In the center, two cells are interacting, which is the rule for all tissues and organs, where stromal and parenchymal cells interact. As an example, we use here the phenomenon of antigen presentation to a naïve T lymphocyte, which is an ADIM cell, by a macrophage, a cell of the INIM system. Macrophages are phagocytic: they recognize infectious agents and foreign materials via innate immune receptors (INIRs), engulf the microbe/foreign material, digest it, and present peptides (epitopes) of the antigen on their MHC-II surface molecules to T cells. T lymphocytes proliferate upon exposure to the antigen (phagocytic pathway). Monocytes, dendritic cells, and B lymphocytes are also antigen-presenting cells. Further, all nucleated cells can present cytosolic antigens to T lymphocytes (cytosolic pathway).
T lymphocytes within the ADIM system. The related dendritic cells are regarded as “professional” antigen-presenting cells of the ADIM system. Monocytes recirculate in the blood and macrophages are distributed in tissues throughout the entire host organism. These phagocytic cells recognize infections and other noxious agents through their INIRs, engulf the microbe/foreign material, digest (process) it, and present peptides via their major histocompatibility-II (MHC-II) surface antigens to T lymphocytes of the ADIM system. This is the phagocytic pathway of antigen presentation. Therefore, monocyte–macrophage activation within the INIM system almost inevitably activates T cells of the ADIM system, so that the entire NISS is mobilized to fight the intruding pathogen/noxious agent. This conclusion is further supported by the fact that all nucleated cells are capable of presenting cytosolic antigen via their surface MHC-I to T cells [20–22].

Once the ADIM system is activated, cytotoxic and helper effector T cells are produced. As a rule, antibody formation also follows after B lymphocytes present antigen to helper T cells and such T cells stimulate B lymphocytes to form antibodies. Immunoglobulins D (IgD) and M (IgM) are formed as surface immunoglobulins by B cells. After an immune response, IgM is secreted first, and later the same B cell may switch to making IgG, IgA, or IgE, depending on the regulatory environment of the cell. All the different classes of antibodies made by one B cell are specific for a single epitope of the antigen to which the cell was responding [21,22].

IgM, IgG, and IgA are able to fix complement after combining with the antigen. Complement is an enzyme system belonging to the INIM system. Moreover, all leukocytes express receptors for fraction C (Fc) the binding of antibodies to their surface, and use such antibodies for better identification of pathogens/noxious agents. Here INIM cells use ADIM antibodies. The complement system has three pathways for activation: by

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**Figure 1.1 (cont.)**

The primary immune response is dependent on pituitary PRL and GH. These pituitary hormones regulate cell growth throughout the entire organism according to this principle. IGF-1 acts like a second messenger in most situations. The HPA axis inhibits ADIM and stimulates NATIM. CATs support the immunoregulating activity of the HPA axis.

Type I CTKs produced within the IS will gradually take over the role of PRL/GH, and the secondary response gradually becomes independent of pituitary hormones. Memory T cells, B lymphocytes, and NK cells survive severe illnesses by HP and regenerate immune function during healing. The hypothalamic hormone VP stimulates PRL and the HPA axis and thereby regulates the process of healing, and maintains ADIM and NATIM in homeostatic harmony.

This figure illustrates that the entire host organism communicates and cooperates with the central regulatory network. Innervation, hormones, CTKs, chemokines, and recirculating leukocytes serve as communication pathways. These pathways of communication are open at all times. No immune response, inflammation, or infection can occur in any tissue/organ in the body without signaling the hypothalamus, PVN about such events. Therefore, infection, inflammation, and immune reactions are under continuous surveillance within the NISS. During homeostasis, this system protects us very efficiently, without disturbing any physiological functions in the body.

*Source:* Adapted from Ref. [124].
antigen–antibody complexes (ADIM, classical pathway); by microbes (INIM, alternative pathway); and by lectins (INIM) [21,22].

We proposed that the Tsrs is a member of the innate INIM. The evidence comes from the activation of Tsrs by CATs and GCs. These hormones activate INIM cells and suppress ADIM cells [31]. During severe trauma or infectious disease, APR develops whereby ADIM is suppressed and the INIM system takes over command for the highly coordinated and very intensive immunological battle to save the host organism [26–28]. The INIM system, which is with us at birth, initiates most of the immune responses against microbes and other noxious agents; it regulates adaptive immunity by antigen presentation and by Tsr cells that suppress ADIM. Therefore, the INIM system is the first to protect the host, and it continues to protect the host till the last moment of survival [27].

1.2.3 The Role of Innervation, Neurotransmitters, and Neuropeptides

Lymphoid organs, such as the spleen, thymus, and bone marrow, are innervated [32]. This fact indicates that the CNS is in permanent contact with the IS. In addition, neurotransmitters and neuropeptides regulate inflammation and immunity [33]. The mediators involved may stimulate or inhibit immune and inflammatory processes. Substance P, neurokinin A and B (collectively known as tachykinins), calcitonin gene-related peptide (CGRP), and vasoactive intestinal peptide (VIP) are pro-inflammatory mediators capable of eliciting an inflammatory response. These mediators also enhance various immune responses. In contrast, somatostatin and galanin are anti-inflammatory and immunosuppressive mediators [31,33,34].

Alpha-adrenergic mechanisms stimulate immunity and inflammation, whereas beta-adrenergic mechanisms inhibit inflammation, allergy, and asthma and exert an immunosuppressive effect. Cholinergic mechanisms are both anti-inflammatory and immunosuppressive [35–37].

1.2.4 Hormonal Immunoregulation

Adaptive immunity is under pituitary control. Growth and lactogenic hormones (GLH) stimulate adaptive immunocompetence, and the HPA axis is inhibitory. The secondary antibody response is partially independent of the pituitary. This observation suggests that memory cells are able to operate independent of pituitary hormones [38]. GCs and CATs promote natural immunity (NATIM) and inhibit the ADIM system by the stimulation of suppressor/regulatory T cells. Numerous other hormones, CTKs, neurotransmitters, and peptides also modulate immune function [28].

Innate immunity functions in the absence of the pituitary gland. From our experiments, it appears that VP stimulates the INIM system, but this result remains to be confirmed. The HPA axis exerts an important moderating effect on CTK production. Excessive CTK production can occur after INIM stimulation if the HPA axis is not functional (see Section 1.2.1).
1.2.5 Immune–Hypothalamic Feedback Signals

Healthy people and animals show only trace amounts of CTKs in their serum, which is not sufficient to deliver immune-derived signals to the hypothalamus. Sensory nerves fulfill this function, as they are stimulated locally by CTKs released in immune organs and at other sites where immune/inflammatory reactions occur. Sensory nerves transmit information about inflammation and immune reactions to the hypothalamic paraventricular nucleus (PVN), which is the center regulating ADIM. The vagus was also shown to have similar capabilities. However, recent results indicate that the vagus contains sensory nerve fibers, which serve as immuno-sensors and transmit information toward the hypothalamus [33,39,125].

1.2.5.1 Feedback by CTKs

CTKs act on the brain; on nerve terminals; on the pituitary, adrenals, gonads, thyroid, epithelium, endothelium, leukocytes, and fibroblasts; and more. In fact, the entire organism uses CTKs to communicate (Figure 1.1). On this basis it is possible to suggest that CTKs are able to activate/suppress the major regulatory circuits in the body, at any level, in case of acute febrile illness.

The stress hormones PRL and GH are elevated soon after LPS injection and during the initial phase of stress [13]. Subsequently, it was discovered that during acute stress, both adaptive and INIM functions are significantly boosted within peripheral tissues, which leads to long-lasting immunity. This was termed the physiological stress response [40]. The enhancement of adaptive immunity is fully justified in this case, as both GH and PRL levels are increased; so is the HPA axis, which explains the augmentation of NATIM.

During acute febrile illness, the concentrations of IL-1β, TNFα, and IL-6 are elevated in the serum, which initiates APR. Later, during the course of the disease, numerous other CTKs will contribute. Additional CTKs that were found to activate the HPA axis are IL-2, -11, -12, and interferon (IFN) [13]. Gp 130 CTKs, such as ciliary neurotropic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin, IL-11, and cardiolipin, potentiated the activation of the HPA axis by IL-1 [39,41]. Leptin plays a role in regulation of the gonadotropic axis [42].

The CTKs IL-1, IL-2, and IL-6 stimulate the pituitary release of PRL, whereas IFN-γ and endothelin-3 are inhibitory [43]. In rats, administration of exogenous IL-1β stimulated the expression of IL-1β mRNA in the hypothalamus by 99%, but not that of IL-6. IL-1 also significantly elevated the plasma levels of adrenocorticotropic hormone (ACTH), PRL, corticotropin, and cortisol production in the adrenal glands [44]. Rats treated with IFN-γ for 5 days (10 IU/kg body weight) showed a 43% increase in circulating PRL [45]. Two of the most potent cytokines regulating anterior pituitary cell function are LIF and IL-6, which belong to the CTK family that uses the common gp130 signal transducer. IL-11 and CNTF exerted a similar stimulatory effect on GH mRNA expression in somatotropic monolayer cell cultures from acromegalic tumors, but these CTKs had no significant influence on GH secretion. CNTF stimulates PRL secretion in lactotropic monolayer cell cultures from patients with prolactinoma. In monolayer cell cultures from normal rat anterior
pituitary, IL-11 and CNTF had no significant effect on the release of either GH or PRL, or on GH mRNA. However, when the cells were cultured in aggregates, in which the three-dimensional structure of the cells is reconstituted, both CTKs, in doses at which they had no effect on monolayer cultures, significantly stimulated both PRL and GH secretion [46].

The presence of L-NAME (1 mM), an inhibitor of NO synthase (NOS), in the incubation medium significantly blunted the inhibition of PRL release produced by TNF-α (50 ng/ml) in female rats. TNF-α increased nitrite release to the incubation medium. The activity of NOS was significantly enhanced when anterior pituitary cells were incubated with TNF-α for 8 hours or more. Also, TNF-α induced iNOS gene expression in anterior pituitary cells [47].

Stromal cell-derived factor-1 (SDF1), via its receptor CXCR4, stimulated the proliferation of the pituitary adenoma cell line GH4C1, and released both PRL and GH through a complex network of intracellular signals [48].

C3a receptors are expressed in pituitary hormone-secreting and nonhormone-secreting (folliculostellate) cells. Both C3a and C3adesArg (a noninflammatory metabolite) stimulate pituitary cell cultures to release PRL, GH, and adrenocorticotropin. Serum levels of these hormones, together with adrenal corticosterone, increase dose dependently with recombinant C3a and C3adesArg administration in vivo [49].

Erythropoietin administration to patients with amyotrophic lateral sclerosis caused a significant reduction of serum PRL levels, maximal 60 minutes after administration [50].

GCs and CATs stimulate IL-4, IL-10, and transforming growth factor (TGFβ), which exert negative feedback on excess CTK production during APR [51]. These CTKs are produced by Tsrs [28].

Several mechanisms have been proposed for conveying the CTK signal across the blood–brain barrier (BBB). Once the signal is transmitted across the BBB, the signal travels through neural pathways as follows: area postrema→nucleus tractus solitarius→ventrolateral medulla→paraventricular nucleus, which initiates HPA axis responses. Descending pathway: PVN→brainstem cell groups→thoracic spinal cord preganglionic neurons→via sympathetic projections to the end organs such as the thymus and spleen [52].

Several of the effects of pro-inflammatory CTKs exerted in a “healthy” brain are amplified in the CNS by the following mechanisms: (a) peripheral CTKs, such as IL-1, have the capacity to elicit their own synthesis in the brain; and (b) a sustained increase in neuronal activity also induces production of physiologically significant amounts of these mediators. The brain-borne IL-1 and IL-6 fulfill physiological roles when their production is not the result of pathological events in the CNS. These CTKs stimulate the HPA axis, and they are involved in physiologic brain mechanisms such as synaptic plasticity, memory formation, and the control of glucose homeostasis [53]. CTKs are now being investigated also for their synaptic and inflammatory action in the CNS. These proteins and their receptors can be synthesized in the brain by glial and neuronal cells and contribute to two main types of action: modulation of neuronal excitability and local inflammatory responses [54].
1.2.6 Neurogenic Inflammation: Neurokinins, Tachykinins, Mast Cells

There is much evidence for the regulation of inflammation and immunity by neuropeptides. Chemosensitive afferent nerves expressing the capsaicin/TRPV1 receptor play important roles in the initiation and modulation of vascular, inflammatory, and immune reactions. These nerves exert their efferent/local regulatory functions primarily via the release of VIP, CGRP, and pain causing substance (SP) from their terminals upon antidromic or orthodromic stimulation. The CTKs induced in the neural microenvironment by tissue injury or inflammation promote the release of pro-inflammatory sensory neuropeptides and lead to augmentation of the inflammatory response. In turn, the release of anti-inflammatory peptides from sensory nerves or from inflammatory cells results in inhibition of the inflammatory vascular reactions [33].

Mast cells are innervated and thus serve as sensory organs for the CNS and function as effector cells in neurogenic inflammation. The complement split products C3a and C5a (anaphylatoxins) cause mast cell discharge and thus signal the brain about complement activation anywhere in the body. Similarly, the inflammatory mediator bradykinin signals the brain by the sensory nerve pathway [31,34].

1.2.7 The Brain as an Immunocompetent Member of the INIM System

TLRs of innate immunity are expressed by neurons and also by their dendrites [55]. This discovery implies that the nervous system expresses INIRs, is capable of sensing directly the penetration of infectious and noxious agents into tissues, and can respond immediately by inducing neurogenic inflammation and/or by the activation of other immune compartments. Thus, the CNS is an immunocompetent organ, as it is capable of recognizing an antigen directly and responding to it in a polyspecific manner characteristic of innate immunity [31].

1.3 The NISS

In addition to the CNS, all leukocytes express TLR [55]. TLR is expressed in the pituitary gland [56], in the adrenal gland [57], in the liver [58], in mucosal epithelial cells [59], in endothelial cells [60], in vascular smooth muscle [61], and also in the cornea [62]. These observations indicate that the entire body participates in INIM reactions. The CNS is capable of directly sensing infectious agents through TLR, and possibly through other INIRs, and can react instantaneously by causing inflammation and mobilizing immune defense mechanisms. Similarly, the pituitary produces propiomelanocortin (POMC) in response to LPS (TLR4 is involved), mucosal epithelial TLR participates in inflammation and responds to pathogens, corneal TLR was found to fight infection, and endothelial TLR was observed to play important roles in homeostasis of the heart [55–62]. Therefore, in addition to participating in NATIM, TLRs fulfill important physiological functions. This is true for CTKs and also for cellular elements of the IS, whether or not natural or ADIM cells are considered [54].
At this stage we may conclude that the entire host participates in APR to noxious stimuli. These stimuli could be immunogenic, but need not be; they may be noxious agents that cause tissue injury, but need not be: it is enough if the host simply senses or anticipates danger. INIM mechanisms have a tremendous capacity to defend the host from physical, chemical, and biological agents that may be harmful to the host. The receptors involved sense antigen presence, inflammation, and injury (e.g., CTKs, nerves), and also receive input from the CNS (e.g., conditioning). The *ADIM system*

![Diagram showing major regulatory circuits in NISS.](image)

**Figure 1.2 Major regulatory circuits in NISS.**

There are hierarchical regulatory circuits in the NISS, which are superimposed on each other. For example, regulatory signals from a higher circuit (such as the CNS) dominate the signals from lower circuits (e.g., pituitary). Pituitary hormones control the function of the adrenals, gonads, thyroid, liver, and other tissues that secrete IGF. CTKs, which are tissue...
is largely, but not entirely, concerned with immunogenic agents. Here antigenic receptors dominate. This system is also subject to conditioning. Therefore, the activation of the INIM system, with or without adaptive immunity, is responsible for the profound host resistance to diverse noxious agents. The NISS is capable of mobilizing the entire organism in the interest of host defense in emergency situations (through APR). The same NISS performs important physiological functions, as is becoming apparent with further research [53,54].

1.3.1 **Hierarchy in Regulation**

The regulatory portion of the NISS consists of *hierarchical regulatory circuits*, which are superimposed on each other. For example, regulatory signals from a higher circuit (such as the CNS) are capable of dominating the signals from lower circuits (e.g., pituitary). As we know, pituitary hormones control hormones secreted by the adrenals, gonads, thyroid, liver, and other tissues that secrete insulin-like growth factor (IGF). CTKs, which are tissue hormones, represent another regulatory circuit. Our experiments indicate that if a higher regulatory circuit fails, a lower circuit will gain dominance and take precedence over lesser signals that still exist in the system; this way, *INIM function is maintained continuously under any circumstance* [63]. This means that the INIM system continues CTK production during acute illness/injury under any conditions, and these CTKs will signal any regulatory circuits of the host organism that remain active. Hence, the INIM system never stops protecting the host organism (Figure 1.2).

**Figure 1.2 (cont.)**

hormones, represent another regulatory circuit. If a higher regulatory circuit fails, a lower circuit will be stimulated via INIRs or by CTKs and thus will continue to regulate the lesser circuits that still exist in the system. *INIM function is maintained continuously under any circumstances* [65]. CTK production continues during acute illness/injury through the INIM system. The INIM cells are capable of performing on their own if necessary; the INIM system never stops protecting the host organism.

INIRs are present throughout the entire organism. Therefore, when infectious/noxious agents are contacted, every regulatory circuit is capable of activating a response independent of the other circuits if necessary. For instance, LPS (which activates TLR4) stimulated ACTH and GC secretion in PVN-lesioned rats [67]. LPS also stimulated natural antibody formation in B lymphocytes, and is able to stimulate many other systems in the body, including the CNS [22].

Abbreviations to Figures 1.1 and 1.2.

ACTH = adrenocorticotropic hormone, ADIM = adaptive immune, ALD = aldosterone, APP = acute phase proteins, CAT = catecholamines, CTK = cytokine, CRH = corticotropin releasing hormone, GC = glucocorticoids, GH = growth hormone, GMCSF = granulocyte–macrophage colony stimulating factor, HP = homeostatic proliferation, HPA = hypothalamus–pituitary–adrenal axis, IGF-1 = insulin-like growth factor 1, IL = interleukin, INIM = innate immunity, INIR = innate immune receptor, INS = insulin, PVN = paraventricular nucleus, PRL = prolactin, NATIM = natural immunity, SH = steroid hormone, MHC-II = major histocompatibility complex-II, NGF = nerve growth factor, NK = natural killer, NPEP = neuropeptide, TNF = tumor necrosis factor, T4 = thyroxin, Tsr = suppressor/regulatory T cells, TSH = thyroid stimulating hormone, VD3 = vitamin D3, VP = vasopressin.
As discussed earlier, most if not all tissues express TLRs. Therefore, if a higher regulatory system, such as the CNS, is injured or paralyzed, for instance, TLR could still signal the system through lower circuits such as the pituitary, adrenals, thyroid, and gonads and thus stimulate host resistance. TLR signaling is also possible at the CTK or even at the cellular level, as all leukocytes express TLR. Thus, even extensive injury to the host organism cannot prevent the INIM system from providing a defense, as TLR could directly signal the immunocytes to respond and they would respond with the function they normally perform. For instance, LPS, which activates TLR4, stimulates natural antibody formation, and is able to stimulate many other systems, including the CNS [22]. The wide spectrum of cells, tissues, and organs that are stimulated by LPS in vivo indicate the extent to which the NISS can be activated by TLR4. Is it possible that during acute illness TLR, or rather INIRs, are able to substitute for regulation by the CNS and in this way promote host defense under circumstances in which the central regulatory system has failed to do so? Indeed, LPS may be used as an immunological adjuvant, and detoxified LPS was found to significantly increase survival in lethally injured animals and patients with severe disease [64].

The CTK response by the INIM system, in rats stimulated with complete Freund’s adjuvant and brain tissue (to induce experimental autoimmune encephalitis (EAE), was not eliminated by anterior pituitary lobectomy (AL), neurointermediate and posterior lobectomy (NIL), or Hypox, although significant changes were seen in the CTK response. AL rats showed significant elevation of IL-1, -2, -6, and -10, whereas NIL animals had the lowest levels of IL-1, -2, -10, -12, and IFN. These results showed that lack of the HPA axis leads to increased CTK production, whereas the intermediate/posterior lobe appeared to be stimulatory for the CTK response. On this basis and that of previous experiments [28], we assumed that VP stimulates the CTK response. However, in Hypox animals CTK levels remained normal in all experimental groups examined. We explain this finding by the activation of lower regulatory circuits. For example, if pituitary hormones are removed completely from the animal, CTK levels are maintained at normal levels by other regulatory circuits, which could be neurotransmitter–peptide circuits, stimulatory and inhibitory CTK circuits, or even cell-to-cell regulation. It should be noted that macrophage numbers in the spleen were proportional to the levels of CTKs in the serum [63].

Elenkov and colleagues lesioned the PVN in rats and observed that such rats responded to LPS. Evidence was obtained that 4 hours after treatment, LPS was able to activate the hypophysial–adrenal system in the absence of hypophysiotrophic neuropeptides of paraventricular origin. It was suggested that, in vivo, LPS could have a direct effect on the pituitary gland or that it acts through a nonparaventricular pathway to activate the HPA axis [65].

These experiments show that elimination of the PVN does not stop the INIM system from responding, either. The PVN is the center of regulation for ADIM function. However, in this case LPS can act directly on TLR4 of the pituitary gland: thus, the HPA axis is still activated, in spite of the regulatory handicap, and GCs can exert their all-important moderating/suppressing action on excess CTK production during endotoxin shock [29].
1.3.2 **Hierarchy of Cellular Receptors**

Hierarchy may also be observed amongst cellular receptors: for example, cell surface receptors operate through long signal-transmission pathways. In contrast, steroid–thyroid hormone and vitamin D receptors are nuclear regulatory proteins. Nuclear receptors are very powerful and are capable of overruling membrane receptors. This is the reason for the tremendous regulatory influence of the HPA axis. Some peptide hormones were proposed to release nuclear regulatory peptides (e.g., PRL) during intracellular processing, but the existence of this phenomenon remains controversial.

At the cellular level, adhesion molecules that deliver cell-to-cell signals are dominant for delivering signals that are specific for the position of a single cell in the body. Such positional signaling consists of adhesion and CTK signals that decide the function of a given cell in the tissues [66]. Each cell is bombarded by a myriad of signals at any time. Activated cells cap the occupied receptors to one pole of the cell where receptor interaction will take place. Positive signals to the cell are transmitted by phosphorylation of receptors by protein kinases, whereas negative signaling involves rapid dephosphorylation of the activated (phosphorylated) sites by phosphatases (Ship). The balance of positive and negative signals decides if the cell will be activated or remain inactive [67].

1.4 **Immunological Memory**

We observed that the secondary antibody response was partially independent of the pituitary. In Hypox rats we obtained a secondary antibody response that was of similar titer to the primary response [38]. This observation shows that memory cells of the ADIM system do not require pituitary hormones for survival and function; they are autonomous. However, their ability to recruit naïve lymphocytes to expand the secondary response was impaired. This observation also showed that memory cells survived the severe adaptive immunodeficiency present in Hypox animals. Others also observed that in immunodeficient (lymphopenic) hosts, memory T cells not only survive, but also undergo antigen and MHC-independent homeostatic proliferation (HP), the biological significance of which is to preserve and regenerate the immunocompetence of the host after severe, debilitating diseases.

1.4.1 **HP of Lymphocytes**

In lymphopenic/immunodeficient hosts, naïve and memory CD4+ and CD8+ T cells, naïve B cells, and natural killer (NK) cells undergo HP. This proliferation is MHC and antigen independent, and B cells share many of the inductive and regulatory characteristics established for naïve and memory T cells and NK cells [68–70].

Naïve T cells can be induced to undergo HP of variable speed with a few members of the common gamma-chain (gamma c) (CD132) family of CTKs. The speed of proliferation depends on the levels of the particular CTK involved [71].
IL-7 was required for homeostatic expansion of naïve CD8+ and CD4+ T cells in lymphopenic hosts and for CD8+ T-cell survival in normal hosts [72]. Unlike naïve T cells, HP memory T cells were largely MHC independent and memory CD8+ cells could utilize either IL-7 or IL-15 to undergo HP. In the absence of both IL-7 and IL-15, HP failed to occur. HP of memory CD4+ cells is independent of IL-7 and IL-15 (and also of IL-4) [73]. Although HP memory cells responded directly to IL-7 and IL-15, naïve T cells required costimulation by dendritic cell-derived CTKs, and selectively respond to IL-4 [74]. All human CD8+ T-cell subsets had the ability to respond to IL-15, which suggests a generic influence of this CTK on CD8+ T-cell homeostasis in humans [75]. IL-15 was important for sustained CD8 T-cell proliferation and accumulation in a lymphopenic setting, as revealed by truncated proliferation in IL-15 (−/−) hosts. At the same time, IL-12 enhanced HP by acting directly on the CD8 T cells, independent of IL-15, suggesting that there are distinct pathways by which CTKs can regulate HP [76]. A novel form of proliferation occurred when naïve T cells encountered raised levels of IL-2 and IL-15 in vivo. In this situation, CD8(+) T cells underwent massive expansion and rapid differentiation into effector cells, thus closely resembling the T-cell response to foreign antigens. However, the responses induced by IL-2/IL-15 were not seen in MHC-deficient hosts, implying that the responses were driven by self-ligands. Hence, HP of naïve T cells can be either slow or fast, with the quality of the response to self being dictated by the particular CTK (IL-7 versus IL-2/IL-15) concerned [77].

In murine allogeneic bone marrow transplantation (BMT) models, two populations of mature donor T cells could be distinguished: (a) alloreactive T cells with decreased expression of CD127 (IL-7 receptor alpha chain) and (b) nonalloreactive T cells, which express CD127 and undergo HP. IL-7 administration increased the HP of nonalloreactive T cells, but had no effect on alloreactive T cells or the development of graft-versus-host disease [78]. There is a prominent, nonredundant role for IL-7 in supporting basal HP of CD8(+)T(M). We propose that homeostatic control of antiviral CD4(+) and CD8(+) T-cell memory is fundamentally similar and characterized by quantitative, rather than qualitative, differences [79]. IL-7 is a CTK produced predominantly by stromal cells of the thymus and bone marrow and is essential for lymphopoiesis. IL-7 is of particular importance in lymphopenic conditions. Its expression is up-regulated in a number of lymphopenic conditions, including marrow ablation prior to BMT, marrow suppression following chemotherapy, and human immunodeficiency virus (HIV) infection. Plasma IL-7 levels inversely correlate with CD4+ T-cell counts in these conditions. Animal models suggest that IL-7 improves immune reconstitution through increasing thymic output and, perhaps more importantly, through antigen-independent homeostatic-driven proliferation in the periphery [80].

Lymphopenia-induced proliferation depends on low-affinity MHC/self-peptide complexes and on IL-7 T cells proliferating in lymphopenic hosts. These cells do not exhibit a unique gene-expression profile, but instead rely on “traditional” signals for this antigen-independent proliferation, which ultimately results in differentiation to “authentic” memory cells [81]. Memory CD8 T cells retain the ability to respond to dendritic cell-mediated stimulation after adoptive transfer into either TAP(-/-) (MHC class I-deficient) or wild-type mice. Surprisingly, naïve CD8 T cells, which
fail to undergo HP and erode in number in the absence of MHC class I, also retain the ability to respond to dendritic cell-mediated antigenic stimulation for at least 1 week after transfer into TAP(−/−) mice [82]. Resting memory CD4(+) cells are dependent on signals from contact with IL-7 and IL-15, but not MHC class II, for their survival and intermittent HP [83].

Thymus and oncostatin M (OM)-dependent HP extrathymic pathways of T cells show how the division of labor between primary and secondary lymphoid organs influences the repertoire and homeostasis of T lymphocytes [84]. CD8(+) T cells express thymic stromal lymphopoietin (TSLP) receptors, and TSLP activates both STAT5 and Akt and induces Bcl-2 in these cells. Correspondingly, TSLP increases CD8(+) T-cell survival in vitro as well as in wild-type and T-depleted mice in vivo, without altering the HP of these cells. Moreover, TSLP can maintain CD8(+) T cells even in the absence of IL-7 [85].

Recent work has shown that two members of the gamma c family of CTKs, IL-7 and IL-15, govern homeostasis of memory T cells. It appears that the two types of memory cells do not display identical homeostatic requirements. For antigen-specific memory CD8+ T cells, IL-7 is primarily important for survival, whereas IL-15 is crucial for their background proliferation. For memory CD4+ T cells, IL-7 has an important role, whereas the influence of IL-15 is still unclear [86]. The activation of STAT5 is the primary mechanism underlying both IL-7- and IL-15-dependent HP of naive and memory CD8(+) T cells and IL-2-dependent development of CD4(+) CD25(+) regulatory T cells (Tregs) [87]. There are distinct functions for IL-7 and IL-15 in T lymphocyte development and homeostasis, and stringent regulation of these processes by suppressor of cytokine signaling-1 (SOCS1) [88].

Dividing HP memory T cells were present in both lymphoid and nonlymphoid tissues. However, the bone marrow was the preferred site for proliferation and contained a major pool of the most actively dividing memory CD8 T cells. Adoptive transfer studies indicated that memory cells migrated through the bone marrow and divided there preferentially [89]. Homeostatic expansion permits T cells to reenter the thymus and deliver antigen in a tolerogenic fashion [90].

In sublethally irradiated lymphopenic mice, HP T lymphocytes were found to arise in neonatal lymphopenia (CD8+ T cells) [91], which inhibited melanoma and colon carcinoma growth; this protection was effective even against established tumors (T cells making IFN) [92]. HP T cells generated autoimmune (IL-21 regulated T cell turnover) [93]. HP T cells appeared 14 days after burn injury. These cells were CD8+ CD44+ IL-7R T cells, which induced enhanced allogeneic skin graft rejection in unburned recipient mice. Treatment with GC abolished both the late homeostatic accumulation of CD8+ memory-like T cells and enhanced the pro-inflammatory CD8+ T-cell response, but not the late enhanced CD8+ anti-inflammatory response [94].

HP T cells transformed chronic rejection to acute rejection of a single MHC class II-mismatched kidney allograft. Such T cells consistently caused reliable rejection even when bona fide memory T cells could not. These functional changes are long-lasting and not restricted to the acute phase of HP [95]. HP cells obtained from T-cell knockout (KO) mice mounted relatively normal acute CD8 T-cell responses to
lymphocytic choriomeningitis virus, but with altered T-cell receptor (TCR) repertoires, and they became functional memory cells capable of recall responses [96].

Current evidence implicates HP in autoimmune diseases and transplant rejection, and suggests that it may represent a barrier to tolerance in protocols that use T-cell depletion [97].

Clonal competition is a component of homeostasis that may contribute to selection of the peripheral T-cell repertoire [98]. Tregs play a major role in the control of HP [99]. HP memory CD8(+) T cells controlled bacterial infection as effectively as “true” memory CD8(+) T cells, but their protective capacity required the presence of CD4(+) T cells during HP. The necessity for CD4 help was overcome, however, if the HP memory CD8(+) T cells lacked expression of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand; also called Apo-2L). Thus, like conventional CD8(+) memory T cells, the protective function of HP memory CD8(+) T cells shows dependence on CD4(+) T-cell help [100].

Similar factors are required for production of protective memory CD8 T cells via both homeostatic and conventional pathways. Induction of protective HP memory CD8 T cells requires CD4(+) T-cell “help,” which we show is antigen nonspecific yet requires CD40L–CD40 interactions with host cells. The functional competence of HP memory CD8 T cells also requires release of endogenous bacterial components (which follows irradiation-induced lymphopenia), potentially mimicking the role of adjuvants in conventional immune responses. Lymphopenic environments lacking these key factors support similar CD8 T-cell HP and the acquisition of memory phenotype, yet the HP memory cells generated are defective in pathogen elimination [101]. HP memory T cells provide protection without compromising the true-memory population. Differences in HP and true-memory T cells may reveal the basis of competition for limited resources within the memory T-cell compartment [102].

The significance of this phenomenon is that memory T lymphocytes are able to survive major disasters and bring back immune function once the crisis is over. During APR, the ADIM system is suppressed by Tsrs and GCs, but not destroyed. The possibility of recovery is present and ADIM function recovers after acute illness. We proposed that healing is under hypothalamic regulation, with VP as the principal regulator [28].

1.5 Autonomy and Redundancy

Immune reactions may be induced in vitro, which argues for the autonomy of immune function. Indeed, the fully differentiated cells of the INIM system can function under diverse conditions with little regulatory input from the host. When it comes to adaptive immunity, the primary response is dependent on pituitary hormones that must be provided. However, the secondary response could develop in the absence of such hormones because memory cells survive hypophysectomy [38].

GH, PRL, erythropoietin, thrombopoietin, and leptin all have structures similar to type I CTKs, and their receptors also belong to the same receptor superfamily. Short-chain type I CTKs include IL-2, -3, -4, and -5; granulocyte macrophage
colony-stimulating factor (GM-CSF); IL-7, -9, -13, and -15; monocyte-CSF (M-CSF); and stem cell factor (SCF). Long-chain hormone/CTKs include GH, PRL, erythropoietin, thrombopoietin, leptin, IL6-11, LIF, OM, CNTF, cardiotrophin-1 (CT1), and G-CSF. Moreover, GH and PRL share the Jak3/Stat5(A,B) pathway of signal transduction with type I CTKs [103–105]. This similarity raises the possibility that GLH could in fact substitute for type I CTKs during immune function.

In contrast with CTKs, which are not present in the circulation in significant quantities during health, PRL and GH are freely available in the serum. Further, the development of naïve lymphocytes in the bone marrow, thymus, and spleen is dependent on pituitary PRL and GH. It was also demonstrated that the primary antibody response in young (100 g body weight) rats was dependent on pituitary hormones when immunization was carried out without the use of immunological adjuvants. This was true for cell-mediated, humoral immunity and autoimmune reactions. The iron levels of anemic Hypox animals were also restored to normal by PRL, GH, and placental lactogen [13,106,107]. Moreover, serum PRL regenerates in young (100 g body weight) Hypox rats and reaches about 40% of normal by 6 weeks after operation. ADIM and bone marrow functions return to normal in such animals [108]. Here it should be noted that during APR, pituitary GH and PRL are suppressed, yet the bone marrow is hyperactive. Elevated levels of type I CTKs, such as GM-CSF and IL-6, could mediate this alternate pathway of activation.

The IS develops memory cells gradually and secondary immune reactions become independent of pituitary hormones. Current evidence suggests that type I CTKs play a key role of maintaining memory cells [71], which in turn play significant roles in the maintenance of immune function during the adult life of animals and humans. Memory cells have a remarkable capacity to survive crisis situations and regenerate immune function after recovery from disease (see Section 1.4.1).

Many cells and tissues other than the pituitary make PRL, including immune cells. PRL is synthesized by various subtypes of immune cells from humans, mice, and rats, and immune-derived PRL is known to play a role in human autoimmune disease [109]. However, lymphocyte-derived PRL is pituitary dependent [110]. It is reasonable to suggest at this stage that in utero the IS relies on placental lactogens for development. After birth, pituitary GH, PRL, IGF-1, and the HPA axis are involved in regulating immune function [111]. This applies to naïve lymphocytes of the ADIM system. After priming with antigen, ADIM cells depend on type I CTKs for their survival and function. This is the case also for memory cells. Type I CTKs also support the HP and survival of naïve and memory T cells, naïve B cells, and NK cells. However, in situations when CTKs are in short supply, such as severe radiation disease for instance, and a number of other “stressful” conditions [112], pituitary GH and PRL are available right in the serum to perform the function of type I CTKs and support the IS until it fully recovers. Clearly, GLH (GH, PRL, and placental lactogens) are involved in the development of the IS, including the bone marrow; in the maintenance of naïve lymphocytes until they are primed with antigen (e.g., maintenance of immunocompetence); and in regeneration of immune function after severe immunosuppressive insults to the body. Compelling experimental and clinical
evidence supports the involvement of PRL in autoimmune disease [109, 113–115] and cancer [116,117].

The INIM system is capable of functioning in the absence of the pituitary gland. As a matter of fact, INIRs function at the cellular level, and after activation of such cells by antigens bearing homologous epitopes (homotopes), the CTKs produced maintain the response at the cellular level, if necessary. However, the HPA axis is invariably activated by INIM-derived CTKs, and GCs act as an important safeguard against exaggerated CTK production. During acute illness, GCs and CATs amplify innate immune function. This is the case under homeostatic conditions as well, where the HPA axis functions in balance with PRL and GH, so both ADIM and INIM functions exist in harmony [13].

Redundancy is the rule within the IS. There are eight major immunoglobulin classes. Each may recognize the same epitope on an antigen, yet the defense mechanisms activated by each are very different. There is significant redundancy in the CTK families and in chemokines. There are multiple alternatives in cell-mediated immunity as well. For instance, a target (cancer) cell may be killed by killer T lymphocytes, by NK cells, by NK T cells, by activated macrophages, antibodies, complement, and so on [118].

It was observed in cancer immunology that a given cancer is attacked by a number of immune mechanisms in the raw, until all mechanisms are bypassed by the cancer and it can progress to clinical cancer [119]. For this reason, the process of oncogenesis may take up to 30 years. Several other diseases show similar pathology. For instance, in asthma the pathological lesions are present at all times, yet symptoms are manifested only periodically during “attacks” [120]. One may propose that during the silent periods the pathological process is suppressed/controlled by regulatory mechanisms available in the host. Similarly, in Alzheimer’s disease numerous plaques form in the brain, and these patients are severely ill; yet indistinguishable plaques are found in asymptomatic normal individuals, but in much lower numbers [121]. Apparently, the brain is able to compensate for injury to a significant degree, but not to the extent that occurs in Alzheimer’s disease.

1.6 Protection for Life by the Neuroimmune Supersystem

All of us suffer from febrile illness on numerous occasions and recover. This indicates that our INIM system is a very important and efficient defense mechanism. This wonderful defense system is one that we have for life: it is always there for us, never lost, and fights for us until the last moment of our existence [122].

The ADIM system does not decline, either. Rather, it undergoes age-related changes: for example, in older individuals the system is maintained largely by memory cells, so the thymus has little to do; hence it involutes. However, the thymus may be reactivated by severe immune depletion, which indicates that the involuted thymus is still a functional organ that is ready to act when necessary [28,123]. The loss of adaptive immunity is not obligatory with aging; it is well preserved in the case of successful aging. Immunosuppression by Tsr during acute illness is reversible, and it occurs during healing [28]. Moreover, the homeostatic survival of T, B, and NK cells
represents a very important and fundamental mechanism of regeneration of immu-
ncompetence during recovery from severe illness. During acute illness, the ADIM
system is suppressed due to amplification of the NATIM system by CTKs, GCs, and
CATs. This occurs in the majority of individuals who do not age successfully and
succumb to various illnesses that activate the NATIM and suppress ADIM. The pro-
longed activation of NATIM may lead to extreme weight loss (cachexia) and eventu-
ally death [26].

The concept of age-related immune deficiency led to doom-and-gloom prospects
concerning aging. However, successful aging, without disease, has been observed.
Therefore, at this time it is clear that disease-free survival is possible until we reach
the natural limit of our lifespan [124].

1.7 Conclusions

Proposals of neuroimmune interaction date back to the late nineteenth century. The
students and followers of Pavlov studied neuroimmune regulation in Russia and con-
cluded that the hypothalamus was involved as a central immunoregulatory organ.
Andor Szentivanyi (1950–51) demonstrated first with objective methodology that the
hypothalamus regulates the anaphylactic reaction and antibody formation in labora-
tory rodents. Korneva and Khai [7] confirmed that antibody formation is regulated by
the hypothalamus. Ader [16] and Gorczynski and Kennedy [17] showed that immune
reactions are sensitive to Pavlovian conditioning.

Hans Selye described the stress syndrome [8], which was characterized by the
activation of the HPA axis and the involution of the thymus and lymphoid organs. He
pointed out that stress leads to the “general adaptation syndrome,” which protected
the animals from “nocuous” agents.

This early scientific inquiry progressed slowly, with only a few laboratories work-
ing on this field. Neither the immune nor the neuroendocrine systems were known
well enough to permit a detailed analysis of neuorimmune interactions. The situation
changed gradually during the mid-1970s when pituitary hormones were studied for
immunoregulation. It was discovered that GH and PRL stimulate ADIM function,
whereas the HPA axis was shown to have immunosuppressive and anti-inflammatory
effect. The initial papers on the subject were followed by investigations in numerous
laboratories and the fundamental nature of neuroimmune interaction has been elic-
ted. It was shown that the HPA axis is stimulated by IL-1, a CTK produced within
the IS. Soon other pituitary hormones and numerous (type I) CTKs were found to be
involved in “feedback” communication of the IS with the neuroendocrine system.

It appears that in utero the IS relies on placental lactogens for development. After
birth, pituitary GH, PRL, IGF-1, and the HPA axis are involved in regulating ADIM
function. This applies to naïve lymphocytes. After priming with antigen, ADIM cells
depend on type I CTKs for their survival and function. This is the case also for mem-
ory cells. Type I CTKs also support the HP and survival of naïve and memory T cells,
B cells, and NK cells. However, in situations when CTKs are in short supply, such
as severe radiation disease for instance, and a number of other “stressful” conditions,
pituitary GH and PRL, which share signal transduction pathways with type I CTKs, are available right in the serum to support the IS until recovery. Clearly, GLH (GH, PRL, and placental lactogens) are involved in the development of the IS, including the bone marrow, thymus, and maintain naïve lymphocytes until they are primed with antigen (e.g., maintenance of immunocompetence). GLH are involved in regeneration of immune function after severe immunosuppressive insults to the body. Compelling experimental and clinical evidence supports the involvement of PRL in autoimmune disease and in cancer.

Much has been established within the first two decades. It became clear that the nervous, endocrine, and immune systems interact continuously and integrate, coordinate, and regulate all bodily functions, including host defense, from conception till death. Hence the term neuroimmune Biology was introduced to name this field.

Meanwhile, a second form of immunity has been recognized in immunology, which is with us from conception till death. This immunity is called INIM or NATIM. Unlike ADIM, where clones of lymphocytes need to proliferate first in order to mount a specific immune response, which takes 5–7 days, the cells of the INIM system are fully differentiated and bare functional innate antigen receptors (INIR), which recognize evolutionarily conserved and highly cross-reactive homologous epitopes (homotopes). Pathogenic microbes degenerated and cancerous cells and even normal tissue components may express homotopes. Thus, the INIM system is the first to defend the host with its instantaneous capacity to respond, and it is with us for our last moment of life.

It has been discovered within the past few years that INIR is expressed in the CNS, in glia cells but also by neurons and their dendrites. This indicates that the CNS is an integral member of the INIM system. It is capable to recognize infection rapidly and respond instantaneously by neurogenic inflammation and by the activation of immunological responses in order to eliminate the problem. Other organs and cells also express TLR, such as leukocytes, the pituitary gland, the adrenal gland, the liver, in mucosal epithelial cells, endothelial cells, in vascular smooth muscle, and in the cornea. Therefore, the entire body participates in INIM reactions. The pituitary produces POMC in response to LPS (TLR4 is involved), mucosal epithelial TLR participates in inflammation and responds to pathogens, corneal TLR was found to fight infection, and endothelial TLR was observed to play important roles in homeostasis of the heart. Therefore, both the INIM and ADIM systems also fulfill important physiological functions.

The activation of the INIM system, with or without adaptive immunity, is capable of exerting a profound host defense response to diverse noxious agents. Clearly this NISS, which is directed by the CNS, is capable of mobilizing the entire organism in the interest of host defense in emergency situations (such as acute illness, APR). NISS also performs important physiological functions, as is becoming apparent.

The regulatory network in NISS consists of hierarchical regulatory circuits, which are superimposed on each other. For example, regulatory signals from the CNS are capable of dominating the signals from lower circuits (e.g., pituitary). As we know, pituitary hormones control hormones secreted by the adrenals, gonads, thyroid, liver, and other tissues that secrete IGF. CTKs represent another regulatory circuit.
Our experiments indicate that if a higher regulatory circuit fails, a lower circuit will gain dominance and take precedence over lesser signals that still exist in the system; this way, INIM function is maintained continuously under any circumstance. The INIM system produces CTKs continuously and defends the host during acute illness/injury under any conditions, and the CTKs will signal any regulatory circuits of the host organism that remain active. The INIM system never stops protecting the host organism (Figure 1.2).

TLRs are expressed in most if not all tissues. Therefore, if a higher regulatory system, such as the CNS, is injured or paralyzed, for instance, TLR could still signal the system through lower circuits such as the pituitary, adrenals, thyroid, and gonads and thus stimulate host resistance. TLR signaling is also possible at the CTK or even at the cellular level, as all leukocytes express TLR. Thus, even extensive injury to the host organism cannot prevent the INIM system from providing a defense, as TLR could directly signal the immunocytes to respond and they would respond with the function they normally perform. INIM always protects the host.

Memory T lymphocytes, B cells, and NK cells are able to survive major disasters and bring back immune function once the crisis is over. During APR, the ADIM system is suppressed by Tsr and GCs, but not destroyed. The possibility of recovery is present and ADIM function recovers after recovery from acute illness. Healing is under hypothalamic regulation, with VP as the principal regulator.

The concept of age-related immune deficiency led to doom-and-gloom for the prospects of aging. However, successful aging, without disease, has been observed. Therefore, at this time it is clear that disease-free survival is possible until we reach the natural limit of our life span.

References


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2 On the History of Immunophysiology: First Steps and Main Trends

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2.1 Introduction

The history of the development of immunophysiology may be divided into several stages. The earliest one, which spans the end of the nineteenth century and the first half of the twentieth century, is the period during which the first facts were discovered and the question was raised as to whether the central nervous system (CNS) can influence immune activity. The second stage was characterized by the accumulation of phenomena and evidence proving the existence of CNS influence on immune function, with special interest in the possibility that conditioned reflexes may modulate immune reactions (1949–1958). The third stage saw further development of immunophysiology and its foundation as a specific, defined scientific field. This developmental stage is best characterized by the broad term *neuroimmune biology*. At present, activity in this field centers on intense analysis of the mechanisms of neural–immune interactions, using sophisticated methodologies that include molecular–biological and genetic analyses.

2.2 Early Investigations

The first investigations—and indeed, the very idea—of possible involvement of the nervous system in the function of the immune system are associated with the Russian physiologists Savchenko, London, and Metalnikov [1–3]. The nonaccidental nature of this association follows from the traditions of the Russian physiological and therapeutic way of thinking, deeply influenced by the concept of *neurism* based on the works of Sechenov, Pavlov, Bekhterev, and Botkin. The scientific foundation created by these eminent scholars and the corresponding viewpoints had a direct impact
on the development of ideas and investigations related to the possible relationships between the central nervous and immune systems.

Despite all the shortcomings of these first steps, these scientists and investigators proposed truly pioneering ideas with lasting value. Due appreciation for their contributions is possible when one looks at them not from a position based on the present level of knowledge, but from the position that obtained a century ago; from that standpoint, one can appreciate how novel and risky was the very posing of this problem. At the time, immunology was in its infancy and appropriate investigative equipment and skills were completely absent, so this foresighted attempt to gain a broader and deeper understanding deserves respect even if the results are not fully consistent with what we now know.

The origin of immunophysiological research may be traced to the 1890s, when the pioneering works of Savchenko [1] and London [2] showed that transection of the brain or removal of some of its parts affects the course of infection. In these studies, major parts of the brains of pigeons were removed surgically, and then anthrax development in the birds was followed. Pigeons are normally resistant to anthrax. However, when the cervical segments of their spines were transected, they became susceptible to this infection. The same was observed in pigeons that had brain hemispheres removed. (It was later shown that transection of the cervical section of the spine impairs antibody production [4].) It should be pointed out that these studies did not address the possible influence of the brain on immunity; however, they were the first to show some involvement of the brain in body responses to infection.

It is generally agreed that the founder of immunophysiological research is Metalnikov [3], a follower of Pavlov, who worked at Pasteur Institute in Paris. Metalnikov was the first to explicitly put forward the idea that the nervous system may influence body defenses and to experimentally check this possibility. In recognition of his contribution, in 1993 the International Society of Neuroimmunomodulation (ISNIM) instituted the Metalnikov Medal for outstanding achievement in this scientific field.

2.3 The Second Period

During the second developmental phase of the field, many researchers were attracted by the idea of discriminating neural influences on the immune system and elucidating their role in the development of immunological reactions. In fact, many different versions of experiments were designed almost simultaneously to answer the most general question: namely, whether the nervous system has an impact on immunological processes. As a rule, the experiments involved the use of drugs that excite or inhibit CNS activity and observation of the effect on the development of immune responses.

These investigations led to the virtually unanimous conclusion that immunological reactions are inhibited by substances, including hypnotic and sedative drugs, that suppress cerebral activity. It should be stressed that the general conclusion drawn from the absolute majority of the studies was at the level of CNS activity, and the functional state of the CNS was found to be a significant factor of the development
of immunological processes as a part of integral body responses in higher animals. CNS activation predominantly stimulated these processes, whereas CNS suppression was associated, in most cases, with inhibition [5–9]. These investigations produced firm evidence for the regulation of immune function by the CNS. Although these studies did not analyze specific mechanisms of neural immunoregulation, they did lay the foundation for further development in the field.

From the modern standpoint, it is easy to find defects in the aforementioned studies. Nevertheless, the intention to study neural influences on immunity is evident in them, and many of them did reveal such influences. At present, a major area of interest is study of the relationship between immune function and CNS activity in newborn animals and babies. Abnormalities have been proven to be of genetic origin.

An unfortunate historical event led to a serious setback in the development of immunophysiology in Russia. The so-called United Session of the USSR Academies of Science and Medical Sciences on Physiology, which was held in 1950, declared only a single line of the development of physiology to be promising, which was Pavlovian physiology. This was indeed a promising and important line of research, and one that is still being developed. However, the session condemned and actually banned other lines of development in physiology. This restriction retarded progress in Russia and severely hampered the development of immunophysiology.

### 2.4 Consolidation of the Science of Immunophysiology

After the United Session, a number of studies focused on the modulation of immune responses by conditioned reflexes. Many of the studies produced crude results; others were carried out inappropriately. An additional difficulty was that the level of knowledge in and development of contemporary immunology could not provide adequate insights into this problem. As a result, a long-term discussion based on facts as well as concepts arose, involving such prominent scientists as Dolin and Krylov, Ado and Ishimova, Gordiyenko and co-authors, Zilber, Zdrodovskiy, and others [10–14].

This turbulent history, and the fact that there are still unresolved questions, attest to the fact that the field of immunophysiology centers on highly complex and exceptionally difficult problems. Such a situation is not unique in science, particularly when a problem is investigated before adequate conceptual and technical means become available to properly address the questions involved. The combination of all these factors led to skepticism that significantly inhibited further developments in the field.

In the 1960s, investigations into the role of the CNS in the regulation of immunological processes intensified, due to several factors:

1. Clinical observations supported the interaction of mental, neurological, and immune mechanisms in patients with autoimmune diseases. American scientist George Solomon was the pioneer in this field [15,16].

2. The development of basic immunology made it possible to design a battery of tests for the determination of various components of the immune system, and to investigate in detail the mechanisms of action of neuroendocrine parameters on immune function both \textit{in vitro} and \textit{in vivo}. 
Physiologists and pathologists created the concept of body integrity, which proposed continuous interactions amongst the various tissues, organs, and systems in a coordinated and regulated manner under both normal physiological and pathological conditions. All this made some scientists realize that the brain must be important for the development of body defenses; because of this recognition, relevant investigations were initiated.

2.5 Modern Immunophysiology

2.5.1 The Role of the Hypothalamus

Studies on the role of the autonomic nervous system in antibody production, which was described as early as 1928 by Gamaleya [17], led to controversial results. The reasons for this may be at least threefold:

1. Different authors used different, incompatible, and often inadequate methods to examine the effects of the autonomic nervous system on immune function.
2. The timing of antigen administration versus the influences exerted on different components of the sympathetic nervous system was not taken into consideration.
3. Various antigens—that is, soluble, corpuscular, toxic, nontoxic, etc.—were used, which variously influenced the outcomes and results and thus complicated the analysis of these experiments.

The focus of scientific investigation shifted from analysis of the effects of excision of cervical sympathetic ganglia on immune function to studies of the effects of lesioning of hypothalamic structures [18,19]. The experiments carried out at Speranskiy’s laboratory [20] provided evidence for the involvement of the hypothalamic area in trophic processes in body tissues.

Studies of hypothalamic influences on immunological reactions using experimental lesions or excitation of the hypothalamus started with the examination of antibody levels and of anaphylactic shock. Fillip and Szentivanyi [21] were able to show thereby that massive lesions of the hypothalamus result in inhibition of the immune response. These findings stimulated studies of the role of hormones in the regulation of immunity [22–25]. The earliest systematic studies along this line were done by Fillip and co-authors [26,27], who showed that massive bilateral lesioning of the tuber cinereum in guinea pigs inhibited the development of anaphylactic shock. It should be stressed that 5% of postoperative experimental animals died of severe impairments of the vegetative and endocrine functions [28]. Other hypothalamic structures have been found to be involved in regulation of NK cell activity, T-helper and T-suppressor cell ratio [29], and macrophage activity [30].

The discovery of specific structures, the lesioning of which drastically reduced immune responses [19,31], initiated a new stage in study of the mechanisms of neuroimmune interactions. The role of hormones in regulation of immunity was examined using the model of local lesioning of the posterior hypothalamic field [32]. Many studies addressed the role of different structures of the brain, including
the hypothalamus, in the activity of separate components of the immune response. These investigations demonstrated the important fact that lesioning or destruction of the hypothalamus and of some other—but by no means all—brain areas affects immune function.

The development of the concept of multilevel hierarchical organization of the system of regulation of immunity opened new prospects for studying the system [33]. This concept stimulated experimental studies of the role of the nervous system in regulation of bone marrow activity. It was shown for the first time that the brain influences colony forming in the spleen; in particular, that lesioning of the posterior hypothalamic field decreases the colony-forming activity of bone marrow [34]. Similar disorders develop following craniocerebral trauma [35]. Some therapeutic approaches were attempted to correct or prevent such disorders. The use of melatonin, delargin, and some other drugs has been proposed [30,34,36].

The possibility of influencing immunological processes by stimulation of hypothalamic structures was demonstrated for the first time by Groot and Harris [37], who stimulated the tuber cinereum and mamillary bodies in the hypothalamus and observed an inhibition of allergic reactions. This was confirmed later by Szentivanyi, who used an anaphylactic shock model [38]. Initial studies investigating the effects of stimulation of the hypothalamic area on blood antibody titers suggested either stimulatory or suppressive (in case of a high current intensity) effects [37–39].

An obvious and logical further step in the development of immunophysiology was to study brain functions during the course of the development of the body’s response to an antigen. The employment of electrophysiological methods in investigation of this problem was a significant achievement. This approach was pioneered by Braun [40], who showed that the number of active neurons in the hypothalamus, along a track passing through the posterior hypothalamic area, increased upon immunization. Subsequent experiments used mainly electrophysiological methods [40–44] and neurochemical methods to detect changes that occurred in the brain after antigenic challenges [45–48]. These investigators primarily studied activity of hypothalamic structures and neurons.

The difficulties of obtaining meaningful results by using extracellular analysis of the impulse activity of neurons made it necessary to use mathematical methods. With these latter methods, huge amounts of data could be processed to elucidate the dynamics of changes in the activity of hypothalamic neurons after immunization. These studies established a spatio-temporal pattern of changes in the electrical activity of defined cerebral structures during the course of the development of body response to antigenic challenge. It has been shown that, under natural conditions, the brain becomes involved in the response to immunization within the first 10–30 minutes after the introduction of an antigen, and that this involvement is followed by further dynamic interactions between the nervous and immune systems [41,43,49,50].

These results are of fundamental importance for the understanding of the interaction of the neuroimmune system, because the initial idea of brain influence on immunity gradually developed into the concept of a *neuroimmune regulatory circuit*. With
regard to a role of the CNS in regulation of immunity, reference should be made to early and successful studies dealing with different neuromediator systems of the brain as they modulate immune function [50–52].

### 2.5.2 Innervation of Lymphoid Organs

A significant advance in NIB was achieved by detailed studies on the innervation of lymphoid organs, including not only their external neural input, but also the internal distribution of nerve fibers and endings within the organs of the immune system [53–56]. The notion of “open” synapses located at nerve endings emerged. Certain stimuli reaching such synapses elicit the release of neuromediators into the immediate vicinity of lymphoid cells and thus bring regulatory information to them via soluble neurotransmitters (e.g., sympathetic outflow) [57].

### 2.5.3 Hormonal Immunoregulation

Much effort was made to elucidate the possible efferent pathways of neural immunoregulation. The possible immunoregulatory role of hormones was studied by numerous investigators. Currently, abundant information is available in the literature on this subject, which is summarized in a number of reviews and monographs [50,58]. The general conclusion is that an excess or deficit of almost any hormone will affect immune function. However, the growth and lactogenic hormone family has emerged as the hormones of immunocompetence. In contrast, the hormones of the hypothalamus–pituitary–adrenal axis were proven to have an immunosuppressive and anti-inflammatory role [59–62]. The concept of an integrated neuroimmune regulatory network gradually emerged on the basis of new knowledge, in the field that later became known as neuroimmune biology.

Blalock [63] and Smith [64] discovered the production of classical hormones by cells of the immune system. Today it is clear that cytokines and chemokines, which originally were thought to be produced by the immune system, are actually produced in the CNS as well, and fulfill both pathological and physiological functions [65,66]. Conversely, hormones, neurotransmitters, and neuropeptides are readily produced within the immune system. However, the production of some soluble mediators of each of these substances does take place in every organ and tissue in the body. This sharing of mediators allows the neuroimmune regulatory network to communicate with each organ and tissue in the body and to coordinate the function of the entire organism in health and disease [67].

### 2.5.4 Some Molecular Mechanisms in Immunophysiology

It is obvious from the evidence discussed in preceding sections that lymphoid cells must have some means of recognizing neural messages. This is fundamental to the whole problem, and is why the discovery of receptors for neuromediators, hormones, and regulatory peptides on immunocyte membranes was a landmark in NIB
development. The identification of neuromediator receptors on the plasma membrane of lymphoid cells explains the ability of these cells to recognize changes in their neuromediator microenvironment [68–72]. The open synapse concept helps to explain how an integrated neuroimmune regulatory circuit is regulated by neuromediators.

Regulatory influences may be transmitted by humoral means, through changes in blood levels of hormones and regulatory peptides, and, to some extent, through changes in the blood supply of lymphoid organs. Neuromediator balance influences the metabolic and functional activity of lymphoid cells [73–76].

It was discovered that immunocytes at different stages of proliferation and differentiation have varying sensitivity to neuromediators. Therefore, the whole process is multivariant by nature. By the end of the 1980s, studies of ligand–receptor interactions in various lymphoid cells provided evidence for the role of specific neuromediators in the activities of lymphocyte populations [68,77–79].

At present, our knowledge of the biochemical mechanisms of signal transmission and of the effects of neurohumoral factors on the metabolic and functional activities of immune cells is incomplete. However, we do know that signal transmission involves the system of cyclic nucleotides, including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), membrane-bound ATPases, changes in calcium fluxes, and the sphingomyelin pathway [80–83].

Recent studies indicate that mediators derived from arachidonic acid are necessary for lymphocyte activation. Thus, the conversion of arachidonic acid into prostaglandins via the cyclooxygenase pathway leads to increased cAMP generation in lymphoid cells. In contrast, the conversion of arachidonic acid into hydroxy- and hydroperoxyeicosatetraenoic acid via the lipoxygenase pathway activates cGMP production.

Neuroimmune interaction is mediated in part by soluble bioregulators, including neurotransmitters and neuropeptides, hormones, cytokines, and chemokines. Interleukin-1 (IL-1) was the first of the interleukins to be found to convey immune-derived messages toward the CNS. IL-1 is also one of the key regulators of host defense reactions, either innate or acquired [84–87].

Virtually, all known mechanisms of signal transduction have been shown to contribute to communication within the neuroimmune regulatory network. For example, IL-1 signals cells in both the immune and nervous systems. The biochemical responses of cells to IL-1 develop within a few minutes. However, neither the exact sequence of signaling reactions nor their complete cascades are known. The classical phosphoinositide pathway, including cyclooxygenase- and lipoxygenase-mediated metabolism of arachidonic acid, has been shown to be implicated in IL-1 signal transduction [88–90]. G-proteins are activated as a result of IL-1 action, but they are not directly involved in IL-1 signal transduction [88,91].

The discovery of the role of ceramides as secondary messengers resulted in the identification of the new (and currently well recognized) pathway of cytokine signal transduction initiated by activation of the membrane-bound neutral sphingomyelinase [92, 93]. This mechanism, which has been named the sphingomyelin pathway, plays a role in IL-1 signaling, in lymphocytes and fibroblasts [94,95] and in neurons [96]. IL-1β action on the CNS involves the type I IL-1 receptor and the sphingomyelin
pathway [97,98]. Membrane-bound sphingomyelinase activity changes in nerve cells and immunocompetent cells in response to stress. Glucocorticoid hormones modify IL-1β signal transduction via the sphingomyelin pathway [99].

The response to infection and severe injury, which is analogous to the stress response, is now known as the acute phase response (APR) [100]. Major contributions were made to the subject of cytokine signaling of the brain during APR by McCann et al. [101] and Bartfay and co-workers [102]. Fever is a hallmark of APR; Blatteis and colleagues rose to excellence in fever research [103].

2.5.5 Biological Rhythms in Neuroimmune Regulation

Living organisms have the capacity to adapt to their environment. In general, daily (diurnal or circadian) and seasonal (circannual) adaptations are distinguished. Melatonin, a pineal hormone, is a key mediator of biological rhythms. Maestroni and co-workers [104] demonstrated first that melatonin is a major immunoregulatory hormone. The circadian organization of the immune response is discussed in this volume by Esquifino and Cardinali [105].

2.5.6 Pavlovian Conditioning of Immune Reactions

An original approach is represented by the studies of Ader et al. [106–109], who investigated the role of Pavlovian conditioning in the suppression of immune reactions. These studies, which used the immunodepressant drug cyclophosphamide (CP) as the unconditioned stimulus, demonstrated that it is possible to suppress humoral and cell-mediated immune responses if CP treatment is paired with a conditioning stimulus. Conditioning could also be used to influence immunopathological processes. Ader chose an exceptionally appropriate model, which made it possible to reproduce the pharmacological effect of CP with the conditioned reflex ultimately resulting in immunosuppression. A key contributor to this area of research is Reginald Gorczynski [110].

2.5.7 Stress and Immunity

An enormous number of publications deal with the clinical relevance of stress to disease processes, which was first pointed out by Solomon [15,16]. Glaser and co-workers [111] contributed significantly to stress research, especially with regard to its clinical applications. Stress is known to alter the neuroimmune relationships in the body [88,89]. The hypothalamus–pituitary–adrenal axis (HPA) is activated not only by IL-1, but also by IL-6 and TNFα, the levels of which increase with stress [112]. Glucocorticoid hormones, which are indispensable components of stress response, suppress the adaptive immune system by inhibiting the secretion of such cytokines as IL-1, -2, -3, -5, -6, -8, -12, and others [113,114]; by influencing the expression of cytokine receptors on immunocompetent cells [113,115]; and by inducing apoptosis
Increased blood levels of glucocorticoids and catecholamines in stress shift the Th1/Th2 balance toward Th2, that is, to increased synthesis and release of anti-inflammatory cytokines (IL-4, -5, -10, -13) responsible for humoral immune reactions and for immunoglobulin (natural antibody) production [117–119]. The intensity of IL-1β signal transduction via the sphingomyelin pathway in cells of the nervous and immune system changes with stress, and may be one of the main mechanisms of the development of stress-induced alterations in immunity [87,97].

Along with glucocorticoids and catecholamines, prolactin is regarded as an indispensable component of stress response [120]. It may function as a stress-limiting factor that prevents stress-induced immunosuppression [121]. The kinase Stat-5, which is activated by prolactin as one of the components of its signal transduction system, interacts with the glucocorticoid–receptor complexes in the cytosol and thereby prevents their binding to specific DNA sites implicated in realization of the immunosuppressive effects of glucocorticoids on lymphocyte functions [122].

Integration of the effects of prolactin, glucocorticoids, and IL-1 occurs at the level of ligand–receptor interactions in immunocompetent cells and is one of the mechanisms that prevent the development of dysfunctions of the immune system in stress [123,124].

Sustained or severe stress suppresses the adaptive immune system, although natural immune mechanisms are augmented at the same time. Short episodes of stress augment peripheral adaptive immune defenses [100,125].

### 2.5.8 Behavioral Immunophysiology

It has been known for a long time that sick animals and human beings do not eat, restrict their movements, and sleep a lot. Recent research has revealed that cytokines are responsible for the development of this behavior. Dancer and co-workers made significant contributions to this field [126].

### 2.6 Historical Note to Figures: Initial Events in the Organization of Research Activities in Immunophysiology

The first meeting of the then relatively sparse numbers of researchers tackling the problem of interactions of the immune and nervous systems occurred at a symposium held in 1982 at the Research Institute of Experimental Medicine (IEM), Soviet Academy of Medical Science, in Leningrad. At the invitation of IEM and the chairperson, E.A. Korneva, the symposium was attended by R. Ader, H. Besedovsky, V. Pierpaoli, I. Solomon, H. Spector, and B. Yankovich, to name a few. This was the seminal event resulting in the ideas to hold the First International Congress and to organize an international society. The First International Congress of Neuroimmunomodulation was held in Dubrovnik under the presidency of
Figure 2.1 Photos of the participants in the Symposium on Immunophysiology held in Leningrad in 1982. These researchers made key contributions to the determination of the further development of immunophysiology in the world and, in fact, were the founders of this field of inquiry: (A) R. Ader (USA), (B) B.D. Yankovich (Yugoslavia), (C) W. Pierpaoli (Switzerland), (D) E.A. Korneva (Russia) and G.F. Solomon (USA) in the forefront, (E) N.H. Spector (USA) and H.O. Besedovsky (Switzerland) in the Peter and Paul Fortress.
B. Yankovich. Thereafter, the International Society of Neuroimmunomodulation (ISNIM) was instituted. Its first elected president was Herbert Spector (USA), to whom the honor of founding the *Journal of Neuroimmunomodulation* belongs. Spector, together with ISNIM vice-presidents B. Yankovich, E. Korneva, and V. Pierpaoli, has significantly contributed to the important task of uniting researchers active in the field of neuroimmunomodulation.

At the same time, due to efforts of R. Ader and I. Solomon, the Society of Psychoneuroimmunology was organized in the USA and the journal *Brain, Behavior and Immunity* was launched. Ader was the first president of the Society and editor-in-chief of the journal (Figure 2.1).

### 2.7 Conclusions

The initial steps in the field that became immunophysiology were difficult in virtually all countries because of a sort of aversion to this research direction. As often happens, progress in the ways of thought lagged behind progress in the methods of science and research. Skepticism was especially typical of immunologists, which is explainable by the fact that most of their studies of processes occurring in the immune system were conducted in test tubes. At the same time, physiologists believed, following the general approach typical for their discipline, that no system in the body functions entirely independently; given this mindset, it was easier for them to accept new ideas. On the whole, all pathfinders in the field of immunology had to be steadfast and inventive to overcome the generally entrenched conservatism of the scientific community.

Recent basic and applied developments in this field have deepened our insights into the mechanisms that are involved in neuroimmune interaction. The signal-receptor problem is being addressed intensively. The use of modern accurate and informative experimental techniques has made it possible to obtain data that provide solid evidence for neuroimmune interaction. Some of the problems are now being addressed by cutting-edge experimental techniques, including the use of DNA microarrays for studies at the genomic level [127].

Many aspects of immunophysiology developed in parallel, though some of them went somewhat ahead. This does not allow presentation of the facts exactly as they occurred in time; however, the logic of their occurrence is reflected, in principle, by this review.

Current progress in the field of immunophysiology (Figure 2.2) is characterized by intense studies of very diverse aspects of the problem, ranging from the molecular mechanisms of interactions between the neuroendocrine and immune systems to the clinical manifestations of neuroimmune disturbances. The search for therapeutic means to correct such disturbances is ongoing. Although it is impossible to present (or even mention) all of the developments and achievements occurring worldwide, the recent book of Neuroimmune Biology, Volume 9 covers a broad spectrum of these studies within the scope of a brief review.
Figure 2.2  World map indicating research centers active in immunophysiology research since 1960 (A) up to 2004 (B).

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3. Selective Pro-Inflammatory Activation of Astrocytes by High Mobility Group Box 1
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3 Selective Pro-Inflammatory Activation of Astrocytes by High Mobility Group Box 1 Protein Signaling

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3.1 Introduction

High mobility group box 1 (HMGB1) protein, originally identified as a chromatin component, is highly conserved in mammals and particularly abundant in developing tissues, in proliferating and transformed cells [1–3]. More recently, HMGB1 has been identified in the cytoplasm and in the extracellular compartment of different activated or dying cells. Specifically, a number of cell types actively export HMGB1 when exposed to inflammatory agents and oxidative stress or when they interact with other cells [4–7]. The active cell release of HMGB1 involves sequential steps. At first, an enhanced nucleocytoplasmic shuttling of the protein is triggered by hyperacetylation and phosphorylation at specific serine residues, mediated by the calcium/calmodulin-dependent protein kinase (CaMK) IV [8,9]. Subsequently, cytosolic HMGB1 is concentrated into secretory lysosomes and released when appropriate stimuli trigger lysosome exocytosis [10]. However, depending on the cell type, a passive release of HMGB1 can occur during the course of apoptosis as well as necrotic death [11,12]. In the last condition, the release of the protein is preceded by a poly-ADP-ribose polymerase (PARP)-dependent nuclear-to-cytosolic translocation of HMGB1 [13]. HMGB1 shows heparan sulfate-rich proteoglycan binding properties that limit its rate of extracellular diffusion [14]. Depending on its local concentration and on the site of cell release, HMGB1 can exert regenerative or inflammatory functions by engagement of some membrane proteins, including the receptor for advanced glycation end products (RAGE) and the toll-like receptors (TLRs) 2 and 4, involved as HMGB1-signaling mediators in different cell types [15,16]. Hence, HMGB1 promotes tissue development and regeneration by activating cell migration, differentiation, proliferation, and immune responses directed against dying cell antigens, and also sustains pathological conditions as an enhancer of inflammatory responses, tumor cell invasiveness, and lethal endotoxemia [17–21].
Recently, emerging roles for HMGB1/RAGE have been proposed in neuropathology and neuroinflammatory responses [22,23]. Normal brain, cultured neurons, endothelial, and glial cells express HMGB1, and the protein level changes in various brain regions in dependence of age [24,25]. A significant up-regulation of brain HMGB1 has been shown to occur in response to cortical impact injury, suggesting that this protein could play a role in the regeneration of the damaged tissue [26]. Because microglia and astrocytes can export HMGB1 when challenged with specific stimuli, and neurons also release this cytokine when exposed to glutamate excitotoxic concentrations, the local amount of extracellular HMGB1 in the CNS can rapidly increase [27–30]. Moreover, HMGB1 receptors undergo up-regulation in inflammatory diseases of the CNS, thus contributing to enhancement of paracrine and autocrine cytokine-like activities exerted by HMGB1 [31,32]. HMGB1 inhibitors can prevent these pro-inflammatory responses, indicating that the cytokine plays a nonredundant role as an inducer of neuroinflammation [33,34]. However, HMGB1 can also bind pro-inflammatory cytokines, such as IL-1β, potentiating their activity [35]. Hence, in the presence of low concentrations of IL-1β, the inflammatory process can be highly amplified by a concomitant release of the HMGB1 costimulus.

Glia l cells of the CNS are implicated in a variety of neurological disorders and are actively involved in local responses, including inflammation and tissue repair processes [36,37]. Recently, microglia have been identified as a target of HMGB1. Specifically, the cytokine released from dying neurons inhibits microglial amyloid beta (Aβ) peptide 42 clearance and enhances the neurotoxicity of Aβ42 [38]. Because astrocytes are equipped with all the surface receptors for HMGB1 and this cytokine activates the release of excitatory amino acids from subcellular particles of astrocyte origin, here we have analyzed the possible functional role of HMGB1 on these glial cells [39–41]. Astrocytes are the most abundant macroglial resident cells of the CNS and play important roles in the homeostasis of the normal brain—but can also assume different active states when exposed to specific insults or chemical stimuli [42]. In keeping with these observations, depending on the inflammatory pattern expressed, these cells are able to produce a plethora of mediators of glial and neuronal cell responses [43]. To determine the specific reactive phenotype induced by HMGB1, we have explored the expression of astrocyte gene markers involved in cell stress and death, immunity, signaling, adhesion, metabolism, and proteolysis and the functional implications of this cell activation. Our results indicate that HMGB1-stimulated astrocytes release several protein factors involved in the progression of inflammatory processes, including chemoattractants, toward immune effector leukocytes.

3.2 Proteomic Analysis of Astrocytes Stimulated with HMGB1

Astrocyte cultures with microglial contamination below 1% were prepared from brains of 1- to 2-day-old rats [44]. The potential contribution of residual contaminating
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Microglia to the inflammatory responses was assessed by measuring nitric oxide (NO) and tumor necrosis factor (TNF-α) production following 72 hours of cell exposure to 10 μg/ml lipopolysaccharide (LPS) [45]. Only LPS-unresponsive astrocyte cultures were used in further experiments. Twenty-four hours before treatments, cells were placed in fresh medium containing 2% fetal bovine serum (FBS). Astrocytes were stimulated with 40 nM purified recombinant HMGB1 for 8 hours or left untreated and lysed (as specified in [46]); 0.7 mg of each sample was submitted to bidimensional (2D) liquid chromatography. Proteomic analysis was carried out by using the ProteomeLab PF2D system (Beckman Coulter, Inc.), which separates proteins according to isoelectric point by means of a chromatofocusing column in the first dimension, and by means of nonporous reversed-phase (RP) high performance liquid chromatography (HPLC) in the second dimension. The first-dimension method was started at a flow rate of 0.2 ml/min by washing the column with 100% start buffer for 45 minutes; 1 ml fractions were collected at 5-minute intervals. The linear pH gradient was generated by running 100% eluent buffer (pH 4.5 ± 0.1; Beckman Coulter, Inc.) for 1 hour; fractions were collected every 0.3 pH units. When the eluent reached pH 4.5, the column was washed with 10 volumes of 1 M NaCl to remove residual proteins. The fractions generated in the first dimension were sequentially injected (250 μl) onto the second-dimension RP HPLC column. The second-dimension method consisted of a 30-minute linear gradient of 5–100% B/A at 0.75 ml/min, where A is 0.10% trifluoroacetic acid (TFA) in water and B is 0.08% TFA in acetonitrile. Data were collected and analyzed by means of ProteoVue/DeltaVue software (Beckman Coulter, Inc.). ProteoVue allows comparison of all second-dimension runs for each sample in a banded map display that represents the UV peak intensity, where each band is a pH fraction. DeltaVue allows side-by-side viewing of the second-dimension runs for two samples. DeltaVue also shows the difference map between two samples from their ProteoVue maps. Figure 3.1 shows the map of HMGB1 up-regulated proteins obtained by comparing the proteomic profile of untreated and of HMGB1-stimulated astrocytes. The map reports the pH gradient versus RP retention time. The band intensity is proportional to the UV absorbance of each protein peak.

At first, we started to analyze protein peaks up-regulated in HMGB1-stimulated astrocytes. Proteins that increased more than twofold were selected for further mass spectrometry analysis and peptide mass fingerprint procedure [46]. Nine of the up-regulated proteins, indicated as numbered spots in Figure 3.1, were identified using a MASCOT search engine (http://www.matrixscience.com). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was adopted. If the proteins were not significantly identified, the mass tolerance was extended to 100 ppm. As shown in Table 3.1, HMGB1 induced a threefold increase in the levels of phosphorylated glial fibrillary acidic protein (GFAP) and vimentin, two glial intermediate filament proteins commonly accepted as astrogliosis parameters [47,48]. HMGB1 also promoted a 2.5-fold up-regulation of alpha enolase, one of the major proteins previously found to be overexpressed in astrocytes stimulated with a complete cytokine mixture (CCM) [43]. Among the other major proteins up-regulated by HMGB1, only heat shock protein (HSP)10 has previously been related to inflammatory processes [49].
This proteomic evidence indicates that HMGB1 induces a pro-inflammatory-like response in primary astrocytes and that its signaling activity promotes changes in the level of several major gene products characteristic of reactive states of these cells.

### 3.2.1 Molecular Mechanisms Involved in HMGB1 Signaling

Because the expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) is relevant to the inflammatory response of astrocytes, the level of these enzymes was evaluated in primary astrocyte cultures exposed to HMGB1 and to a complete cytokine mixture previously characterized as an artificial approximation of a full inflammatory condition [45]. As reported in Figure 3.2(A), resting astrocytes
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did not express detectable amounts of the inflammation-specific proteins COX-2 and iNOS, but the CCM induced both gene products. In contrast, HMGB1 only induced a dose-dependent up-regulation of COX-2, suggesting that this cytokine displays a selective pro-inflammatory activation of astrocytes.

Moreover, according to the lack of HMGB1 effect on the induction of iNOS, we did not observe any NO\textsubscript{2} production in astrocytes cultured for 24 hours in the presence of the cytokine (data not shown). COX-2 plays a major role in the inflammatory reaction and has previously been characterized as a cytokine-responsive gene that is induced by several pro-inflammatory factors, including IL-1\textbeta, TNF-\alpha, and LPS. This enzyme is expressed in activated astrocytes following CNS injury and has been proposed as a marker for in vivo astrocyte activation. Starting from a previous observation showing that freshly collected in situ-matured astrocytes express RAGE [41], we analyzed the involvement of this receptor as an upstream mediator of the HMGB1-stimulated production of COX-2. As shown in Figure 3.2(B), a RAGE-blocking antibody prevented the HMGB1-promoted induction of COX-2, indicating a crucial role for this receptor in the inflammatory response of astrocytes to HMGB1. It has previously been

Table 3.1 Major Proteins Up-Regulated by HMGB1 in Primary Astrocytes\textsuperscript{a}

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>Protein</th>
<th>Score</th>
<th>Coverage (%)</th>
<th>Peptides Matched</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HSP10 (GI: 461731)</td>
<td>67</td>
<td>58</td>
<td>6</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>Ubiquitin and ribosomal protein L40 precursor (GI: 13928952)</td>
<td>73</td>
<td>26</td>
<td>3</td>
<td>n.d.\textsuperscript{b}</td>
</tr>
<tr>
<td>3</td>
<td>Histone H2B1 (GI: 12025526)</td>
<td>73</td>
<td>61</td>
<td>9</td>
<td>n.d.\textsuperscript{b}</td>
</tr>
<tr>
<td>4</td>
<td>Alpha enolase (GI: 56757324)</td>
<td>101</td>
<td>34</td>
<td>16</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>GFAP (GI: 5030428)</td>
<td>161</td>
<td>50</td>
<td>25</td>
<td>3.1</td>
</tr>
<tr>
<td>6</td>
<td>Golgi phosphoprotein3 (GI: 62461580)</td>
<td>1.58\textsuperscript{d}</td>
<td>13</td>
<td>4</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>Lipase A precursor (GI: 109460058)</td>
<td>88</td>
<td>20</td>
<td>5</td>
<td>8.1</td>
</tr>
<tr>
<td>8</td>
<td>Vimentin (GI: 14389299)</td>
<td>119</td>
<td>24</td>
<td>11</td>
<td>3.5</td>
</tr>
<tr>
<td>9</td>
<td>Vimentin (GI: 14389299)\textsuperscript{c}</td>
<td>73</td>
<td>54</td>
<td>19</td>
<td>3.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Protein scores greater than 59 are significant (p > 0.05) using MASCOT search engine.
\textsuperscript{b}n.d., not detectable in unstimulated cells.
\textsuperscript{c}Phosphorylated protein form.
\textsuperscript{d}This protein was recognized (p > 0.05) with ALDENTE search engine (www.expasy.org).
reported that up-regulation of COX-2 is also mediated by RAGE in microglia stimulated by S100B, through activation of a Cdc42–Rac1–JNK and a Ras–Rac1–NF-κB pathway [50]. RAGE can interact with several protein ligands that are accumulated in the extracellular matrix by dying cells or following tissue injuries and show the common characteristics of multiple β-sheets [51]. Although the wide expression and the ability to function as a sensor for the environmental cues suggest the implication of RAGE in inflammatory tissue responses, the relevant downstream signal pathways are not fully elucidated. Astrocytes stimulated with HMGB1 showed that the COX-2 immunoreactive signal disappeared in the presence of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK) kinase (MEK) inhibitor PD098059 (see Figure 3.2(B)), indicating that COX-2 induction requires activation of the MAPK/ERK1/2 pathway. A time-course analysis of ERK1/2 activation was carried out on HMGB1-stimulated astrocytes by using an antibody that recognizes phospho-42 ERK (p-ERK2) only when phosphorylated at Thr183 and Tyr185. As shown in Figure 3.3(A), HMGB1 promoted rapid activation of p-ERK2, reaching a maximum at 10 minutes; this effect subsided almost completely within 2 hours. This finding is in agreement with previous evidence showing that in neuron/astrocyte cell cultures and organotypic hippocampal slice cultures, the activation of ERK1/2 is necessary for the induction of COX-2 [52]. In contrast, HMGB1, even at a concentration up to 400 nM, did not trigger either activation of p38 MAPK or NF-κB, or degradation of IkBα (data not shown), events that are often related to the generation

Figure 3.2 Levels of the pro-inflammatory markers COX-2 and iNOS in astrocytes stimulated with HMGB1. Astrocyte proteins (20 μg) were subjected to Western blotting. β-Actin was used as a control for protein loading. (A) Astrocytes were left untreated (ctrl), or treated for 16 hours with the indicated additions; (B) astrocytes were left untreated, or pretreated for 15 minutes with the indicated additions and then stimulated with HMGB1 for 9 hours. Blots are representative of three separate experiments.

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It has been shown previously that the expression of iNOS is dependent on p38 MAPK and NF-κB activation in rat cortical astrocytes [54,55]. The inability of HMGB1 to activate both p38 MAPK and NF-κB in these cells suggests a reason for its ineffectiveness as an inducer of iNOS (see Figure 3.2(A)). Furthermore, pretreatment of astrocytes with an RAGE-blocking antibody prevented the HMGB1-promoted ERK2 phosphorylation (Figure 3.3(B)). Taken together, these results provide clear evidence of the involvement of the MAPK/ERK1/2 cascade as a downstream transducer of HMGB1/RAGE signaling in primary astrocytes. In keeping with this result, it has been demonstrated previously that ERK-1/2 could be involved downstream of RAGE signal transduction [56,57], and that the binding of HMGB1 to the cell surface induced intracellular ERK–RAGE interactions and enhanced ERK activity in the cell [58].

**Figure 3.3 Effect of HMGB1 on phosphorylation of ERK2.** Astrocyte proteins (20 μg) were subjected to Western blotting. (A) Upper panel: Time-course analysis of phospho-ERK1/2 (p-ERK1/2) in astrocytes stimulated with 40 nM HMGB1. The total amounts of ERK1/2 are shown in the lower panel. (B) Quantification of p-ERK1/2 in astrocytes untreated, or treated for 10 minutes with HMGB1. Where indicated, a monoclonal RAGE antibody (mAb anti-RAGE) was added 15 minutes before HMGB1 cell stimulation. Upper panel: Numbers under the p-ERK2 bands refer to the relative quantification of the immunoreactive signals. The total levels of ERK1/2 are shown in the lower panel. Blots are representative of three separate experiments.

3.2.2 Pattern of Inflammatory Genes Induced by HMGB1 on Primary Astrocytes

The profile of astrocytes activated by HMGB1 was further defined by the semi-quantitative reverse transcription-polymerase chain reaction reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of several genes that are up-regulated in the fully inflammatory transcriptome of these cells [43]. As shown in Figure 3.4, HMGB1 induced a significant increase in the levels of CCL5 and the ELR-chemokines CXCL1 and CXCL2. However, the level of the monocyte chemoattractant protein CCL7 transcript was not affected, suggesting that HMGB1 is a specific, rather than a general, activator of CC chemokine transcription in primary astrocytes. This specificity is further supported by the fact that HMGB1 induced a twofold increase in the level of the matrix metalloproteinase (MMP)-9 transcript, leaving MMP-2, MMP-3, and the tissue inhibitor of the matrix metalloproteinase (TIMP)2 unaffected. Because MMP-3 transcription increases in rat brain astrocytes stimulated with LPS [59], the lack of MMP-3 induction by our recombinant HMGB1 also confirms the functional insignificance of possible contaminating LPS. Moreover, LPS did not induce expression of the chemokines genes up-regulated by HMGB1, and the increase of MMP-9 gene expression promoted by LPS was also significantly lower than that obtained with HMGB1 (Figure 3.4).

As expected, HMGB1 induced a twofold increase in the level of the COX-2 transcript. However, this cytokine did not change the expression level of several

![Figure 3.4](image_url)

**Figure 3.4** Effect of HMGB1 on the transcriptional regulation of genes related to inflammatory activation of astrocytes. Total RNA was isolated from astrocytes stimulated with 40 nM HMGB1 (white bars) or CCM (gray bars) or 0.3 pg/ml LPS (checked bars) for 5 hours. Quantification of RT-PCR products relative to the indicated target genes was carried out by the Quantity One Software. The expression of Glycer aldehyde 3-phosphate dehydrogenase was used as an internal control. Three independent experiments were performed. *p < 0.05; #p < 0.01 versus untreated cells (solid horizontal line).

inflammatory genes related to immunity (caspase 4, interferon-induced protein with tetratricopeptide repeat (IFIT)3, and IL-6), signaling (interferon regulatory factor-1 (IRF1) and TNF-α), and vascular cell adhesion molecule-1 (VCAM1). It has been shown previously that IL-1 is an inducer of VCAM1 in astrocytes [60], so the pattern of genes induced by HMGB1 is consistent with activated transcription of a restricted number of inflammatory genes, different from those induced by classic pro-inflammatory cytokines. It is noteworthy that recent observations indicate HMGB1 as a potentiating factor for the pro-inflammatory functions of IL-1β [35]. The lack of VCAM1 induction in astrocytes exposed to HMGB1 indicates that our astrocyte cultures do not contain even low amounts of IL-1β. It has also been demonstrated previously that some of the pro-inflammatory functions of HMGB1 can derive from various contaminants bound to this sticky protein [61]. The ability of HMGB1 to interact with inflammatory agents, such as LPS, phosphatidylserine, DNA, and some inflammatory cytokines, indicates that if we are to dissect the direct signaling role of HMGB1 on different cell models, we will have to utilize the cytokine in a highly purified form and to consider possible effects played by suboptimal amounts of HMGB1-binding molecules rather than by HMGB1 itself. In conclusion, in primary astrocytes HMGB1 induces a specific pattern of gene expression, which includes chemotactic molecules and a proteolytic enzyme that facilitates tissue infiltration by inflammatory cells.

3.2.3 Analysis of Chemokines and Metalloproteinases Released by HMGB1-Stimulated Astrocytes

To determine whether induction of specific chemokines was parallel to their enhanced release, the conditioned media of primary astrocytes stimulated with HMGB1 were subjected to ELISA assays. As reported in Figure 3.5, the release of CCL5 was highly up-regulated by HMGB1, and the extracellular amounts of this chemokine depended on the time of stimulation and on the concentration of HMGB1 added.

Owing to the role of CCL5 as an inducer of multiple chemokines and cytokines in astrocytes [62], it has been suggested that this chemokine could initiate inflammatory cascades in the CNS. Hence, the release of CCL5 by HMGB1-stimulated astrocytes can contribute to the amplification of inflammatory events by autocrine/paracrine signaling. This assumption is suggested by the presence of several types of redundant receptors for CCL5 on the surface of astrocytes. Furthermore, HMGB1 stimulated the cell production of CCL2, CCL20, and CCL3. CCL3 and CCL5 recognize the CCR5 receptor and operate as stimulators of immature dendritic cell migration. An important role for CCR5 and its ligands has been suggested by experimental evidence indicating that desensitization of this receptor abrogates the recruitment of dendritic cells to inflamed regions of the CNS [63]. Both CCL5 and CCL2 have also been identified as chemokines that guide macrophages/microglia and T cells to sites of injury/inflammation [64]. Because astrocytes are abundant in the CNS and are localized in proximity to the blood–brain barrier (BBB), their up-regulation of CCL5 and CCL2 could directly contribute to rapid pro-inflammatory responses by attracting both resident microglia and circulating monocytes. Astrocytes and microglia/macrophages express the receptor for CCL2, named CCR2 [65]. Hence, this chemokine
can directly activate astrocytes, through autocrine or paracrine mechanisms, stimulating reactive gliosis but also promoting migration and activation of effector cells in demyelinating lesions. Of note, previous observations showed that cortical injection of HMGB1 in mouse brain did not increase the level of pro-inflammatory mediators such as iNOS and IL-1β, but did increase the sensitivity to ischemic injury [29]. Hence, HMGB1 seems to require the presence of substimulatory concentrations of other glia-activating factors to display neuroinflammatory properties in vivo.

Recently, we demonstrated that HMGB1 elicits the release of excitatory amino acids from astrocyte-derived subcellular particles (gliosomes), but not from nerve endings (synaptosomes), by altering the activity of the glutamate–aspartate transporter [41]. It has been proposed that glial CCL5 facilitates the spontaneous release of glutamate from human nerve endings [66]; hence, we hypothesize that the concomitant increase of glutamate and CCL5 release from astrocytes exposed to extracellular HMGB1 can contribute to the amplification of both neurodegenerative and neuroinflammatory processes in acute and chronic CNS diseases.

To assess the role of the MAPK–ERK1/2 cascade in the induction of astrocyte CC chemokines, cells were stimulated with 40nM HMGB1 for 24 hours in the presence of the MEK inhibitor PD095058. In this condition, the release of the various CC

Figure 3.5 Quantification of CC chemokines released by astrocytes stimulated with HMGB1. Astrocytes were cultured in the presence of the indicated amounts of HMGB1, and quantification of each chemokine in the conditioned media was carried out by ELISA. Where indicated, 50μM PD098059 was added to the cell culture 15 minutes before HMGB1. Experiments were performed twice. *p < 0.05; #p < 0.01 versus cells stimulated for 24 hours with 40nM HMGB1.

chemokines decreased markedly. Concerning the CXC chemokines transcriptionally induced by HMGB1 (see Figure 3.4), we observed that CXCL1 and CXCL2 were actively released by astrocytes stimulated with HMGB1 in a time- and concentration-dependent manner (Figure 3.6).

Although astrocytes are probably the only source of chemokines containing the ELR motif in the CNS, both neurons and astrocytes can respond to this chemokine family because both express specific receptors for these chemokines [67]. The CXC chemokine pathway has been associated with BBB compromise and the clinical manifestation of autoimmune demyelination [68]. CXCL1 and CXCL2 seem to play important roles in multiple sclerosis as chemoattractants of destructive immune cells that form the characteristic infiltrates in areas of demyelination. Specifically, CXCL1, a functional homologue of human IL-8, is mainly associated with neutrophil recruitment from the blood, an early step of acute inflammation. Concurrence of CXCR2
on oligodendrocytes and induced CXCL1 on hypertrophic astrocytes in multiple sclerosis have also been proposed as a novel mechanism for recruitment of oligodendrocytes to areas of damage—an essential prerequisite for lesion repair in this devastating human pathological condition [69]. Interestingly, it has recently been shown that the levels of HMGB1 correlate with active inflammation lesions of multiple sclerosis and experimental autoimmune encephalomyelitis [32]. Altogether, these findings suggest that HMGB1 could be involved in the molecular mechanisms responsible for disease progression.

Finally, we detected an HMGB1-dependent astrocyte release of the CX₃CL1 chemokine, the only member of the δ-chemokine subfamily. CX₃CL1 is almost exclusively expressed in the brain, where it plays an important role in neuroinflammation and neurodegeneration [70]. It has been demonstrated previously that this chemokine is induced in astrocytes within inflammatory lesions and plays a critical role in neuron-to-glial communication after peripheral nerve injury and inflammation [71]. A membrane-bound form of this chemokine has been interpreted as a cell adhesion molecule for circulating inflammatory cells [72]. Conversely, the soluble form of CX₃CL1 can create a chemotactic gradient for natural killer (NK) cells in the CNS [73]. However, this chemokine plays multiple roles. At low concentration, it seems to be predominantly involved in cell regulation, survival, and proliferation, though it also reduces the excitotoxicity caused by excessive neuronal exposure to glutamate [74]. Hence, it has been proposed that this chemokine can play a general physiological function and can behave as an anti-inflammatory mediator. Following the identification of a proteinase involved in the cleavage of CX₃CL1 from the cell membrane, it has been postulated that the regulation of this cleavage may affect the biological functions of this chemokine. The present finding, showing that HMGB1 induces astrocytes to express and release CX₃CL1, is consistent with a very complex role played by this cytokine in the CNS, as a modulator of cell mechanisms underlying both neurodegenerative and protective functions.

The release of CXC and CX₃C chemokines was markedly reduced by PD098059, providing further evidence that the MAPK/ERK1/2 cascade is a fundamental mediator in the astrocyte activation program promoted by HMGB1 signaling. It has been shown previously that reactive astrocytes, in response to inflammatory agents, are able to secrete a set of extracellular matrix-degrading enzymes that include the matrix metalloproteinases [75,76]. Increased MMP proteolytic activity contributes to the pathogenesis of many neuroinflammatory and neurodegenerative conditions in the CNS. Early and late induction of different MMPs has been proposed to play a role in neuronal death as well as in repair processes following hypoxia–ischemia insults [77]. Zymography analysis (Figure 3.7(A)) revealed that the conditioned media of astrocytes subjected to stimulation with HMGB1 for 24 hours contained increased amounts of MMP-9. The proteinase was released in an HMGB1-dose-dependent manner and again, this cell response to HMGB1 was prevented by PD098059 (Figure 3.7(B)).

In contrast, MMP-2 was not modulated by HMGB1, and the MEK inhibitor PD098059 did not affect the release of this metalloproteinase. The ability of HMGB1 to induce MMP-9 release further supports the idea that HMGB1 displays signaling properties quite different from those typical of classical inflammatory agents such
Selective Pro-Inflammatory Activation of Astrocytes by High Mobility Group 65 as LPS or TNF-\(\alpha\) [78]. Taken together, these results corroborate the transcriptional profile and further extend the number of chemokines that are actively released by astrocytes exposed to nanomolar concentrations of HMGB1.

3.3 Functional Properties of HMGB1-Activated Astrocytes

It has previously been shown that astrocytes regulate the homing of different leukocyte subsets to the inflamed CNS [63]. To establish the functional properties of HMGB1-activated astrocytes, following cell exposure to HMGB1 we tested the conditioned medium for its capacity to induce chemotaxis on the monocytic cell line Mono Mac 6. This cell target was selected because (i) it is known that nanomolar concentrations of extracellular HMGB1 do not affect monocyte chemotaxis [79], (ii) CCL2 is one of the chemokines most represented in the conditioned medium of HMGB1-activated astrocytes, and (iii) CCL2 behaves as a monocyte chemoattractant. As shown in Figure 3.8, we confirmed that the cell line Mono Mac 6 is insensitive to 40 nM HMGB1. We also demonstrated that the conditioned media, from astrocytes

![Figure 3.7](image_url)
stimulated with 4 and 40 nM HMGB1, show a threefold and sixfold increase, respectively, in chemotactic activity in comparison with the medium of unstimulated cells. Moreover, the chemotactic activity of the conditioned medium from astrocytes exposed to 40 nM HMGB1 was similar to that obtained upon cell exposure to the CCM. Of note, the conditioned medium of astrocytes treated with 0.3 pg/ml LPS, corresponding to the amount of contaminating LPS present at the maximal concentration of recombinant HMGB1 utilized in this experiment, did not display chemotactic activity on the monocytic cell line Mono Mac 6.

Although the complex mixture of bioactive molecules released by HMGB1-stimulated astrocytes has not as yet been fully characterized, these results indicate that it displays potent monocyte chemoattractant properties. We can speculate that CCL2, present at high concentrations in the conditioned medium of HMGB1-stimulated astrocytes, could also promote migration of neural progenitor cells (NPCs), thus contributing to the neurogenesis that occurs during development of, and throughout adult life in, the CNS [80]. Moreover, the mixture of factors released by astrocytes stimulated with HMGB1 also contains CX3CL1, a chemokine able to protect neurons from glutamate-induced toxicity [81]. Hence, the concomitant presence of CCL2 and CX3CL1 could exert a positive effect on neuron survival. In contrast, it has been reported that CCL2 is also involved in neurotoxic microglia activity.
Selective Pro-Inflammatory Activation of Astrocytes by High Mobility Group 67. Thus, the final tissue response to HMGB1 is the result of a complex network of interactions among several signaling molecules, relevant receptors engaged, and different sensitive cell types. A number of important parameters converge to determine a protective or a detrimental response, including the effective concentrations of cytokines and chemokines involved, the duration of these stimuli, and their capability to interact with each other in a synergistic or antagonistic manner.

## 3.4 Conclusion

Early studies on the extracellular signal properties of HMGB1 indicated that this protein is a potent immediate and late mediator of inflammation [6,12]. However, experimental evidence reported in this review, and in other recent reports, supports the assumption that HMGB1 behaves as a key regulator of CNS functions, but indicate that it is not endowed with classical pro-inflammatory properties both in \textit{in vitro} and \textit{in vivo} conditions [22,58]. This discrepancy can be attributed to the remarkable ability of HMGB1 to interact with several unrelated protein molecules, resulting in the formation of protein complexes that acquire new signaling properties. Specifically, it has been shown that the high inflammatory activities previously attributed to HMGB1 could be due to a highly enhanced pro-inflammatory function displayed by IL-1β and IFN-γ bound to HMGB1 [35]. Hence, we know that HMGB1 can directly activate different cell programs, depending on the specific receptors expressed and on the intracellular cascades involved, but it can also amplify inflammatory responses through binding to pro-inflammatory mediators. An emerging field of research is aimed to shed light on the molecular mechanisms that modulate the inflammatory processes in the CNS. Exploration of the involvement of HMGB1 in specific physiopathological conditions is needed if we are to understand the contribution of this cytokine in neuroinflammation, neurological disorders, and postischemic damage, as well as in regulation of the neuroimmune system. Moreover, the potential roles of HMGB1 as an inducer of tissue reparation in mild acute inflammatory diseases, and also as a mediator of damage in chronic inflammatory conditions, pose several challenging questions about the possibility of exploiting this versatile cytokine as a target of new therapeutic strategies.

## Acknowledgments

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## References


Section D

Physiology

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Choroid Plexus and Immune Response of the Brain

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Department of Neurosurgery, University of South Florida, Tampa, FL, and
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4.1 Introduction

The choroid plexuses (CPs), located within the ventricles of the brain, produce the cerebrospinal fluid and form an interface between the peripheral blood and the CSF. Key functions related to brain homeostasis that have been ascribed to CP include processes that establish, survey, and maintain the biochemical and cellular status of the brain under normal and pathological conditions. Recent evidence implies that CP is a suitable source of transplantable immune factor- and tropic factor-secreting cells with neuroregenerative and neuroprotective capabilities. Indeed, conditioned media from CP promotes neurite outgrowth and prevents the death of cultured embryonic neurons [1–3]; furthermore, CP transplants promote regeneration of damaged spinal cord and reduce the functional and structural consequences of brain trauma in animal models of stroke [1,4]. In parallel, transplanted encapsulated neonatal porcine CP also exerts neuroprotective effects in rat [5] and monkey models of Huntington’s disease (HD) [3]. In this chapter, we describe the role of the CP in brain development, normal brain homeostasis, immune surveillance, and aging, and also discuss representative CNS disorders affected by abnormal CP functions. Finally, we draw from recent laboratory findings demonstrating the efficacy and safety of transplanting CP cells for treating brain disorders.

4.2 Choroid Plexus: Basic Structure and Function

Gross examination of the CP reveals its basic structure as lobulated with a single continuous layer of cells derived from the ependymal lining of the ventricles (Figure 4.1). The intricate morphology of the CP originates from its fronds projecting into the fluid-filled ventricles, which is thought to allow the CP to perform complex functions. The inner layer of the projections contains a densely enriched bed of vascular supply interspersed between connective tissue and epithelium. On basal lamina rest choroidal epithelial cells, which form a large central spherical nucleus with abundant cytoplasm possessing numerous villi on their luminal surface (Figure 4.2(A)).
The lined epithelial cells are connected by tight junctions that physically restrict the movement of substances between blood and cerebrospinal fluid, fittingly called the blood–CSF barrier (Figure 4.2(B)). The complex configuration of the CP is further revealed by the capillaries of the vascular bed, which are large, with thin fenestrated endothelial walls and bridging diaphragms overlying the fenestrations. This specialized CP structure resembles that of the gastrointestinal tract, characterized by successively smaller folds that provide increased surface area and thereby enhance mixing of fluids. The abundance of organelles may be related to the CP’s multiple secretory functions, such as metabolism and protein synthesis (Figure 4.2(C)).

Foremost among the many established functions of the CP is CSF production [6]. The importance of this CP role cannot be understated, in view of the fact that humans require maintenance of CSF volume at about 80–150 ml, requiring new CSF production at a rate of approximately 500 ml/day. The blood–CSF barrier is critical to CSF production, in that CSF is generated primarily by active secretion, with water entering the CSF from the blood along an osmotic gradient or via specific water channels such as aquaporin. The barrier function of CSF containment transfers to the CP as CSF shifts from the vasculature to the epithelium, where tight junctions form between the epithelial cells to confer the permeability properties of the individual cells [7]; this further exemplifies the prominent role of the CP in CSF production.

An equally major role of the CP in brain functioning relates to guarding the CSF from, and monitoring for, the presence of noxious compounds or potentially damaging cellular invasion [8,9]. The highly selective permeability of the CP protects the brain...
by monitoring the overall biodistribution of drugs and toxic compounds, for which it uses a full complement of metabolizing enzymes (including Phase I–III enzymes) for functionalization, conjugation, and transport of drugs. Examples of detoxifying mechanisms of the CP include the utilization of (a) high concentrations of glutathione, cysteine, and metallothioneins that potently sequester toxic agents circulating in the CSF; (b) protective enzymes such as superoxide dismutase, glutathione-s-transferase, and glutathione peroxidase and reductase to provide a barrier protecting against free-radical oxidative stress; and (c) organic ion transport systems and multidrug resistance proteins for filtering detrimental substances from the CSF.

### 4.3 Choroid Plexus and the Immune System

Recently, maintenance of a tolerant or responsive immunological status of the brain has been designated as another important neural homeostasis function of the CP [10]. Although the CNS has long been thought of as an immune-privileged organ, such immunity is not absolute. Indeed, it is well documented that the CNS can mount an immune response within the brain tissue. The immune reaction is tightly controlled by the CNS via blockade of immune-cell entry into the brain, with such responsibility largely borne by the blood–brain barrier (BBB) and CP blood–CSF barrier. Whereas immune-cell migration into the CNS is almost absent under physiological conditions, immunocompetent cells are able to breach the BBB and CP blood–CSF barrier and subsequently gain access to the brain [10]. CNS pathogenic conditions, such as viral or
bacterial infections, or those that occur during inflammatory diseases, such as multiple sclerosis and stroke, are characterized by immune-cell entry across the blood–CNS barriers. In the animal model of experimental autoimmune encephalomyelitis, which is considered the prototype model for the human disease multiple sclerosis, T cells are found to penetrate the CNS and initiate the molecular and cellular events leading to edema, inflammation, and demyelination in the CNS [10]. Here, in addition to the BBB as the entry point for the circulating immune cells into the CNS, the CP blood–CSF barrier is established as an equally critical brain passage for circulating lymphocytes. During experimental autoimmune encephalitis (EAE), the CP undergoes deleterious ultrastructural changes, including up-regulated expression of VCAM-1, ICAM-1, and MAdCAM-1 localized on the apical surface of CP epithelial cells, but completely lacking on the fenestrated endothelial cells. The increased ICAM-1, VCAM-1, and MAdCAM-1 expression in CP epithelium mediates binding of lymphocytes via their known ligands. Parallel in vitro studies revealed that CP epithelial cells can be induced to express ICAM-1, VCAM-1, MAdCAM-1, and, additionally, MHC class I and II molecules on their surfaces [10], further advancing the notion that CP is closely associated with CNS immunosurveillance. Taken together, investigations into the mechanisms underlying immune-cell entry into the CNS, especially the sustained integrity of the CP blood–CSF barrier during pathological conditions, may reveal potential treatments that can be directed against CNS immune or inflammatory diseases.

### 4.4 Choroid Plexus and Brain Development

As noted in Section 4.3, the strategic location of the CP makes it an excellent brain structure for distributing molecules to the brain. In studies following this logic, CP has been demonstrated to be a major source of biologically active compounds (Table 4.1). These capabilities allow the CP to monitor and respond to the biochemistry of the brain by manipulating and maintaining baseline levels of the extracellular milieu throughout the CNS. The molecules secreted by the CP gain access to the brain parenchyma via volume transmission, convective distribution, and intraparenchymal diffusion/receptor-mediated retrograde transport [1–5,11].

During development, CPs form early during embryogenesis, assisting in control of the developing extracellular environment [12] by secreting morphogens, mitogens, and trophic factors that guide and pattern both the general and specific growth of the brain [9,13]. In particular, the embryonic CP contains high levels of insulin-like growth factor (IGF)-II localized in the floor plate of the hindbrain, which prompted speculations that CP-derived IGF-II diffuses to and binds to IGF receptors on the floor plate cells and activates their role in guiding spinal axon growth [14]. Further support for CP contribution to morphogenesis comes from demonstrations that the radial migration of cerebral cortical neurons from the ventricular and subventricular zone to the cortical plate is governed by gradients of soluble factors, such as CP-secreted Slit proteins [15–17]. In vitro, CP is found to secrete a soluble factor, related to Slit2, that diffuses through the CSF and aids in establishing a gradient of a repulsive cue guiding cortical neurons away from the ventricular surface [17].
In vivo, CP is implicated in the modulation of neurite outgrowth in the developing cerebellum [18]. Using co-cultures of explanted cerebellum and fourth-ventricle CP from fetal and infant rats, CP was shown to secrete a soluble neurite-growth factor exhibiting a biphasic feature that correlates with the major milestones of cerebellar

Table 4.1 Gene Array Analysis Showing Examples of the Diversity of Genes Expressed in High Abundance within Porcine CP

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selected therapeutic proteins of interest</td>
<td></td>
</tr>
<tr>
<td>Transthyretin</td>
<td>23,478</td>
</tr>
<tr>
<td>Connective tissue growth factor</td>
<td>20,951</td>
</tr>
<tr>
<td>Transforming growth factor (\beta)1</td>
<td>9,545</td>
</tr>
<tr>
<td>Neuronatin</td>
<td>8,420</td>
</tr>
<tr>
<td>Osteoclast stimulating factor</td>
<td>7,242</td>
</tr>
<tr>
<td>Tissue factor</td>
<td>6,557</td>
</tr>
<tr>
<td>Axotrophin</td>
<td>5,179</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>4,260</td>
</tr>
<tr>
<td>Neuronal endocrine protein</td>
<td>3,636</td>
</tr>
<tr>
<td>Neuronal protein 3</td>
<td>2,781</td>
</tr>
<tr>
<td>Pigment epithelium derived factor</td>
<td>1,352</td>
</tr>
<tr>
<td>Matrix and adhesion factors</td>
<td></td>
</tr>
<tr>
<td>Integrin, (\beta)1 binding protein 1</td>
<td>23,753</td>
</tr>
<tr>
<td>Laminin receptor 1</td>
<td>15,791</td>
</tr>
<tr>
<td>Matrix metalloproteinase 9</td>
<td>9,399</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>8,039</td>
</tr>
<tr>
<td>Metalloproteinase inhibitor 1 (TIMP-1)</td>
<td>7,983</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>7,451</td>
</tr>
<tr>
<td>Type III collagen</td>
<td>4,709</td>
</tr>
<tr>
<td>Integrin, (\beta)5</td>
<td>2,776</td>
</tr>
<tr>
<td>Integrin, (\beta)4</td>
<td>2,430</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1,737</td>
</tr>
<tr>
<td>Integrin, (\alpha)__</td>
<td>1,847</td>
</tr>
<tr>
<td>Cytoskeletal components</td>
<td></td>
</tr>
<tr>
<td>(\beta)-actin</td>
<td>19,715</td>
</tr>
<tr>
<td>Vimentin</td>
<td>17,416</td>
</tr>
<tr>
<td>Tubulin, (\alpha)2</td>
<td>15,099</td>
</tr>
<tr>
<td>Tubulin, (\beta)1</td>
<td>14,403</td>
</tr>
<tr>
<td>Villin 2</td>
<td>11,168</td>
</tr>
<tr>
<td>Dynemin</td>
<td>10,093</td>
</tr>
<tr>
<td>Actinin, (\alpha)1</td>
<td>8,919</td>
</tr>
</tbody>
</table>
morphogenesis. Additionally, rats with hydrocephalus, and thus impaired CP structure and function, also have impaired cortical development, suggesting that the factors in circulating CSF are vital for development [19].

### 4.5 Choroid Plexus and Aging

As with developmental effects, CP also directly influences aging. In humans, the height of CP epithelial cells decreases by about 10–11% during life [20]. The aged epithelial-cell cytoplasm becomes rich with Biondi Ring tangles and lipofuscin deposits [21], and the nuclei appear irregular and flattened as the basement membrane thickens [20]. The stroma thickens and collects collagen fibers, hyaline bodies, calcifications, and psammoma bodies, and the infiltrating arteries become thicker and fragmented [22,23]. Analogous aberrant changes occur in aged mouse and rat choroid epithelial cells [24,25].

Because the overall functions of CP are energy dependent, and with energy metabolism compromised in aging, the CP cannot maintain its normal energy output in the aged brain. Synthesis of enzymes needed for anaerobic respiration and oxidative phosphorylation decline in aging rats, with lactate dehydrogenase and succinate dehydrogenase decreasing 9% and 26%, respectively [26]. As a consequence of the aging-mediated reduction in energy metabolism, there is a surge in the number of epithelial cells deficient in cytochrome C oxidase, which alters the respiratory mitochondrial chain and decreases ATP production [27]. Reductions in Na\(^+\)/K\(^+\)-ATPase and the Na\(^+\)/K\(^+\)–2Cl\(^–\) co-transporter also occur [28]. The combined anatomical and enzymatic deteriorations could lead to a diminution of CSF secretion that translates to about a 45% decrement in aged animals [29]. As a result of reduced secretion and the simultaneously increased CSF volume caused by brain atrophy, CSF turnover is significantly longer in elderly rats (7.9 hours) than in young rats (2.2 hours). In agreement with the aged rat research, CSF production has been reported to diminish with aging in humans, from 0.41 ml/min at 28 years to 0.19 ml/min at 77 years [9,22]. Together with age-related cerebral atrophy, the turnover of CSF decreases to less than 2 times daily in elderly subjects, versus 3–4 times per day in young adults. These significant alterations in CP and CSF have been postulated to result in inadequate distribution of nutritive substances, additional cellular stress, and reduced clearance of toxic compounds. The functional consequences of an aged CP are also recognized at the level of clinical symptoms, including accelerated cognitive and motor decline and/or the development of certain neurological disorders.

### 4.6 Choroid Plexus and Neurodegeneration: Alzheimer’s Disease as a Case in Point

In an effort to elucidate the influence of an aging CP on CNS disorder, we discuss laboratory evidence demonstrating CP alterations in Alzheimer’s disease (AD). The age-related deficiencies of CP are exacerbated in AD, and are characterized
by significantly greater epithelial-cell atrophy, with cell height decreasing up to 22% relative to age-matched controls [20]. Moreover, AD leads to a greater intracellular distribution of lipofuscin vacuoles and Biondi Ring tangles in the CP [21]. Concomitant to late-onset AD, the CP epithelial basement membrane becomes very irregular and thickens an additional 28% beyond that seen in age-matched controls [20]. Additional pathological features reminiscent of AD pathology that occur in CP include fibrotic stroma of the villi, with extensive vascular thickening and numerous hyaline bodies, and calcifications with deposits of IgG, IgM, and C1q along the epithelial basement membrane [21,27]. These structural confounds lead to reduced CSF secretion, reducing turnover to 36 hours in AD patients [22].

The progression of atrophy in choroidal epithelial cells in AD is associated with pronounced reductions in secretory activity and transport functions. Levels of transthyretin (TTR), a CP-synthesized molecule that associates with β-amyloid peptide to form complexes, are more than 10% lower in AD [30]. Levels of ascorbic acid and α-tocopherol, the two major scavengers of free radicals in CSF, are decreased in AD, likely adding to oxidative stress [31,32]. CSF folate and vitamin B12 (important for methylation of numerous molecules) are significantly lower [33–35], while homocysteine, which mediates lipid peroxidation and increases the production of toxic (E)-4-hydroxy-2-nonenal, is increased in AD CSF. The impaired ability of the CP to clear molecules from the CSF has profound implications [22]. In rats, clearance of intraventricularly injected β-amyloid peptide decreases from 10.4 μl/min at 3 months of age to 0.71 μl/min at 30 months. Consequently, in young rats the brain content of amyloid peptide increases from 7% at the end of CSF perfusion as compared to 49% in old animals [29]. The increase of β1-40 and β1-42 amyloid peptide levels in elderly humans could be related to decreased clearance from the CNS.

Decreased CSF production could also enhance protein glycation and the formation of β-amyloid oligomers [22]. The AD brain contains elevated levels of glycation products and deposits of amyloid peptide; senile plaques and fibrillar tangles contain advanced glycation products [22]. Glycation promotes protein aggregation, the polymerization of tau microtubule associated proteins, and protein β-amyloid peptide aggregation. The reduced CSF turnover, the increase of protein glycation, and the diminution of β-amyloid clearance, taken together, could induce oligomer formation and retention. More importantly, the compromised ability of the CP to perform its CNS homeostasis functions could exacerbate AD progression, leading to methylation problems, increased oxidative stress and lipid peroxidation, decreased amyloid clearance, augmented tau protein polymerization, and formation of amyloid peptide oligomers and fibrils [22].

4.7 Choroid Plexus as Transplantable Cells for Brain Repair: Pilot Studies

Cell therapy has emerged as an experimental treatment for CNS disorders. The observation that CP function deteriorates as a result of aging and pathologic condition provides the impetus to rescue the damaged CP by transplanting healthy CP [9].
The diminished function of the aged/diseased CP may respond much like other diseases characterized by secretory cell dysfunction, where the principle of transplanting or replacing a failing organ (such as CP) or specific cell type is a logical means of restoring lost function. Based on the finding that CP secretes a multitude of growth factors, we advanced the novel concept that the CP is a potential source of stable, dose-controlled polypeptide delivery [36].

In vitro studies demonstrate that CP isolated and maintained in culture exerts potent neuroprotective effects [1–3]. Conditioned media from alginate-encapsulated CP promoted the survival and extension of neurites from embryonic cortical neurons against serum deprivation-induced cell death. This effect was dose dependent and nearly complete with 10–30% conditioned media. These data dovetail nicely with a study in which mouse CP epithelial cells were cultured with dorsal root ganglion (DRG) neurons [2]. After 4–5 hours of co-culture, the DRG neurons developed elongated neuronal processes with elaborate branching patterns over the surface of the epithelial cells. The ability of CP cells to provide a scaffold for the extension of neurites is consistent with its known production of extracellular matrices including laminin and fibronectin [37,38]. The trophic and tropic effects of CP establish potentially excellent circumstances for the protection and repair of damaged CNS architecture.

In a similar fashion using in vivo models of brain disorders, the delivery of neurotrophic factors via CP transplants to the site of injury also offers theoretical promise for treating spinal cord trauma. In a rat model of damaged spinal cord, syngeneic fragments of CP grafted into the dorsal funiculus (C2 level) showed that epithelial cells of the grafted CP survived well and induced robust regeneration of the damaged axons of the spinal cord [11]. Injections of horseradish peroxidase into the sciatic nerve labeled regenerating fibers extending from the fasciculus gracilis into the graft within 7 days after transplant. This effect was evident for at least 10 months. Some axons elongated rostrally into the dorsal funiculus and long-duration evoked potentials were recorded 5 mm rostral to the lesion 8–10 months after grafting. This study demonstrates that CP transplants afford robust therapeutic benefits in a model of spinal cord injury.

4.8 Encapsulated Choroid Plexus Cells as Immunoisolated Transplant Approach

In an effort to apply the concept of CP transplantation in the clinic, we recognized the limitation of harvesting of CP from humans. The alternate approach of harvesting CP from other species carries its own technical problems, especially the issue of graft-versus-host diseases. In this regard, the idea of immunoisolation is appealing, as it may circumvent the limitations inherent in cross-species (xenogeneic) transplantation of CP. Immunoisolation is based on the observation that xenogeneic cells can be protected from host rejection by encapsulating, or surrounding, them within an immunoisolatory, semipermeable membrane. Single cells or small clusters of cells can be enclosed within a selective, semipermeable membrane barrier that admits
oxygen and required nutrients and releases bioactive cell secretions, but restricts passage of larger cytotoxic agents from the host immune defense system.

To date, all studies using encapsulated CP for CNS transplant studies have utilized microcapsules formed using alginate. Alginate—one of the most frequently investigated biomaterials for cell encapsulation—is a polysaccharide composed of guluronic (G) and mannuronic (M) acid linked by α- and β-glycoside bonds. The ratio of these monomers contributes directly to certain physical characteristics of the polysaccharide. Once cationically crosslinked, materials high in G are more brittle, because of a more networked structure resulting from α bonds, whereas those high in M, with more linear β linkages, exhibit decreased three-dimensional crosslinking and greater elasticity [36,39,40].

Prior to cell encapsulation, alginate powder is typically reconstituted in a suitable buffer, and a variety of purification techniques are employed to rid the solution of proteins, endotoxins, and polyphenols. These techniques include solvent extraction, sequential filtration, charcoal extraction, dialysis, and others. Contaminant removal is essential to maintaining the optimal balance of hydrophilicity as well as to preventing inflammation related to endotoxin. The purification process ultimately determines the final physical and chemical characteristics of the encapsulated cell product, as fine variations in copolymer ratio, molecular weight, and purity can all be controlled at this step. Following purification and reconstitution of the alginate solution at a suitable pH, quality control analysis is carried out to maintain optimal operating specifications for encapsulation and subsequent in vivo longevity. The final purified alginate can be characterized both as a raw material and as a formed capsule using the analytical techniques shown in Table 4.2.

For the xenogeneic transplantation paradigm, neonatal porcine CP (7–14 days of age) is isolated from the lateral ventricles and dissociated using conventional collagenase digestion procedures. The resulting cell clusters are groupings of epithelial cells ranging from 50 to 200 μm in diameter. Prior to encapsulation, viability, which is typically greater than 95%, is confirmed by staining the cells with a vital dye. The encapsulation process does not affect cell viability, so these cells can be maintained in culture for months if needed or desired. The cultured CP clusters maintain the typical genotypic and phenotypic characteristics of the native, undigested tissue. Based on the knowledge that the CP epithelium is rich in tight junctions and lined with microvilli, we have used immunocytochemical techniques (zonula occludens; ZO-1) to identify tight junctional complexes and the tubulin associated with the cytoskeleton of the microvilli (Figure 4.3).

Following confirmation of cell viability and phenotype, CP cells are encapsulated in alginate microcapsules by extruding a mixture of cells dispersed in 1.7% sodium alginate through a droplet-generating apparatus into a bath of cations. This process is typically performed at an encapsulation density of 10,000–50,000 clusters or 200,000–5M cells/ml alginate. The cells, entrapped in the calcium-alginate gel, are coated twice with poly L-ornithine (PLO), followed by an outer coat of alginate. The central core of alginate is liquefied by chelation. The resulting microcapsules have a diameter of between 500 and 750 μm.

The alginate/PLO microcapsule appears to be very stable when implanted into the brain relative to a commonly used transplant site such as the peritoneum. Using
Fourier-transform infrared spectroscopy (FTIR), the surface of explanted capsules (up to 6 months in the brain or peritoneum) was analyzed for the relative proportion of alginate (outer coat) and PLO (middle coat). Using a mathematic relationship between FTIR peaks related to these two material components, an index was generated to compare the stability of the microcapsules. A notable difference was observed: breakdown in the peritoneum was rapid, whereas identical alginate capsules transplanted into the brain were completely stable for the duration of the six-month study [39,40].

<table>
<thead>
<tr>
<th>Analytical Method</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bulk Material Analysis</strong></td>
<td></td>
</tr>
<tr>
<td>$^1$H-NMR</td>
<td>Uronic acid ratio (M:G). Critical for physical and chemical stability of formed capsules.</td>
</tr>
<tr>
<td>SEC-MALLS</td>
<td>Weight-average molecular weight ($M_w$) calculations for overall chain length and sample homogeneity (polydispersity).</td>
</tr>
<tr>
<td>Dynamic viscosity</td>
<td>Used to calculate intrinsic viscosity and molecular weight. Useful for controlling droplet formation and encapsulation procedure.</td>
</tr>
<tr>
<td>Protein concentration</td>
<td>Bulk quantification of alginate purity and potential for degradation/host response.</td>
</tr>
<tr>
<td>FTIR</td>
<td>Alginate purity and comparison against a standard. Useful for characterizing stability based on ratio of alginate to polycation peaks.</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>Quantification of endotoxin impurities per FDA guidelines and to minimize tissue response.</td>
</tr>
<tr>
<td><strong>Alginate-Polycation Microcapsules</strong></td>
<td></td>
</tr>
<tr>
<td>Viability</td>
<td>Vital dye for determination of dosing, vitality, and biomass of encapsulated product.</td>
</tr>
<tr>
<td>Diffusion</td>
<td>Predictor of isolation capability and pharmacokinetic potential.</td>
</tr>
<tr>
<td>Burst</td>
<td>Bulk modulus of material and ultimate physical strength of microcapsule.</td>
</tr>
<tr>
<td>Postencapsulation phenotype</td>
<td>Confirmation of potential cell functionality and morphology.</td>
</tr>
<tr>
<td>Microbiology/virus screening</td>
<td>Screen for product acceptance and lot release.</td>
</tr>
<tr>
<td>Morphometry</td>
<td>Batch polydispersity and average size of capsules and their respective wall thickness.</td>
</tr>
</tbody>
</table>
4.9 Encapsulated Xenogeneic Choroid Plexus Transplants for Stroke Therapy

Stroke is the third leading cause of death and a leading health care burden in developed countries. There are no effective treatments for mitigating the neuronal loss following stroke, although neural transplantation may be one means of repairing the stroke-ravaged brain. Delivery of therapeutic molecules via cell transplantation soon after stroke might be useful for reducing or preventing the disease pathology. Based on these considerations, isolated CP obtained from rodents was tested for its neuroprotective effects in a conventional rodent model of stroke [1,4]. Rats received a 1-hour middle cerebral artery (MCA) occlusion immediately followed by transplantation of alginate-encapsulated CP on the cortex overlying the brain region (striatum) that would be normally infarcted following the MCA occlusion. Behavioral testing on days 1–3 following surgery, using the elevated body swing test and Bederson neurological examination, revealed profound motor and neurological impairments in control animals that were significantly improved in animals receiving alginate-encapsulated CP transplants. Histological analysis revealed that the behavioral improvements were accompanied by a significant decrease (~35–40%) in the volume of striatal infarction. This paradigm might have actually underestimated the therapeutic potential of CP grafts, as the therapeutic molecules were required to diffuse out of the capsules and through several millimeters of cortical tissue. Accordingly, the concentration of the cocktail reaching the infarcted region was modest compared to local concentrations. Future studies should carefully consider alternative transplant sites as well as the possibility of using single-cell suspensions of epithelial cells to potentially augment the benefits obtained to date.

Figure 4.3 Porcine CP epithelial clusters, stained with ZO-1.
4.10 Encapsulated Xenogeneic Choroid Plexus Transplants for Huntington’s Disease Therapy

Huntington’s disease is a devastating, autosomal-dominant neurodegenerative disorder characterized by an intractable course of mental deterioration and progressive motor abnormalities that invariably results in death. There are no effective treatments. Unlike many other neurodegenerative diseases, the polyglutamine expression in HD permits an unequivocal diagnosis of HD early in life, even in utero. The ability to identify presymptomatic individuals provides the opportunity to design interventions that could be applied before the development of substantial neurodegeneration and expression of the behavioral changes. Accordingly, the neuronal cytoarchitecture and physiology of the striatum could be maintained or preserved, while forestalling the debilitating consequences of the disease.

To determine if CP transplants have therapeutic potential in HD, neonatal porcine CP was encapsulated within alginate microcapsules and tested for its neuroprotective potential in a rat model of HD [5]. In these studies, the animals received stereotaxic transplants of either empty capsules or CP-loaded capsules directly into the striatum. Three days later, the same animals received unilateral injections of the excitotoxin quinolinic acid (QA; 225 nmol) into the ipsilateral striatum. After surgery, transplanted animals gained body weight more rapidly than controls. After surgery, animals were also behaviorally tested for function of their forepaws, using the placement test. When given 10 trials on the behavioral test, the control rats were able to make only 1–2 directed motor responses. In stark contrast, the rats receiving CP transplants were virtually indistinguishable from normal animals on this task, as they made greater than 9 out of 10 correct responses. Nissl-stained sections further demonstrated that CP transplants significantly reduced (by more than 80%) the volume of the striatal lesion produced by QA.

Based on the benefits of CP transplants in the QA rodent model of HD, a similar experiment was conducted using young adult cynomolgus monkeys [3]. Using stereotaxic techniques, 20 cell-loaded capsules were loaded into a cannula and implanted into the head of the caudate and the right putamen. A total of four monkeys received cell-loaded implants; three monkeys served as controls and received implants of empty capsules. Seven days following capsule implantation, each monkey received an injection of QA (5 μl for a total of 900 nmol of QA) approximately 2 mm posterior to the previous implant site. All monkeys were sacrificed four weeks after the QA lesion. The brains were removed and frozen sections (40 μm) were cut on a sliding microtome. A mouse anti-neuronal nuclei (NeuN) monoclonal antibody was used to label striatal neurons for determination of striatal cell counts and lesion volumes. The number of NeuN immunoreactive (NeuN-ir) neurons within the caudate and putamen nuclei was estimated stereologically using an optical fractionator unbiased sampling design. The volume of intact striatum was also estimated on a series of equispaced NeuN-ir sections along the striatum.

The histological results paralleled those observed in the previously described rodent studies. In controls (animals receiving QA and empty capsule implants), QA
administration produced a large lesion in both the caudate and putamen nuclei as shown in NeuN stained sections. The lesion site encompassed much of the caudate and putamen nuclei before the anterior commissure. With the exception of some occasional NeuN-positive debris and shrunken neurons, the lesion core was virtually devoid of NeuN-positive neurons. In contrast, the lesion size was notably reduced in animals receiving implants of encapsulated CP. In these animals, the core of lesion was minimal and limited to a small, defined area at the tip of the injection site. Immediately outside of this central core, but still adjacent to the needle tract, numerous healthy NeuN-ir neurons with dendritic NeuN immunoreactivity were observed. Stereological counts of NeuN-ir neurons confirmed the gross histological assessment, revealing that, relative to the intact striatum, QA produced a marked loss of NeuN-ir striatal neurons (43%) that was significantly prevented by prior implants of encapsulated choroid plexus (only an 8% loss of neurons) (Table 4.3). Results from the volumetric analysis of intact striatum also paralleled the cell counts. Relative to the intact striatum, animals receiving QA and empty capsules exhibited large lesions characterized by a 40% decrease in striatal volume (745.508 mm³ versus 446.825 mm³). Conversely, the striatal volume was 672.228 mm³ in animals previously implanted with encapsulated CP, which did not differ significantly from the volume of the intact striatum. Both rodent and monkey studies reveal that implants of CP can provide trophic influences to degenerating striatal neurons and suggest that this strategy may ultimately prove relevant for the treatment of HD.

4.11 In Vitro and In Vivo Determinations of the Effect of Age on CP Function

Given the profound changes in CP function that occur during aging, we conducted a series of studies to determine if (a) encapsulated CP can be maintained in vitro for extended periods of time without losing its therapeutic activity, and (b) if encapsulated CP derived from aged animals is less potent than CP from young animals. To begin to answer the first question, neonatal porcine CP was encapsulated within alginate microcapsules and maintained in vitro for 1, 2, or 7 months [41]. The encapsulated cells remained viable (>80%) at all time points and were transplanted unilaterally into the rat striatum. Seven days later, the same animals received unilateral injections

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact Striatum</th>
<th>Lesion/Implanted Striatum</th>
<th>Cell Loss (%)</th>
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<tr>
<td>QA + empty capsule implant</td>
<td>41959437 ± 1309554</td>
<td>23965075 ± 1557936^a</td>
<td>43</td>
</tr>
<tr>
<td>QA + choroid plexus implant</td>
<td>42031113 ± 409306</td>
<td>38615375 ± 6012797^b</td>
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^a p < 0.0001 versus intact striatum.
^b p < 0.001 versus QA lesioned striatum.
of QA adjacent to the implant site. Separate groups of animals served as controls and received QA alone. In controls, QA lesions produced a significant loss of body weight and impaired function of the contralateral forelimb. In contrast, implants of CP were potently neuroprotective, as rats receiving CP transplants did not lose body weight and were not significantly impaired when tested for motor function. These benefits were independent of the length of time that the cells were held in vitro.

A second set of studies determined whether age-related impairments occur in the neuroprotective capacity of CP. Choroid plexus was isolated from either young (3–4 months) or aged (24 months) rats [42]. In vitro, young CP epithelial cells secreted more vascular endothelial growth factor (VEGF) and were metabolically more active than aged CP epithelial cells. Additionally, conditioned medium from cultured aged CP was less potent than young CP at enhancing the survival of serum-deprived neurons. Finally, encapsulated CP was tested in the QA model of HD as described in Section 4.10. Animals were tested for motor function 28 days after CP implantation (21 days post-QA administration). In the control group, QA lesions severely impaired function of the contralateral forelimb. Implants of young CP again prevented the impairments in motor function. In contrast, implants of CP from aged rats were only modestly effective and were much less potent than young CP transplants. These data demonstrate that alginate-encapsulated CP cells can be retained for extremely long periods of time in vitro, but they also directly link the natural aging process with a diminished neuroprotective capacity. Additional studies are warranted in improving the long-term potency of encapsulation when chronic neurodegenerative disorders are being contemplated as targets for CP cell therapy.

4.12 Conclusions

Maintenance of CNS homeostasis is vital to brain function. The CP appears to play vital roles in the stable integrity of the CNS, via its main secretory function that allows therapeutic molecules to penetrate the brain, but guards against the entry of immune cells. Therapeutic strategies exploiting the growth factor secretion and immune regulatory properties of CP may prove directly relevant to treating brain disorders. Indeed, the transplant studies conducted to date lend support to the use of CP for repairing the diseased and/or aging brain. Subsequent studies are needed to clearly elucidate the mechanisms of action by which these therapeutic benefits are achieved with CP, in order to further improve the functional outcomes. Envisioned mechanism-driven experiments include determining whether CP functions within parenchymal tissue in the same manner as within the CSF, and conducting vis-à-vis comparisons between native ability of CP to secrete a physiologically balanced and temporally adjusted cocktail of bioactive compounds versus delivery of single agents. Equally critical to advancing CP cell therapy are translational research issues such as examining the potential clinical indication with emphasis on optimizing the donor source and age of the transplanted cells, determining whether specific cell types within the CP (i.e., purified epithelial cells) are most beneficial, and identifying the optimal postinjury timing, transplant location and dosage, of cells to be grafted into the CNS.
References


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5 Role of the Sympathetic Nervous System in Immunity

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5.1 Early Evidence for Nervous System–Immune System Communication

Early in the history of medicine, it was noted that disease development and/or progression was associated with a change in an individual’s behavior, which was often induced by life circumstances and/or environment. Because behavior was associated with the nervous system and certain diseases were associated with the immune system, clinicians believed that one system could influence the other. However, finding definitive proof for such an association took almost a century of research. One of the earliest studies to suggest that a relationship might exist between the immune status of an individual and his or her behavioral state was a 1919 study, which reported that one’s mental state was associated with the incidence of tuberculosis infections [1]. In 1925, another study reported that inducing a state of fatigue in rabbits increased disease susceptibility to, and mortality from, *Streptococcus pneumoniae* [2]. In 1936, Selye [3,4] described a myriad of structural changes that occurred in various organ tissues during the biological response to stress, including the appearance of lymphoid organ atrophy. By 1960, studies were specifically designed to show the existence of an association between the psychological and immunological profiles of individuals afflicted with rheumatoid arthritis, which is an immune-mediated disease [5,6]. The scientific basis for a possible association between behavior and immunity remained unknown until more data accumulated to show that the behavior associated with disease states, as referred to in the earlier studies, could affect, and be changed by, the activity of cells within the immune system. Therefore, these early studies provided a basis for the suggestion that changes in behavior might influence the level of immunity, which subsequently would affect the development and/or progression of disease. If true, then some mechanism had to exist by which the center for coordinating behavior, that is, the brain, influenced the center for coordinating health, that is, the immune system. It then took almost 15 years for a series of experiments to be designed to prove that such a connection truly exists.
In 1975, using a classical conditioning paradigm, Ader and Cohen [7] showed for the first time that behavior could influence an immune response. This finding also suggested that such an association might have biological relevance for the cause and treatment of disease, such as was reported for lupus [8]. Besedovsky and DelRey [9,10] later showed that the sympathetic nervous system (SNS) played a role in modulating the magnitude of a primary antibody response, suggesting one possible mechanism by which behavior might influence the level of immunity. Besedovsky and DelRey [11,12] also showed that antigen exposure caused a change in the firing rate of neurons within the hypothalamus, which allowed activation of the SNS. We now know that the brain communicates with cells in the periphery through two well-characterized mechanisms: namely, the SNS and the neuroendocrine system. Felten and his colleagues [13,14] provided an anatomical basis for the association between immunity and SNS activity by showing that every lymphoid organ is innervated with sympathetic nerve fibers and that nerve terminals are located within the area where immune cells reside. Radioligand binding analysis and pharmacological approaches confirmed that adrenergic receptors that bind norepinephrine (NE) are expressed on immune cells [15]. These findings and many others set the stage for the establishment of an entirely new field of study focused on the role played by the SNS in modulating the immune response (Figure 5.1). It has been proposed that a dysfunction in any part of the communication between the nervous and immune systems might eventually mediate the development and/or progression of a disease state, but such an association remains inconclusive [16,17]. The latter limitation is further complicated by the fact that the nervous and immune systems are often studied as separate disciplines, which contributes much to an understanding of how each system functions autonomously, but not to an understanding of how a communication between the two systems might be clinically relevant.

This chapter specifically focuses on the association that exists between the SNS and the immune system. Documentation for such an association includes data to show that lymphoid tissue is innervated with sympathetic nerve fibers containing NE that is released when antigen enters the immune system, that immune cells express receptors for NE, that engagement of these receptors on immune cells activates a cascade of signaling intermediates to affect gene expression, and that changes in gene expression are associated with changes in immune-cell activity and function.

5.2 Innervation of Lymphoid Tissue

The SNS is typically associated with the physiological “fight or flight” response, because it is involved in the regulation of cellular activity in all major organ systems, especially during times of critical need. Neuroanatomical and neurochemical data confirmed a structural basis for sympathetic neural input into the immune system (reviewed in Ref. [18]). Using a retrograde transport technique, it was found that sympathetic innervation of lymphoid tissue stemmed from sympathetic
premotor neurons located in the brainstem, pons, and hypothalamus, which are areas within the CNS that are known to be activated by immune stimuli [19,20]. In bone marrow, these neurons project to sympathetic preganglionic neurons in the intermediolateral cell column of the T8-L1 spinal cord, which sends extensions to thoracolumbar paravertebral sympathetic ganglionic neurons [21]. In the thymus gland, sympathetic innervation is received from the cervical and upper thoracic sympathetic chain ganglia [22,23]. In the spleen, sympathetic preganglionic neurons arise from the T1–T12 region of the thoracic spinal cord [24], and provide input to the postganglionic splenic nerve [25]. Finally, individual lymph nodes receive sympathetic input from postganglionic neurons that are associated with specific regions of the body where the lymph node is located [26,27].

In contrast to sympathetic innervation of lymphoid tissue, evidence to date shows no parasympathetic or sensory input from either the vagus nerve or dorsal root ganglia [25,28,29], except in lymph nodes, where they may receive neural sensory afferents [30]. Thus, the SNS appears to provide the only pathway for direct neural modulation of immune-cell activity.

Figure 5.1 Communication between the nervous and immune systems. Upon antigen exposure, immune cells are activated to secrete cytokines that deliver a signal to the brain. The SNS is activated centrally to send a signal back to the periphery via sympathetic nerves that release NE in close proximity to the immune cells that express an adrenergic receptor to bind it. The binding of NE to an adrenergic receptor on an immune cell modulates the level of signaling intermediates that regulate gene expression, which in turn changes the level of immune cell activity and function.
5.3 Release of Norepinephrine

Electron microscopic studies of the splenic white pulp showed that sympathetic nerve terminals were in direct apposition to T cells and adjacent to interdigitating dendritic cells and B cells [13,14]. Within 9–18 hours after antigen administration to mice, NE was released from nerve terminals in the spleen, as measured by NE turnover analysis [31], and was found to reach an estimated synaptic concentration of $3 \times 10^{-3}$ M [32]. Upon release from the nerve terminal, NE is either metabolized by catechol-O-methyltransferase and/or monoamine oxidase, taken back up into the nerve terminal, lost by diffusion, or bound to a receptor on a target cell. A recent report showed that, in addition to sympathetic nerve terminals, phagocytes may also release NE and express the enzymes for NE synthesis and degradation [33], providing another mechanism by which NE could be released within a microenvironment during an immune response to modulate immune-cell activity [34]. Human CD4+CD25+ T cells were found to express tyrosine hydroxylase and to synthesize catecholamines that may provide an auto-inhibitory signal [35]. Endogenous catecholamines were found to accelerate lymphocyte apoptosis via a mechanism that includes regulation of anti-apoptotic and pro-apoptotic gene and protein expression, providing a mechanism for controlling immune-cell activity [36]. In addition, immune-cell-derived neurotrophic factors may direct innervating fibers to the site of the response [37,38] to release NE to bind to beta-adrenergic receptors ($\beta$ARs) expressed on immune-cell populations. Thus, the initiation of an immune response in the periphery sends signals to the CNS, resulting in subsequent regulation of the immune response via activation of the SNS (reviewed in Ref. [39]).

5.4 Expression of the Beta-2-Adrenergic Receptor

Norepinephrine binds to adrenergic receptors (ARs) that are either of the alpha ($\alpha$) or beta ($\beta$) subtype. These two subtypes of adrenergic receptors are further distinguished pharmacologically and biochemically as $\alpha_1$, $\alpha_2$, and $\alpha_3$ or $\beta_1$, $\beta_2$, and $\beta_3$. Most innate immune cells, excluding natural killer (NK) cells [40], are known to express both families of adrenergic receptors. For example, bone-marrow-derived dendritic cells express not only the $\beta_1$AR and $\beta_2$AR, but also the $\alpha_1$AR and $\alpha_2$AR subtypes [41,42]. Likewise, monocytes/macrophages express the $\beta_2$AR subtype [43], as well as the $\alpha_1$AR and $\alpha_2$AR [33,44]. In contrast, NK cells [40] and adaptive immune cells express the $\beta_2$AR exclusively (reviewed in Ref. [15]). This dichotomy is interesting, given that adaptive immune cells and NK cells both develop from lymphoid precursors in the bone marrow, as opposed to most innate immune cells, which develop from myeloid precursors.

Radioligand binding analyses have identified the $\beta_2$ARs on both human and murine T cells and found that, on average, CD4+ T cells express approximately 200–750 binding sites per cell [45–50]. However, resting clones of murine Th1 cells, but not clones of Th2 cells, express a level of $\beta_2$ARs detectable by both radioligand
binding analysis and immunofluorescence, and are able to accumulate adenosine-3′-5′-cyclic monophosphate (cAMP) upon receptor engagement [51]. These findings raised the possibility that the β2AR could be differentially expressed on CD4+ T cell subsets. Similar studies using human Th1 and Th2 cells have either confirmed [52–54] or refuted [55,56] the findings with the murine effector T cell subsets. Although we await studies at the gene level, we do know that naïve CD4+ T cells, which are the precursors of both Th1 and Th2 cells, are likely to express the β2AR, because the mRNA for the β2AR has been shown to be expressed in a purified naïve CD4+ T cell population [57], and the earlier radioligand binding studies were likely performed using the naïve CD4+ T cell. Differential expression of the receptor on CD4+ T cell subsets has important implications for how NE might regulate the generation of a Th1 response, although more work must be done at the gene expression level as the naïve CD4+ T cell differentiates.

Radioligand binding analyses showed that B cells expressed approximately twice the number of β2ARs than T cells, although the Kd value of the βAR was similar; that is, the B cell expressed approximately 620 βAR binding sites per cell with an affinity of approximately 0.1 nM [58–62]. Adrenergic receptor selective agonists and antagonists, as well as immunofluorescence, were used to confirm expression of the β2AR on B cells, while the accumulation of intracellular cAMP was used to measure signaling of the β2AR in B cells [63]. Similar data have accumulated to show that such changes occur in both T and B cells following exposure to NE or a β2AR agonist, confirming expression of a functional receptor on both cell types (reviewed in Ref. [15]).

It will be important to keep in mind that the level of adrenergic receptor expression varies among the different immune-cell types and is regulated by a variety of factors, including the activation state of the cell, cytokines, and neurotransmitters (see Figure 5.2 and reviewed in Ref. [15]). Findings also suggest that the timing of receptor expression may play an important role in mediating neurotransmitter regulation of immune cells [64]. Therefore, when sympathetic innervation of a secondary

![Figure 5.2 Variables that determine the effect of norepinephrine on immune cell activity and function.](image_url)

**Figure 5.2** Variables that determine the effect of norepinephrine on immune cell activity and function. Norepinephrine stimulation of an adrenergic receptor expressed on an immune cell has the potential to modulate the activity and effector function of that immune cell. However, the physiological status of the immune cell and/or the microenvironment in which the immune cell resides determine the level of modulation mediated by NE, resulting in either positive or negative modulation of gene expression and subsequent effector function.
lymphoid organ exists, the $\beta_2$AR is expressed on the surface of immune cells, and immune cells are exposed to a $\beta$AR agonist, the level of intracellular cAMP and protein kinase A (PKA) activity increases. The following sections present the findings that have identified the changes induced in immune-cell activity following $\beta_2$AR stimulation and a change in cAMP and PKA activation, as well as the mechanisms by which these signaling intermediates affect gene expression and immune-cell function.

5.5 Evidence That Norepinephrine Regulates Immune-Cell Function

The innate immune system protects in an antigen-nonspecific manner and does not generate memory cells. In contrast, the adaptive immune system protects in an antigen-specific manner and generates long-term protection in the form of memory cells. Pharmacological evidence shows that the SNS is involved in regulating the level of response in both the innate and adaptive immune systems. Early studies used the chemical neurotoxin 6-hydroxydopamine (6-OHDA) to reversibly deplete NE from peripheral sympathetic nerve terminals in adult mice. Depending on the model system and antigen used, such NE-depleted mice had either enhanced, suppressed, or unaltered immune responses when compared to mice in which NE remained intact (reviewed by Ref. [65]), suggesting that NE released from nerve terminals within the microenvironment of immune cells regulated the response to antigen. These data set the stage for many studies designed to determine the mechanisms by which NE and $\beta_2$AR engagement on the cell surface of various immune cells affecting these cells’ activity. The subsequent sections summarize these findings.

5.6 NK Cells

Natural killer cells are innate immune cells that mediate killing of microbe-infected cells by lysis and production of interferon-gamma (IFN-$\gamma$). Acute stress or the infusion of an adrenergic agonist has been reported to cause a rapid and transient increase in the number of circulating NK cells [66–71] that is prevented completely by administration of a $\beta_2$AR antagonist [67,70,71]. There is evidence from an in vitro study that NK cell adherence to cultured vascular endothelium is inhibited in the presence of a $\beta_2$AR agonist [72], and that NK cell activity is reduced [73–76]. Likewise, NE reduces the ability of NK cells to bind to a target cell, and the mechanism appears to involve a reduction in CD16 expression on NK cells [76]. In addition, NE inhibited NK cell release of IFN-$\gamma$ and tumor necrosis factor-alpha (TNF-$\alpha$), and this effect prevented NK cell maturation into a functional cytotoxic cell [76]. Thus, the effect of NE and $\beta_2$AR stimulation on NK cell number and activity appears to be primarily inhibitory.
5.7 Macrophages

*Monocytes/macrophages* are innate immune cells whose primary role in immune protection is phagocytosis of antigens and presentation to other immune cells such as the CD4+ T cell, as well as the production of cytokines. Cytokine gene expression in a macrophage appears to be a primary target for NE. Interleukin-10 (IL-10) production was enhanced by the addition of a βAR agonist to lipopolysaccharide (LPS)-activated human monocytes or mouse peritoneal macrophages [77–79], while IL-12 and TNF-α production by monocytes was inhibited [80–83]. There is also evidence that macrophages respond to αAR agonists. For example, macrophages responded to an α2AR agonist with increased secretion of TNF-α [84]. In addition, when a human monocytic cell line was exposed to an α1AR agonist, the cell produced IL-6 [85]. However, *in vitro* stimulation of resting monocytes with LPS induced expression of the α1AR, and subsequent agonist exposure caused an increase in the phosphorylation of extracellular signal-related kinase (ERK) [85]. Cells from children with juvenile idiopathic arthritis expressed mRNA for the α1AR and responded to agonist exposure with an increase in IL-6 and TNF-α secretion [86]. NE also modulated the expression of matrix metalloproteases: expression was inhibited by addition of a β1AR, but not a β2AR, antagonist, suggesting that NE may play a role in joint destruction in arthritic patients or macrophage-assisted tumor cell invasion into surrounding normal tissue [87]. These data suggest that monocytes/macrophages express multiple adrenergic receptor subsets, and that the inflammatory cytokine response is inhibited by β2AR simulation, but enhanced by α1AR stimulation, whereas the anti-inflammatory cytokine response by a macrophage appears to be enhanced by NE.

5.8 Dendritic Cells

*Dendritic cells*, another antigen-presenting cell (APC) of the innate immune system, presents antigen to T cells and mediate their activation. The administration of a β2AR antagonist *in vivo* enhanced the contact hypersensitivity response, suggesting that NE stimulation of the β2AR exerts an inhibitory effect on the cells that participate in the hypersensitivity response, including dendritic cells [88]. This conclusion was supported by a finding that NE inhibited Langerhans cell migration from the site of antigen deposition to the site of antigen presentation in the draining lymph nodes, and that this was an α1-β2AR-dependent effect [41,89]. This study proposed that NE might act as either a chemotactic factor to direct dendritic cell (DC) migration or a stimulant to affect DC motility. The latter possibility was supported by an acute restraint stress paradigm in mice that showed an increase in DC migration to the skin that was prevented by NE depletion [41]. In addition, exposure of bone-marrow-derived DC to NE or a β2AR agonist reduced either LPS- or protein antigen (KLH)-induced IL-12 release [80,81,90], but facilitated IL-10 release [90]. Thus, NE appears to suppress DC migration as well as inhibiting inflammatory cytokine release and enhancing anti-inflammatory cytokine release, both of which effects are seen in the macrophage, as described in Section 5.7.
5.9 CD4+ T Cells

CD4+ T cells are adaptive immune cells that produce cytokines to help activate and regulate other T cells, macrophages (Th1), or B cells (Th2). NE depletion, either before or after sensitization in a contact hypersensitivity response, decreased the Th1 cell-mediated response [91], suggesting that NE might play a role in enhancing Th1 cell development or Th1 cell activity. This possibility was tested when mice became available that were genetically deficient for the enzyme dopamine beta-hydroxylase, which is required for the synthesis of NE [92]. These NE-deficient mice produced less IFN-γ and were more susceptible to infection with *Listeria monocytogenes* and *Mycobacterium tuberculosis*, suggesting that NE may play a role in enhancing the level of IFN-γ produced and the subsequent role that this cytokine plays in protection through a Th1 cell-mediated immune response.

However, it is still not clear whether NE mediates the effect of IFN-γ level enhancement by affecting naïve CD4+ T cell activation, naïve CD4+ T cell development into a Th1 cell, or the level of IFN-γ produced by the resulting Th1 cells. Naïve CD4+ T cells activated in vitro produced less IL-2 after exposure to NE, and this effect was prevented when a β2AR, but not a β1AR or αAR, antagonist was added [93]. In addition, NE and β2AR stimulation on a CD4+ T cell was reported to inhibit the activation of NF-κB by stabilizing its inhibitor protein IκBα [94], which caused apoptosis and cell death [95,96]. These findings suggest at least two mechanisms by which NE could diminish IL-2 production by a naïve T cell, but fail to explain the mechanism by which a NE-exposed naïve T cell produces a higher level of IFN-γ upon reactivation. Further experiments showed that NE did not affect the number of Th1 cells that developed into Th1 cells, but did prepare those Th1 cells that developed to produce more IFN-γ per cell when reactivated [97]. In this study, NE did not affect the level of IL-12 secreted by the APC, as has been reported by others [80,81,98–100], but this may be due to a difference in experimental design for APC activation. Thus, the effect exerted by NE to increase the level of IFN-γ produced, as suggested by both in vivo and in vitro results, may involve an effect on the naïve CD4+ T cell to prepare the T cell to make more IFN-γ after it differentiates into a Th1 cell. It is also possible that NE might affect a CD4+ T cell at the effector stage, as opposed to the naïve T cell stage. In Th1 cells, an increase in intracellular cAMP inhibited the production of IL-2 [101–103] and IFN-γ [103]. However, it soon became clear that the timing of β2AR stimulation on a Th1 cell in relation to the time of Th1 cell activation might play an important role in resolving the effect of NE on Th1 cell activity. For example, exposure of Th1 cells to NE or a β2AR-selective agonist before their activation decreased both IL-2 and IFN-γ production [51]. However, stimulation at either the time of, or after, cell activation appeared to be without effect or to induce a small increase in IFN-γ production [104].

NE may indirectly exert a suppressive effect on Th1 cell development via a direct effect on either the level of IL-12 produced by dendritic cells or on the ability of Langerhans cells to migrate to the lymph node [41,105]; both of these occurrences would severely hamper naïve T cell activity and differentiation along the Th1 cell pathway. *In vitro* exposure of human peripheral blood mononuclear cell (PBMC) to
NE or a $\beta_2$AR agonist induced a decrease in IFN-$\gamma$ production, primarily due to a decrease induced in IL-12 production by APC [80,81,98–100]. These findings indicate that a decrease in IL-12 induced by either NE or a $\beta_2$AR agonist prevented Th1 cell differentiation, resulting in a possible shift to Th2 cell development.

The clinical relevance of such an effect of NE on the level of IFN-$\gamma$ produced by Th1 cells is unclear. When human HIV-infected PBMCs were exposed in vitro to NE, stimulation of the $\beta_2$AR decreased production of IFN-$\gamma$, IL-10, and IL-4, but enhanced HIV replication in T cells [106], suggesting that NE stimulation of the $\beta_2$AR on T cells may enhance HIV pathogenesis by inhibiting the production of all cytokines that would prevent HIV replication. Also, it has been reported that T cells infiltrating the inflamed synovium of rheumatoid arthritis (RA) patients and mice are of the Th1 cell phenotype and produce IFN-$\gamma$ [107,108]. In addition, the administration of a $\beta_2$AR antagonist prior to, and during, the disease process appears to delay the onset and reduce the severity of joint injury [109], suggesting that NE stimulation of the $\beta_2$AR on either a naïve CD4$^+$ T cell or a Th1 cell itself might play a role in increasing IFN-$\gamma$ and thereby exacerbate this autoimmune disease process [110]. This finding was recently expanded to the CD4$^+$CD25$^+$ T cell subpopulation of cells isolated from collagen-immunized mice. When these T cells were isolated from immunized NE-depleted mice and adoptively transferred to mice with established collagen-induced arthritis, the cells secreted less cytokine and decreased disease severity in comparison to cells from immunized NE-intact mice, suggesting that NE may increase disease severity by increasing cytokine release in the early phases of disease induction [110,111].

In contrast, it was thought that NE has no effect on Th2 cell activity because the Th2 cell did not express the $\beta_2$AR [51]. While the effect of NE and $\beta_2$AR stimulation on naïve T cell differentiation to Th2 cells, and the resulting function of the Th2 cell, remain unknown, one study suggested that NE may hinder the development of a normal Th2 response, because cells from an immunized NE-deficient mouse produced significantly higher levels of IL-2 and IL-4 following reactivation in vitro as compared to cells isolated from immunized NE-intact control mice [112]. However, an effect on Th2 cell activity could be caused indirectly by a NE-mediated effect on another cell that influences Th2 cell activity. For example, in mice receiving thermal burn injuries, elevated plasma NE levels were associated with increased macrophage production of the Th2 chemokine CCL2 and the subsequent development of a predominant Th2-like response, which was prevented by NE depletion [113,114]. This finding suggested that NE regulated macrophage chemokine secretion and the subsequent increase in Th2 cell accumulation at the site of injury, which possibly might influence susceptibility to infections after a thermal burn, as has been described previously [115].

Thus, the effect of NE and $\beta_2$AR stimulation on CD4$^+$ T cell activity remains controversial. There appears to be an inhibitory effect on naïve CD4$^+$ T cell IL-2 production, but an enhancing effect on the amount of IFN-$\gamma$ that is produced by Th1 cells that develop. Therefore, NE and $\beta_2$AR stimulation of a naïve CD4$^+$ T cell does not appear to affect the number of Th1 cells that develop, but appears to affect the amount of IFN-$\gamma$ secreted by those Th1 cells. The effect of NE on effector Th1 and
Th2 cells is even less well understood, but it is possible that an elevation in the level of intracellular cAMP within any CD4+ T cell subset is able to affect the cellular activity of any subset, whereas NE and β2AR stimulation affects only naïve CD4+ T cell and Th1 cell activity, since these subsets express the β2AR and Th2 cells do not. In addition, the timing of β2AR stimulation on a Th1 cell in relation to the time of cell activation appears to be an important factor affecting cellular activity, with stimulation prior to activation inhibiting IFN-γ, but stimulation at the time of, or later than, activation having no effect or enhancing the level of IFN-γ secreted.

5.10 CD8+ T Cells

CD8+ T cells are adaptive immune cells that mediate specific killing of microbe-infected cells or tumor cells. Few studies have shown a direct correlation between the level and timing of NE exposure and/or β2AR stimulation on CD8+ T cell activity, but a few findings have provided some insight. Exposure of mice to restraint stress to increase NE levels decreased the generation of a CD8+ T cell response to either herpes simplex virus (HSV) or influenza virus infection, which was partially mediated by a βAR-induced mechanism [116,117]. In contrast, certain types of physical and psychological stress in humans elevated NE levels and induced an increase in CD8+ T cell numbers that was blocked by administration of a βAR antagonist [71,118]. Likewise, acute administration of a β2AR agonist to healthy subjects for 7 days caused cell numbers to increase; in contrast, chronic administration caused a decrease [119]. However, chronic exposure to a β2AR agonist in asthmatic individuals did not change the number of bronchial CD8+ T cells [120], whereas chronic exposure in HIV-infected individuals increased CD8+ T cell numbers [121], suggesting that a disease process may also influence a CD8+ T cell response to β2AR stimulation when compared to normal cells.

The timing of exposure to NE in relation to the stage of CD8+ T cell differentiation may be relevant, just as it seems to be for the CD4+ T cell. For example, if adrenergic ligands were added after the generation of cytolytic T lymphocyte (CTL), that is, during the effector stage of the response to antigen, a decrease in CTL activity occurred [122,123] that may have been due to a cAMP-induced decrease in the T cell receptor (TCR)-dependent release of cytotoxic granules [124]. In one study, administration of a monoamine oxidase inhibitor to tumor-bearing rats increased both NE levels and the percentage of CD8+ T cells in the spleen [125]. When mice were depleted of NE before sensitization with trinitrochlorobenzene (TNCB), the generation of hapten-specific CD8+ T cell cytotoxicity decreased upon TNCB challenge in comparison to controls [91], suggesting that NE is required for the generation of cytotoxicity after initial antigen exposure. Thus, the role of NE and/or β2AR stimulation in modulating CD8+ T cell activity remains uncertain in both humans and animals, being both inhibitory and stimulatory, though these changes may be influenced by the time of β2AR stimulation in relation to the stage of CD8+ T cell differentiation or the time of adrenergic receptor stimulation in relation to T cell activation.
5.11 B Cell

The primary role of the B cell is to produce antigen-specific antibodies that will increase cell-mediated clearance of the antigen. The role played by NE in regulating the magnitude of an antibody response has been indicated primarily by studies conducted in NE-depleted mice. Most data showed that NE depletion decreased the Th cell-dependent antibody response (reviewed in Ref. [126]), suggesting that NE might exert an enhancing effect on the endogenous activity of immune cells that generate the antibody response. A plethora of *in vitro* studies followed and showed that NE did indeed affect B cell activity directly. For example, β2AR stimulation and elevation of cAMP affected B cell proliferation by either inhibiting [127–129] or enhancing [130–132] B cell proliferation. The reader is referred to the following comprehensive review of all of the early history in this area of defining the effects of NE on B cell activity [126,133]. It is now clear that many of these findings were important in laying the groundwork for the studies described in this section, which have started to identify the molecular mechanism responsible for mediating the enhancing effect of NE on the antibody response (Figures 5.3 and 5.4).

Pharmacologic characterization of the NE effect on the antibody response showed that NE produced an enhanced response [134], a result supported by the finding that selective β2AR stimulation enhances the antibody response with a magnitude and kinetics similar to that produced by NE, and this enhancement was blocked with the β2AR antagonist propranolol [135]. These results suggested that NE stimulates the

![β2AR expression by murine CD4+ T cell subsets.](image)

*Figure 5.3* β2AR expression by murine CD4+ T cell subsets. The murine naïve CD4+ T cell expresses the β2AR, and this expression is maintained as the naïve T cell differentiates to a Th1 cell. However, as the naïve T cell differentiates to a Th2 cell, β2AR expression is lost; the mechanism by which this occurs remains unknown.
β₂AR specifically to mediate the enhancing effect on the antibody response. With the development of a model system of purified splenic naïve B cells cultured in the presence of CD40L and IL-4, the key stimuli required for a B cell to become activated to make antibody, researchers were able to conduct more mechanistic studies that bypassed the complicated and uncontrollable model system involving macrophages, dendritic cells, T cells, and antigen. Data generated using this model system revealed that β₂AR stimulation activates the classical signaling pathway, which involves an association of the receptor with stimulatory G-proteins, activation of adenylyl cyclase, increased intracellular accumulation of adenosine-3’-5’-cyclic monophosphate, and increased PKA activity (reviewed in Refs [136,137]). Upon activation, PKA regulates the activity of multiple targets via phosphorylation, including various transcription factors, such as CREB [138], which translated into a higher level of OCA-B binding to the 3’immunoglobulin(Ig)H-enhancer region of the IgH locus to increase the level of IgG₁ produced per B cell, without affecting the number of cells that switched to produce IgG₁ (see Figure 5.5 and Refs [139–141]).

Via an indirect pathway, NE stimulation of the β₂AR on a B cell up-regulated expression of the costimulatory molecule CD86 (also known as B7-2), which upon
stimulation with CD28 activated another signaling pathway in the B cell that up-regulated the transcription factor Oct-2, which also regulated the level of 3′-IgH enhancer activity [141,142]. CD86 stimulation was also shown to increase the level of Oct-2 binding to the 3′-IgH enhancer [141], allowing a cooperative binding of the elevated levels of both OCA-B and Oct-2 to the 3′-IgH enhancer region to further increase the rate at which IgG1 mRNA was produced [141]. For a more detailed description of how an understanding of the role played by the β2AR in enhancing the IgG1 response led to the discovery of the signaling pathway activated by CD86 (which had been considered devoid of signaling ability), please refer to the following review [143] and subsequent signaling studies [144,145].

Taken together, these findings suggested that NE exerts a regulatory effect on the level of IgG1 produced by enhancing the endogenous activity of the B cells that were activated to generate the response. These findings also suggested that signaling pathways

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**Figure 5.5** Signaling intermediates activated in a B cell by β2AR stimulation. In the absence of β2AR stimulation, B cell activation induces class switch recombination that results in the production of IgG1. When NE is released, the β2AR is stimulated and activates a signaling pathway inside the cell. This pathway includes the activation of the enzyme adenylate cyclase, an increase in intracellular cAMP levels, and activation of PKA, which allows the targeted phosphorylation of downstream signaling intermediates and transcription factors that alter gene expression to regulate the activity of the 3′IgH-enhancer. This enhancer region controls the rate at which antibody transcription occurs to increase the level of IgG1 or IgE produced per cell, without affecting the number of cells that switch to secrete IgG1.
activated in a B cell through stimulation of an immunoreceptor (CD86) and a neuroreceptor (β2AR) converge to regulate the magnitude of an IgG1 response. These findings were confirmed in vivo when B cells alone were adoptively transferred to NE-depleted or NE-intact immunodeficient mice and the mice were administered an intraperitoneal injection of anti-CD40 antibody and IL-4, an anti-CD86 antibody, and/or the β2AR agonist terbutaline. The level of serum IgG1 was increased almost twofold following β2AR stimulation and almost threefold following CD86 stimulation, and this level was further increased by almost sixfold when both CD86 and the β2AR were stimulated. Likewise, the level of mature IgG1 transcript and Oct-2/OCA-B transcript and protein produced by splenocytes from these mice positively correlated with the increased level of serum IgG1 protein. Therefore, the level of mature IgG1 transcript and IgG1 protein increase after β2AR and/or CD86 stimulation on a B cell, and this change appears to be associated with a similar increase in the level of Oct-2 and OCA-B transcript produced in vitro and in vivo. Thus, for one antibody isotype, that is, Th2/IL-4-dependent IgG1, we now know the mechanism by which NE and β2AR stimulation exert their enhancing effect.

An understanding of the regulatory mechanism by which CD86 and/or β2AR stimulation increases the level of IgG1 produced by a murine B cell may help to explain the linkage of acute stress to an enhancement of IgG immunity in humans. For example, the drug ephedrine exacerbates lupus symptoms in lupus-prone mice, via a mechanism that appears to involve stimulation of the β2AR on a B cell to increase the level of IgG [146], suggesting that NE stimulation of the β2AR on a B cell could potentially regulate the severity of this disease process. In acute stress, an increase in the level of NE released from lymphoid organ nerve terminals might overstimulate the β2AR on a B cell to induce a higher level of IgG, as has been reported in mice [63,140,141]. While in chronic stress, the β2AR may be down-regulated, thus preventing optimal β2AR stimulation on a B cell and, consequently, preventing optimal IgG production. However, chronic stress in mice was shown to be associated with no change in NE levels, but associated with an increase in β2AR expression on immune cells, which could explain the increased sympathetic effect on T and B cell responses [147]. The latter findings may have relevance for vaccination protocols in which the goal might be to raise the level of humoral immunity in patients dealing with undue stress; for example, cancer patients who need IgG1 to fight against pneumonia, but who cannot respond well to a vaccine.

The information available regarding IgG2a is virtually nonexistent, and information concerning IgE is only now being reported. Although one might have predicted that the mechanism responsible for the β2AR-mediated increase in the Th2/IL-4-dependent IgE response would be similar to that for the Th2/IL-4-IgG1 response, the signaling intermediates used to induce the enhancing effects are quite different (see Figure 5.6 and Ref. [148]). In contrast to PKA-dependent activation of camp response element binding (CREB) that mediates an increase in IgG1, PKA instead regulates a phosphatase called hematopoietic protein tyrosine phosphatase (HePTP), which is involved in p38 MAPK regulation, to mediate an increase in the level of IgE [149]. The resulting β2AR-induced increase in p38 MAPK activity regulates the level of IgE produced in a CD23- and CD21/CD19-dependent manner that increases the rate of IgE mRNA transcription and the amount of IgE produced per cell, without affecting class switch recombination [150]. Thus, β2AR-mediated enhancement of the IgG1 response appears to use the
Figure 5.6 The $\beta_2$AR activates unique signaling intermediates to regulate the level of IgG$_1$ or IgE produced by a B cell. Although stimulation of the $\beta_2$AR results in an increase in the production level of either IgG$_1$ or IgE, a unique signaling pathway is activated to modulate the level of antibody produced. Norepinephrine stimulation of the $\beta_2$AR activates the classic signaling pathway, which involves activation of the enzyme adenylate cyclase to elevate the level of intracellular cAMP and subsequently activate the level of PKA, which is involved in modulating the level of both IgG$_1$ and IgE. However, the pathways appear to diverge at this point. To modulate the level of IgG$_1$, PKA phosphorylates the transcription factor CREB, which translocates to the nucleus and increases expression of the transcriptional co-activator protein OCA-B, which binds to the transcription factor Oct-2. The OCA-B/Oct-2 complex then binds to the 3′IgH enhancer to increase the rate of IgG$_1$ mRNA transcription. To modulate the level of IgE, PKA phosphorylates and inactivates hematopoietic protein tyrosine phosphatase (HePTP), which frees bound p38 MAPK. Inactivation of HePTP allows the released free p38 MAPK to become activated, resulting in a higher level of activated p38 MAPK to increase gene expression of several intracellular and surface molecules that are proposed to play a role in regulating the rate of IgE mRNA transcription, which is also regulated by the 3′IgH enhancer.
PKA-dependent CREB-mediated pathway to do so, whereas enhancement of the IgE response appears to use the PKA-dependent HePTP/p38 MAPK-mediated pathway.

An understanding of the mechanism by which β2AR stimulation regulates IgE may be useful for developing therapies to treat allergic asthma, a disease that involves IgE. At present, all therapies used to treat allergic asthma target some biological mediator along the allergic response cascade. For example, a β2AR agonist targets the bronchial smooth muscle cell to relieve bronchoconstriction, an antihistamine targets histamine to prevent an inflammatory response, a glucocorticoid receptor agonist targets immune cells to suppress cell reactivity, and an anti-IgE antibody targets free IgE to prevent IgE-induced symptoms in general and lung pathology in particular [151]. When glucocorticoids are used with one of the other therapies, IgE levels decrease and asthmatic symptoms disappear, most likely because of a global suppression of immune-cell activity [152]. However, the major disadvantage of the glucocorticoid co-therapeutic approach is that the side effects from long-term use are severe [153], particularly because the glucocorticoid-induced immune suppression renders an individual prone to infections. Also, if the key to the success of the glucocorticoid co-therapy is an overall suppression of immune cell activity, then it is possible that β2AR agonist therapy alone contributes to asthma pathology by enhancing B-cell activity that would be suppressed by glucocorticoids. The need to determine if this possibility is true became paramount when the FDA issued an alert, in January of 2006, regarding the use of long-acting β-agonists, which were found to increase the severity of asthma responses and the number of asthma-related deaths [154]. Although the causes of and treatments for allergy/asthma are varied and controversial, the symptoms associated with allergic asthma appear to be precipitated by the presence of IgE [155–157]. Because anti-IgE monoclonal antibodies are an effective therapy for inhibiting both early and late allergic reactions in humans [158], it would be beneficial for us to identify a homeostatic mechanism that targets the B cell itself to regulate IgE so that we can modulate the activity of this cell therapeutically. The finding that NE and β2AR stimulation use different signaling intermediates to regulate the level of IgE versus IgG1 [148,150] suggests that we may be able to locate such a sensitive target. Such findings might also explain why long-term conventional β2AR agonist therapy stops working after a few years and/or why stress exacerbates an asthmatic episode.

5.12 Concluding Remarks

In this chapter we focused on the association that exists between the SNS and the immune system.

We know that lymphoid tissue is innervated with sympathetic nerve fibers that contain NE, which is released when antigen enters the immune system. We know that most immune cells express b2AR for NE, but that the Th2 cell represses expression as it differentiates from a naïve T cell. We know that engagement of b2AR on immune cells activates a cascade of distinct signaling intermediates that affect gene expression for certain antibody isotypes. And finally, we know that b2AR-induced changes in CD86 gene expression on a B cell are associated with changes in CD86 function in the B cell, which is responsible for changes in immune-cell activity and function.
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6 Circadian Organization of the Immune Response: Rat Adjuvant Arthritis as a Model

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6.1 The Circadian Clock Has Indispensable Biological Functions

Organisms populating the Earth are under the steady influence of daily and seasonal changes resulting from the planet’s rotation and orbit around the sun. This periodic pattern is most prominently manifested by the light–dark cycle and has led to the establishment of endogenous circadian timing systems that synchronize biological functions to the environment. This is the basis of predictive homeostasis [1], which evolved as an adaptation to anticipate predictable changes in the environment, such as light and darkness, temperature, food availability, or predator activity. Therefore, the circadian clock is one of the most indispensable biological functions for living organisms, because it acts as a multifunctional timer to adjust the homeostatic system, including sleep and wakefulness, immune function, hormonal secretions, and various other bodily functions, to the 24-hour cycle [2–4].

In mammals, the circadian system is composed of many individual, tissue-specific cellular clocks. To generate coherent physiological and behavioral responses, the phases of this multitude of cellular clocks are orchestrated by a master circadian pacemaker residing in the suprachiasmatic nuclei (SCN) of the hypothalamus. At a molecular level, circadian clocks are based on clock genes, some of which encode proteins able to feed back and inhibit their own transcription. These cellular oscillators consist of interlocked transcriptional and posttranslational feedback loops that involve a small number of core clock genes (about 12 genes identified currently). The positive drive of the daily clock consists of two basic helix–loop–helix, PAS-domain-containing transcription factor genes, called Clock and Bmal1. The protein products of these genes form heterodimeric complexes that control the transcription of other clock genes, notably three Period (Per1/Per2/Per3) genes and two
Cryptochrome (Cry1/Cry2) genes, which in turn provide the negative feedback signal that shuts down the Clock/Bmal drive to complete the circadian cycle [4,5]. Per and Cry messenger RNAs peak in the SCN in mid- to late circadian day, regardless of whether an animal is nocturnal or diurnal. Other clock genes provide additional negative and positive transcriptional/translational feedback loops to form the rest of the core clockwork, which has been characterized in rodents by a transgenic gene-deletion methodology. Clock-gene expression oscillates because of the delay in the feedback loops, regulated in part by phosphorylation of the clock proteins that control their stability, nuclear reentry, and transcription complex formation [3,4,6].

Clock genes are expressed in a tissue-specific fashion, often with unknown function. Although a substantial number of genes are rhythmic (about 10% in the SCN or peripheral tissues), the rhythmic genes tend to be different in the different tissues. For example, in comparisons between heart and liver, or between the SCN and liver, only a 10% coincidence was seen [5,7,8]. The phase of the peripheral clock oscillations is delayed by 3–9 hours as compared to that of SCN cells, suggesting that the peripheral tissues are receiving timing cues from the master SCN oscillator. Furthermore, oscillations in isolated peripheral tissues dampen rapidly, unlike the persistent rhythms in isolated SCN neurons [5,7–9].

Sorting of the cycling transcripts into functional groups has revealed that the major classes of clock-regulated genes are implicated in processes specific to the tissue in which they are found. For example, many cycling transcripts in the liver are involved in nutrient or xenobiotic metabolism. It is also of interest that many of the regulated transcripts correspond to rate-limiting steps in their respective pathways, indicating that control is selective and very efficient. Indeed, about 10% of the genome is under the control of the circadian clock [10].

As noted, the trillions of cellular clocks in primates are synchronized by a few thousand neurons located in the SCN. It is remarkable that such a small group of neurons displays the properties of a central clock. Indeed, these “neuronal oligarchies,” like the human ones, control trillions of cells in the body by (a) taking control of the major communication channels (the endocrine and autonomic nervous systems), and (b) concentrating the relevant information in a private way (e.g., light information arriving via the retino-hypothalamic tract). Thus, it is not surprising that anatomical studies have shown that the SCN projects to at least three different neuronal targets: endocrine neurons, autonomic neurons of the paraventricular nucleus (PVN) of the hypothalamus, and other hypothalamic structures that transmit the circadian signal to other brain regions [2]. The SCN projections are generally indirect, via the sub-PVN zone [11]. Through autonomic nervous system projections involving the superior cervical ganglia, the SCN controls the release of a major internal synchronizer, the pineal substance melatonin [12].

Although circadian rhythms are anchored genetically, they are synchronized by and maintain certain phase relationships to external factors [13]. These rhythms will persist with a period different from 24 hours when external time cues are suppressed or removed, such as in complete social isolation or in constant light or darkness. Research in both animals and humans has shown that only a few such environmental cues are effective entraining agents for the circadian oscillator (“Zeitgebers”).
Indeed, temporal organization is an important feature of the biological systems, and its main function is to facilitate adaptation of the organism to the environment. The light–dark cycle, food, ambient temperature, scents, and social cues have all been identified as Zeitgebers in rats [9]. Stress is also capable of perturbing temporal organization by affecting the shape and amplitude of a rhythm or by modifying the intrinsic oscillatory mechanism itself. In particular, social stress in rodents has been found to cause disruptions of the body temperature, heart rate, and locomotor activity rhythms (see, e.g., Refs [14–16]).

An entraining agent can actually reset, or phase-shift, the internal clock. Depending on when an organism is exposed to such an entraining agent, circadian rhythms can be advanced, delayed, or not shifted at all. Therefore, one factor involved in adjusting the daily activity pattern to the appropriate time of day is a rhythmic variation in the influence of the Zeitgeber as a resetting factor [13]. In humans, light exposure during the first part of the night delays the phase of the cycle; a comparable light change near the end of the night advances it. At other times during the day, light exposure has no phase-shifting influence [17,18].

In the case of the immune system, our work concentrated on the role of the autonomic nervous system (parasympathetic and sympathetic) in providing the anatomical basis for the circadian control of lymph node function [19,20]. These studies continued our former studies on the role of sympathetic and parasympathetic nerve terminals in thyroid follicular and C cell and parathyroid cell regulation [21,22]. The concept that autonomic nerves are a very efficient avenue for conveying time-of-day information to the periphery has since been generalized to tissues like the adrenal glands, pancreas, liver, ovaries, and many other organs [2,23].

6.2 The Immune System Shows a Circadian Organization

Light and daily rhythms have a profound influence on immune function. Many studies have described circadian variations of immune parameters such as lymphocyte proliferation, antigen presentation, and cytokine gene expression. The number of lymphocytes and monocytes in the human blood reaches maximal values during the night and is lowest after waking. Natural killer (NK) cells, by contrast, reach their highest level in the afternoon, with a normal decrease in number and activity around midnight [24–28].

Immune cells have been checked for the presence of clock genes [29–33]. In a study intended to determine whether circadian clock genes function in human peripheral blood mononuclear cells, the circadian clock genes human Per1, Per2, and Per3 were found to be expressed in a circadian manner in human peripheral blood mononuclear cells, with a peak level occurring during the second part of the active phase [34,35]. To investigate the presence of molecular clock mechanisms in NK cells, as well as the circadian expression of critical factors involved in NK cell function, Arjona and Sarkar [36] measured the circadian changes in the expression of clock genes (Per1, Per2, Bmal1, Clock), Dbp (a clock-controlled output gene), cAMP (response element-binding) CREB (involved in clock signaling), cytolytic factors
(granzyme B and perforin), and the cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α in NK cells enriched from the rat spleen. They discovered that the existence of molecular clock machinery is conserved across different lymphocyte subsets and peripheral blood cells. Moreover, these cells may share common entrainment signals. Emerging data in the literature suggest that circadian regulation may be crucial for host defenses against cancer [37,38].

Both the humoral arm and the delayed (cellular) arm of the immune system function in a rhythmic manner. Indeed, circadian variations in immunocompetent cells in peripheral blood are of a significant enough magnitude to require attention in medical diagnostics [39,40]. Researchers have reported circadian changes in the circulation of T, B, or NK lymphocyte subsets in peripheral blood, and in the density of epitope molecules at their surface, which may be related to cell reactivity to antigen exposure. Changes in lymphocyte subset populations can depend on time-of-day-associated changes in cell proliferation in immunocompetent organs and/or on diurnal modifications in lymphocyte release and traffic among lymphoid organs. Circadian rhythmicity is revealed in circulating cells; lymphocyte metabolism and transformability; circulating hormones and other substances that may exert various actions on different targets of the immune system; cytokines, receptors, and adhesion molecules; cell cycle events in health and cancer; reactions to antigen challenge; and disease etiology and symptoms [29–35,41,42].

It must be noted that the role of the SCN, the central circadian pacemaker, in entrainment of lymphocyte function and in coordination of signals by which circadian information is conveyed to the immune cells, remains unsettled. Rhythms in the number of circulating T cells persisted in rats with disrupted circadian output [43]. Similarly, SCN ablation did not affect the 24-hour rhythms in cell cycle phase distribution in bone marrow cells [44], suggesting that some rhythms in the immune system are SCN independent. It is known that circadian gene expression can be maintained in vitro [45]. Thus, some peripheral clocks may be able to independently generate circadian oscillations, and this could be also the case for lymphocytes. Rather than a mere rhythm generator for the periphery, the SCN should be envisioned as a transducer for light entrainment. However, there are entrainment signals other than light that may be coordinating the rhythm in NK cell function and other immunological parameters. For example, feeding is an important Zeitgeber for peripheral clock-gene expression [43] and, interestingly enough, internal desynchronization produced by restricted feeding during the light period slowed down tumor progression in mice [46]. Daily activity rhythms are also considered to act as entrainment cues for peripheral tissues [47], and may also influence the molecular clock in lymphocyte cells. In addition, intrinsic immunological outputs such as cytokine secretion could function as entrainment factors for immune cells. Indeed, interleukin (IL)-6 has been shown to induce Per1 expression in vitro [48].

Several studies have investigated the changes in cytokine levels that occur during the 24-hour sleep–wake cycle in humans; however, it is difficult to measure these changes because endogenous cytokine levels are low (for references see [49]). Plasma TNF-α levels peak during the dark phase of the cycle, and the circadian rhythm of TNF release is disrupted by sleep pathology such as obstructive sleep
apnea. Plasma IL-1β levels also show a diurnal variation, being highest at the onset of non-REM sleep. The levels of other cytokines (including IL-2, IL-6, IL-10, and IL-12) and the proliferation of T cells in response to mitogens also change during the 24-hour cycle. Although the production of macrophage-related cytokines (such as TNF-α) increases during sleep (in response to in vitro stimulation), this occurs in parallel with a rise in monocyte numbers in the blood. The production of T-cell-related cytokines (such as IL-2) increases during sleep, independent of migratory changes in T-cell distribution [49]. All of these observed diurnal changes could be specific to the effects of sleep, or could be associated with the circadian oscillator. To dissociate the effects that result from the sleep–wake cycle from those due to the endogenous circadian oscillator, experimental procedures such as constant routine or forced desynchrony must be used. At present, there are no reports of studies using these methods to elucidate the effects of sleep on immunity.

6.3 “Sickness Behavior” Includes Changes in Circadian Rhythms

Circadian neuroimmune connections imply that the immune cells provide a very important feedback component to the brain. Indeed, there are several mechanisms by which the immune system can modify central clock structures [50–53]. In the case of rheumatoid arthritis, inflammation is characterized by increased local synovial and systemic levels of the pro-inflammatory cytokines IL-1, IL-6, IFN-γ, and TNF-α, which are directly involved in the pathophysiology of this disease [54]: such increased cytokine production plays a key role in neuroendocrine activation pathways in arthritis [55]. As large, hydrophilic proteins, cytokines can only cross the blood–brain barrier at leaky points (the circumventricular organs) or via specific active transport mechanisms [56]. Cytokines act at the level of the organum vasculosum laminae terminalis, a circumventricular organ located at the anterior wall of the third ventricle. IL-1 binds to cells located on the vascular side of this circumventricular structure, thereby inducing synthesis and release of second messenger systems, such as nitric oxide (NO) synthase (NOS)/NO and the cyclooxygenase/prostaglandin systems [57]. It must be noted that a central compartment for cytokines exists and that there are data indicating that an increase in peripheral cytokines can evoke a mirror increase in brain levels of cytokines (for references see [50,53]).

Inflammatory stimuli can also induce central nervous system (CNS) stress response through afferent peripheral neural signaling. This was shown mainly for cytokines from the peritoneum, which can cause early rapid activation of the nucleus tractus solitarius in the brainstem via the vagus nerve [53,58]. Experimental evidence suggests that symptomatology after antigen administration, like anorexia and depressed activity, is part of a defense response to antigenic challenge and is mediated by the neural effects of cytokines. These changes are known generally as “sickness behavior,” that is, the “nonspecific” symptoms (anorexia, depressed activity, loss of interest in usual activities, disappearance of body care activities) that accompany
the response to infection [59]. These nonspecific symptoms of infection include fever and profound psychological and behavioral changes in circadian structure [51]. Sick individuals experience weakness, malaise, listlessness, and inability to concentrate [52]. They consistently show evidence of decreased amplitude of circadian rhythmicity, like superficial sleep at night and hypersomnia, loss of interest, and depressed activity during the day. The link between the immune system and sleep was first identified in the 1970s, when a sleep-inducing factor was isolated and chemically characterized from human urine: factor S, a muramyl peptide derived from bacterial peptidoglycan (for references see [60]). Subsequently, muramyl dipeptide and factor-S-related peptidoglycans were all shown to induce the key immunoregulatory cytokine IL-1. IL-1β is a potent somnogen, as well as a potent pyrogen. In fact, IL-1β is one of the most neurologically active molecules known. Subsequent studies revealed that bacterial lipopolysaccharide (LPS), LPS components, and viral synthetic dsRNA, as well as killed and living bacteria, can induce IL-1, TNF-α, IL-6, and IL-10. The presence of systemic inflammation, characterized by an elevation of certain potent pro-inflammatory cytokines such as IL-1, IL-6, IL-10, and TNF-α, may predispose patients to develop cardiovascular complications.

In addition to acute inflammation, there is a range of other clinical conditions in which peripheral cytokine signals might modulate brain function. Numerous studies have shown that the therapeutic administration of cytokines for the treatment of hepatitis, cancer, multiple sclerosis, or rheumatoid arthritis induces depressive symptomatology, which widely overlaps with the syndrome of sickness behavior observed in animal models of acute inflammation [59]. However, during acute infection and inflammation the amounts of circulating inflammatory cytokines are huge, usually two orders of magnitude or more above baseline levels. In contrast, circulating levels of cytokines are only moderately increased in the most frequent clinical situations in which cytokines play a role in inducing symptoms of depression, such as chronic infection or inflammation, stress, alcoholism, aging, cancer, cardiovascular disease, or autoimmune disorders. Slightly increased TNF-α and possibly also IL-6 levels are often found in patients with these diseases [61].

It is important to note that a clinically relevant immune circadian component is the T helper 1 (Th1)/T helper 2 (Th2) balance [62]. Both branches support different defense functions. Th1 responses include cell-mediated reactions that are important for cellular pathogens, whereas Th2 responses regulate production of antibodies in response to extracellular pathogens and mediate allergic processes. Moreover, the effects of IFN-γ, a major Th1 cytokine, and IL-4, a major Th2 cytokine, are antagonistic. Thus, the cytokine balance, which determines the selection of the effector mechanisms of type 1 or type 2 immunity [62,63], is a factor critical for the development of an effective immune response.

Aside from other cytokines, including IL-2 and TNF-α, Th1 cells releasing mainly INF-γ become activated in response to intracellular viral and bacterial challenges and support various cellular (type 1) responses, including macrophage activation and antigen presentation. In contrast, the cytokines typical of Th2 immunity—IL-4 as well as IL-5, IL-10, and IL-13—tend to drive humoral (type 2) defense by stimulating mast cells, eosinophils, and B cells against extracellular pathogens. Nocturnal
sleep favors a shift toward Th1-mediated immune defense. A circadian peak of the ratio of IFN-γ/IL-10 production in whole blood samples is found during nocturnal sleep. This peak was completely abolished after the administration of cortisone at 21:00 hours in the preceding evening, suggesting that the suppression of endogenous cortisol release during early sleep plays a mediating role in the Th1 shift [62,63]. However, slow-wave sleep not only suppresses the release of glucocorticoids, but also promotes the release of growth hormone (GH) and prolactin, which support Th1 cell-mediated immunity.

In the past few years, a number of studies have started to unravel the basis for immune-factor circadian modulation of the circadian system itself [53]. Several reports indicate a possible immune feedback regulation of the circadian clock. For example, immunosuppressant drugs such as cyclosporine affect the phase of locomotor activity [64] and of hormone secretion [65,66]. Moreover, immune-related transcription factors are present and active in the SCN, and SCN activity is partially necessary for light-induced phase shifts [64].

Introduction of gram-negative bacteria into the body causes the liberation of toxic, soluble products of the bacterial cell wall, such as LPS, also known as endotoxin. Peripheral administration of LPS exerts profound effects on the sleep–wake cycle and sleep architecture and may produce, at higher doses, fever and the characteristic sickness behavior observed during inflammatory diseases, including sleep-pattern changes and fever oscillations during the day [60,67]. In mice, susceptibility to lethal doses of endotoxin increases dramatically during the resting period [68], and a similar temporal pattern of induced mortality has also been established for TNF-α [69].

Results in hamsters indicate that LPS treatment induces changes in the phase of locomotor activity rhythms in a manner similar to light-induced phase delays [70]. The phase-shifting response to LPS was reduced when the activation of NF-κB, a transcription factor reported to play a role in the photic input of the circadian system [64], was prevented. LPS treatment stimulates the dorsal area of the SCN, as assessed by c-Fos activation [70]. Astrocytes have been shown to be mediators of immune mechanisms in several experimental models. Indeed, these cells express cytokines and their receptors in diverse cerebral structures, as well as subunits of the immune-related transcription (NF-κB), and they respond to stimulation with LPS and pro-inflammatory cytokines [53].

Data from our laboratory indicate that melatonin, administered in the drinking water, has the capacity to counteract the effect of LPS on body temperature in hamsters, when injected at “Zeitgeber” time (ZT) 0 (ZT12 defined as the time of light off) [71]. Evidence that melatonin improves survival from endotoxin shock has also been published [72,73].

Therefore, one possible mechanism through which infection-related changes in circadian rhythms can occur is by direct modification of the activity of cells in the SCN [53]. Cytokine receptors (e.g., IFN-γ receptors) have been detected in neuronal elements of ventrolateral SCN [74]. Expression of SCN IFN-γ receptors followed a 24-hour rhythm, coinciding with the expression of Janus kinase 1 and 2 as well as the signal transducer and activator of transcription factor 1, the main intracellular signaling pathway for IFN-γ. In an ontogeny study, SCN IFN-γ receptors were found
to reach their adult pattern between postnatal day 11 and 20, at a time when capacity for photic entrainment of the pacemaker becomes established [75]. Indeed, high doses of an IFN-γ/TNF-α cocktail disrupt electrical activity of SCN neurons [76,77].

The capacity of intracerebroventricular administration of IFN-γ to modify 24-hour wheel-running activity was assessed in golden hamsters [78]. Animals received IFN-γ or saline at ZT 6 or ZT 18. Intracerebroventricular administration of IFN-γ at ZT 6 produced a significant phase advance in acrophase of rhythm, an effect not seen with injection at ZT 18. IFN-γ depressed the mesor value of rhythm significantly; the effect was seen with both ZT 6 and ZT 18 injections [78]. IFN-γ was very effective at disrupting circadian rhythmicity of pituitary hormone release [79]. These results support the view that the circadian sequels arising during the immune reaction rely partly on central effects of IFN-γ [78]. A disruptive effect of systemic administration of IFN-α on the circadian rhythm of locomotor activity, body temperature, and clock-gene mRNA expression in SCN has also been documented in mice [80]. Moreover, LPS incubation modified the circadian arginine–vasopressin release from SCN cultures [81]. Motzkus et al. [48] demonstrated that IL-6 induced murine Per1 expression in SCN cell cultures. Day/night variations of transcripts encoding cytokine receptors and suppressors of cytokine signaling were correlated in groups of mice of different ages, with Fos induction elicited by intracerebroventricular injections of TNF-α and IFN-γ [82]. Cytokine-elicited Fos induction was high at early night, when suppressors of cytokine signaling levels were low. Such Fos induction was significantly reduced in the older SCN at early night, and paralleled by reduced expression of IFN-γ receptor transcripts as compared to the younger SCN.

Most of the neuroendocrine effects of cytokines have been examined at single time point in the day–night cycle, thus overlooking the intricacies of significant daily variation in pituitary hormone release. Because of this, we measured the circadian pattern of plasma adrenocorticotropic hormone (ACTH), GH, prolactin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) at six different time points within a 24-hour cycle in adult male Wistar rats that received five daily injections (intraperitoneal) of human IFN-γ (105 U.I./kg body weight) or saline at 08:30 hours [79]. A factorial ANOVA for main effects indicated a significant (43%) increase of circulating prolactin in IFN-γ-treated rats. Time-of-day changes were significant for the five hormones examined, and these diurnal variations became altered by IFN-γ administration, with a phase-advance of the ACTH peak, a suppression of the rest-phase peak of GH, the appearance of a second peak of prolactin at an early phase of daily photoperiod, and blunting of the 24-hour variations of plasma FSH [79]. The data further pointed to an effect of IFN-γ on the mechanisms responsible for the circadian organization of pituitary hormone release. Indeed, severe immune challenges, such as animal models of sepsis [83] or infection with blood-borne parasites such as Trypanosoma cruzi or Trypanosoma brucei [84], or HIV-infected animals or patients [85] display different levels of circadian disruption, including complete arrhythmicity, suggesting that circadian rhythms can be considered a good indicator of quality of health.

In recent years we have examined the circadian disruption of hormone release and immune-related mechanisms in several animal models in which circulating cytokines are increased, including rat adjuvant arthritis, alcoholism, calorie restriction, social
isolation in rats and rabbits, and the aging process [86]. The underlying rationale for
the experimental approach used was that most published studies dealing with hor-
mone or immune changes in the above-mentioned situations were performed at sin-
gle time points in the 24-hour span—an important drawback in view of the circadian
nature of hormone release and immune function; also, most of the preceding manipu-
lations employed disrupt circadian rhythmicity. The results obtained in the rat model
of adjuvant arthritis are reviewed in section 6.4.

6.4 Rat Adjuvant Arthritis as an Experimental
Model of Rheumatoid Arthritis

Rheumatoid arthritis is a systemic inflammatory disorder that mainly affects the dia-
throdial joint. It is the most common form of inflammatory arthritis and affects about
1% of the population, in a female/male ratio of 2.5/1. The disease can occur at any
age, but it is most common among those aged 40–70 years. The geographic distri-
bution of rheumatoid arthritis is worldwide, with a notably low prevalence in rural
areas [87–89].

Although it initially presents as a symmetrical polyarticular synovitis with promi-
nent hand involvement, rheumatoid arthritis has multiple potential systemic manifes-
tations. The clinical course of the disorder is extremely variable, ranging from mild,
self-limiting arthritis to rapidly progressive multisystem inflammation with profound
morbidity and mortality. Fever and weight loss can be part of the acute symptoms,
while splenomegaly, vasculitis, neutropenia, and amyloidosis are some of the com-
lications that may occur in patients with long-standing disease [87,90,91].

Rheumatoid arthritis is a T-cell-driven autoimmune process associated with the
production of autoantibodies. Rheumatoid arthritis is initiated by CD4+ T cells,
which amplify the immune response by stimulating other mononuclear cells, syn-
ovial fibroblasts, chondrocytes, and osteoclasts. The release of cytokines, especially
TNF-α, IL-1, and IL-6, causes synovial inflammation. In rheumatoid arthritis, the
inflammatory process, usually tightly regulated by mediators that initiate and maintain
inflammation and mediators that shut the process down, becomes imbalanced, leaving
inflammation unchecked and resulting in the destruction of cartilage and bone.

Efforts to develop safer and more effective treatments for rheumatoid arthritis rely
heavily on the availability of suitable animal models [92,93]. Among these models, rat
adjuvant arthritis is widely employed [94]. Hallmarks of this rat model are reliable onset
and progression of easily measurable, polyarticular inflammation, marked bone resorp-
tion, and periosteal bone proliferation. Induction of adjuvant disease can be done either
with Freund’s complete adjuvant supplemented with mycobacterium, or by injecting
synthetic adjuvants [92–94]. The pathogenesis for development of adjuvant disease fol-
lowing injection of mycobacterial preparations is not fully understood, although a cross-
reactivity of mycobacterial-wall antigens with cartilage proteoglycans occurs.

After FCA injection into rats, the inflammatory disease of the joints shows four stages
in its time course: preclinical (first week), acute (weeks 2–4), postacute (weeks 5–8),
and recovery (weeks 9–11) [95]. The preclinical stage of FCA arthritis (first week) is characterized by discrete radiological lesions of the forepaws and a slight increase in the threshold for struggle triggered by foot pressure, presumably due to an impending, initially painless, stiffness. The acute stage of arthritis (weeks 2–4) is defined by signs of hyperalgesia, lack of mobility, and a pause in body weight gain; during the acute period, hindpaw and forepaw joint diameters increase [95]. In the later, acute, stages of disease (day 12+), adjuvant arthritis rats are often relatively immobile due to severity of paw swelling. At day 18th, an increase in scratching behavior and signs of hyperalgesia are clearly established as compared with the adjuvant’s vehicle-injected group. FCA arthritis is induced most easily in inbred Lewis rats; it is also produced, to a milder extent, in Wistar and Sprague-Dawley rats [93,96–100].

Use of the adjuvant arthritis model offers an opportunity to study pathological changes in a variety of tissues other than the joints [53]. Among these, CNS changes are most relevant [50–52]. The major objective of our work during the last years has been to examine several circadian correlates of both the preclinical and acute phases of arthritis in rats.

6.4.1 Adjuvant Arthritis Disrupts Normal Chronobiological Organization

We have examined a number of immune and neuroendocrine circadian rhythms in FCA-injected rats by looking for changes in the preclinical phase of arthritis (2–3 days after FCA injection), as well as in the acute phase of the disease (18 days after FCA injection) (Tables 6.1–6.3).

Generally, changes in circadian rhythms in lymph node immune function tended to be more profound at the preclinical phase of the disease. For example, B-cell- and T-cell-mediated mitogenic activity of LPS and concanavalin (Con A), respectively, were modified in amplitude or acrophase during the preclinical phase [101] but exhibited few or no changes during the acute phase of experimental arthritis [102] (Table 6.1). Similarly, 24-hour variations of B and T cells, as well as of CD4+ (T helper) and CD8+ (T cytolytic) cells, became significantly changed during the preclinical phase [101,103] but showed an absence of changes during the acute phase [102]. In the case of lymph node cell proliferation and local autonomic nerve activity, the increase in amplitude and mesor of rhythms found in the preclinical phase of arthritis was higher than that observed as the disease progressed [104]. Therefore, the results suggest that some sort of homeostatic compensation for initial changes in circadian rhythmicity of immune changes occurs with the development of arthritis (Table 6.1).

Regarding changes in neuroendocrine rhythmicity during rat arthritis, early data from FCA-injected rats had indicated that the 24-hour organization of the biologic responses was altered. For example, morning–evening differences in circulating ACTH and corticosterone disappeared by days 7–21, and between days 6 and 8 after FCA injection a loss of the adrenocortical ornithine decarboxylase (ODC) circadian rhythm of activity was found [105]. In our own studies conducted during the preclinical phase of arthritis, we found a significant effect of immune-mediated inflammatory response on diurnal rhythmicity of circulating ACTH, GH, prolactin, and thyrotropin
Table 6.1 Summary of Changes in Circadian Rhythms of Submaxillary Lymph Node Immune Responses during the Preclinical (3rd day) and Acute (18th day) Phases of Freund’s Adjuvant Arthritis in Rats

<table>
<thead>
<tr>
<th>24-Hour Rhythms</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preclinical Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>Mitotic response to Con A</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Mitotic response to LPS</td>
<td>Unchanged</td>
<td>Changed</td>
<td>Unchanged</td>
</tr>
<tr>
<td>NK cells</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>B cells</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>T cells</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>B–T cells</td>
<td>Induction of rhythm</td>
<td>Induction of rhythm</td>
<td>Increased</td>
</tr>
<tr>
<td>CD4+ cells</td>
<td>Induction of rhythm</td>
<td>Induction of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>CD8+ cells</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>CD4+–CD8+ cells</td>
<td>Induction of rhythm</td>
<td>Induction of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Noradrenergic activity</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>Cholinergic activity</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Acute Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>Mitotic response to Con A</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Mitotic response to LPS</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>B cells</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>T cells</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>CD4+ cells</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>CD8+ cells</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Noradrenergic activity</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>Cholinergic activity</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>5-HT</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Inhibitory amino acids</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Excitatory amino acids</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
</tbody>
</table>

\(^a\) Estimated from ornithine decarboxylase activity changes.

\(^b\) Estimated from tyrosine hydroxylase activity and neuronal NE uptake.

\(^c\) Estimated from \(^3\)H-acetylcholine synthesis and neuronal choline uptake.

\(^d\) Aspartate, glutamate.

\(^e\) GABA, taurine.
(TSH) release, and further found that this effect was partially sensitive to immunosuppression by cyclosporine [65] (Tables 6.2 and 6.3). Further experiments indicated that hypothalamic levels of corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), GH-releasing hormone (GHRH), and somatostatin were altered in the preclinical phase of arthritis [106]. In the median eminence, rats injected with the adjuvant vehicle exhibited significant 24-hour variations for the four hypophysiotropic hormones examined, with maxima at noon. These 24-hour rhythms were inhibited or suppressed 3 days after FCA injection. Administration of the immunosuppressant drug cyclosporine impaired the depressing effect of FCA injection on CRH, TRH, and somatostatin content in median eminence, but not on GHRH. The activity of cyclosporine was less evident in other hypothalamic regions examined [106]. Generally, a decrease in amplitude or mesor of transmitter rhythms was detectable, mainly in anterior and medial hypothalamic regions [107] (Table 6.2).

Table 6.2 Summary of Changes in Circadian Rhythms of Hypothalamic and Hypophysial Hormones and Neuropeptides, Pineal Melatonin, and Plasma Proteins during the Preclinical (3rd day) Phases of Freund’s Adjuvant Arthritis in Rats

<table>
<thead>
<tr>
<th>24-Hour Rhythms</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preclinical Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Augmented</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Augmented</td>
</tr>
<tr>
<td>GH</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>TSH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>LH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>Albumin</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>α-1 globulin</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>α-2 globulin</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>β-globulin</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>γ-globulin</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Median eminence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>TRH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>GHRH</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>NE</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>DA turnover</td>
<td>Unchanged</td>
<td>Changed</td>
<td>Unchanged</td>
</tr>
<tr>
<td>5-HT turnover</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

(Continued)
We also examined the changes in circadian rhythms of CNS and hypophysial hormones and neurotransmitters during the acute phase of Freund’s adjuvant arthritis (i.e., 18 days after FCA administration) (Table 6.3). Differing from the relative compensation of circadian immune changes seen at this time of arthritis, changes in 24-hour rhythms of neuroendocrine parameters persisted during the clinical phase of the disease [108]. Daily rhythms in plasma LH, testosterone, and TSH became

<table>
<thead>
<tr>
<th>24-Hour Rhythms</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anterior hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>TRH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>GHRH</td>
<td>Unchanged</td>
<td>Changed</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Unchanged</td>
<td>Changed</td>
<td>Unchanged</td>
</tr>
<tr>
<td>NE</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>DA turnover</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>5-HT turnover</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td><strong>Medial hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>TRH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>GHRH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>NE</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>DA turnover</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>5-HT turnover</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td><strong>Posterior hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>TRH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>GHRH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>NE</td>
<td>Unchanged</td>
<td>Changed</td>
<td>Unchanged</td>
</tr>
<tr>
<td>DA turnover</td>
<td>Decreased</td>
<td>Unchanged</td>
<td>Decreased</td>
</tr>
<tr>
<td>5-HT turnover</td>
<td>Unchanged</td>
<td>Changed</td>
<td>Unchanged</td>
</tr>
<tr>
<td><strong>Pineal gland</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melatonin</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
</tbody>
</table>
suppressed or disrupted in arthritic rats. Concerning GH, the depressed mean values found in the preclinical phase of arthritis also persisted during the acute phase, as was the case for the changes in catecholamine transmitter activity [109]. Twenty-four-hour variations in dopamine (DA) content were blunted in the anterior hypophysial lobe, but remained unaltered in the neurointermediate lobe [109]. Disruption of

### Table 6.3 Summary of Changes in Circadian Rhythms of Hypothalamic, Hypophysial, and Pineal Hormones and Neurotransmitters during the Acute (18th day) Phase of Freund’s Adjuvant Arthritis in Rats

<table>
<thead>
<tr>
<th>24-Hour Rhythms</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute Phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Augmented</td>
</tr>
<tr>
<td>FSH</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>LH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Testosterone</td>
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<td>Suppression of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>GH</td>
<td>Absence of rhythm</td>
<td>Absence of rhythm</td>
<td>Decreased</td>
</tr>
<tr>
<td>TSH</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
<td>Unchanged</td>
</tr>
<tr>
<td><strong>Anterior hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>Suppression of rhythm</td>
<td>Suppression of rhythm</td>
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<td>5-HT turnover</td>
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endocrine circadian rhythms of plasma prolactin, insulin-like growth factor-1, LH, and testosterone, and of pituitary prolactin mRNA, was recently reported in male Long-Evans rats injected with FCA 23 days earlier [110].

6.4.2 Melatonin as a Circadian Immunoregulatory Signal in Adjuvant Arthritis

Melatonin was first isolated and identified by Lerner and his associates in 1958 [111]. It is the major neurohormone secreted by the pineal gland during the dark hours at night. In addition, melatonin has been demonstrated to be produced by many organs other than the pineal gland, including the retina [112], the gastrointestinal tract [113], skin [114], lymphocytes [115], the thymus [116], and bone marrow cells [117].

Circulating melatonin derives almost exclusively from the pineal gland. Once formed, melatonin is released into the capillaries, and in higher concentrations into the cerebrospinal fluid [118], and is then rapidly distributed to most body tissues [119]. Circulating melatonin is metabolized mainly in the liver, where it is first hydroxylated by cytochrome P450 monooxygenases and then conjugated with sulfate to form 6-sulfatoxymelatonin. Melatonin is also metabolized in tissues by oxidative pyrrole-ring cleavage into kynuramine derivatives. The primary cleavage product is \( N^1\)-acetyl-\( N^2\)-formyl-5-methoxykynuramine (AFMK), which is deformylated either by arylamine formamidase or hemoperoxidase to \( N^1\)-acetyl-5-methoxykynuramine.

It has been proposed that AFMK is the primitive and primary active metabolite of melatonin [120]. The SCN regulates pineal gland function through a polysynaptic network involving the subparaventricular zone in the hypothalamus. Descending polysynaptic fibers from these regions project through the medial forebrain bundle and the reticular formation to the intermediolateral horns of the cervical segments of the spinal cord [121]. Postganglionic sympathetic fibers from the superior cervical ganglia reach the pineal gland and regulate melatonin biosynthesis through the presynaptic release of norepinephrine (NE). NE release occurs during the “night” portion of the circadian pacemaker cycle, provided that this occurs in a dark environment [122].

Plasma melatonin exhibits a circadian rhythm with high levels at night and low levels during the day, attaining peak concentrations of plasma melatonin between 02:00 and 04:00 hours. The timing of melatonin secretion is closely associated with the timing of sleep, and it also coincides with decreases in core body temperature, alertness, and performance [123]. Melatonin has the capacity to alter the timing of circadian rhythms, and functions in concert with light to synchronize circadian rhythms with the prevailing light–dark cycles. Melatonin has been used therapeutically in treating various circadian-rhythm sleep disorders, such as the delayed sleep phase syndrome, shift-work sleep disorder, jet lag, and blindness [124–127].

Melatonin exerts many physiological effects by acting on membrane and nuclear receptors, although other actions are receptor-independent (e.g., scavenging of free radicals or interaction with cytoplasmic proteins). The two melatonin receptors cloned so far (\( MT_1 \) and \( MT_2 \)) are membrane receptors that have seven membrane domains and belong to the superfamily of G-protein-coupled receptors [128].
Melatonin receptor activation induces a variety of responses that are mediated both by pertussis-sensitive and -insensitive \(G_i\) proteins. In the cytoplasm, melatonin interacts with calmodulin [129] or tubulin [130]. Nuclear receptors of the retinoic acid receptor superfamily (RZR/ROR \(\alpha\)) have been identified in several cells, among them human lymphocytes and monocytes [131].

The immunomodulatory role of melatonin is related in part to its action on specific melatonin receptors located in immunocompetent cells. In a study on two human lymphocytic (Jurkat) and monocyctic (U937) cell lines, the addition of melatonin was found to enhance IL-2 and IL-6 production by acting primarily through nuclear RZR/ROR \(\alpha\) receptors [132]. Human lymphocyte-synthesized melatonin may play a crucial role in modulating the IL-2/IL-2 receptor system, as indicated by studies showing that when melatonin biosynthesis is blocked by the tryptophan hydroxylase inhibitor parachlorophenylalanine, both IL-2 and IL-2 receptor levels fall, an effect counteracted by the addition of exogenous melatonin [131]. Similarly, prostaglandin E\(_2\)-induced inhibition of IL-2 production increased when MT\(_2\) membrane receptors were blocked by luzindole. Taken together, these data indicate that melatonin synthesized in human lymphocytes is involved in the physiological regulation of the IL-2/IL-2R expression through a mechanism comprising both membrane and nuclear melatonin receptors [131].

The contribution of MT\(_1\) and MT\(_2\) receptors in mediating the melatonin-induced enhancement of cellular and humoral immune function was explored in mice [133]. The effect of melatonin-enhanced splenocyte proliferation in both wild-type and MT\(_1\) \(-/-\) mice was blocked by the MT\(_2\) antagonist luzindole, indicating that the melatonin-induced enhancement of immune function was mediated via MT\(_2\) receptors [133].

Repeated stimulation of T helper (Th) cells in the presence of IL-12 causes Th cells to differentiate into Th1 cells, which produce IL-2 and IFN-\(\gamma\) and are particularly effective in enhancing immune responses that involve macrophages and other phagocytes. Melatonin augments IFN-\(\gamma\) production by Th1 cells. The enhancement of NK cell activity by melatonin is attributed to the increased production of IL-2 and IL-12 [131]. Physiologically, the nocturnal melatonin peak has been associated with a high IFN-\(\gamma\)/IL-10 ratio [134]. Melatonin’s immunoenhancing effect depends on its ability to enhance the production of cytokines, as well as its antiapoptotic and antioxidant action.

Our studies on the role of melatonin in arthritis have been mainly addressed to examination of the participation of melatonin in regulation of circadian rhythmicity of immune parameters in rats [107]. Pretreatment for 11 days with a pharmacological dose of melatonin (100\(\mu\)g) affected some aspects of the early phase of the immune response elicited by FCA injection, at the preclinical phase of disease. Cell proliferation in rat submaxillary lymph nodes and spleen during the immune reaction (as assessed by ODC activity) exhibited a pineal-dependent diurnal rhythmicity, as it was reduced by pinealectomy or pineal sympathetic denervation [135,136]. This effect was counteracted by a pharmacological melatonin dose (100\(\mu\)g/day). Exogenous melatonin also restored the reduced amplitude in diurnal rhythms of lymph node or splenic tyrosine hydroxylase (TH) activity and lymph node acetylcholine synthesis [135,136].
Further examination of melatonin activity on circadian rhythmicity of cell proliferation in submaxillary lymph nodes and spleen at the clinical phase of arthritis was conducted in young and old Sprague-Dawley rats [137]. Pineal melatonin content was measured, as well as the efficacy of melatonin treatment to recover modified circadian rhythmicity of submaxillary lymph node and splenic ODC and TH activities and of lymph node \(^3\)H-acetylcholine synthesis. After 17 daily injections of 10 or 100 \(\mu g\) of melatonin in the evening, the treatment restored the inflammatory response in old rats (assessed plethysmographically in hind paws) to the level found in young animals. In young rats, an inflammation-promoting effect of 100-\(\mu g\) melatonin was demonstrated. As a consequence of the immune reaction, submaxillary lymph node and splenic lymph cell proliferation augmented significantly, with acrophases of 24-hour rhythms in the afternoon for lymph nodes and in the morning for spleen. Mesor and amplitude of ODC rhythm were lowest in old rats, although melatonin injection generally augmented its amplitude. Lymph node and splenic TH activity attained maximal values at early night, while maxima in lymph node \(^3\)H-acetylcholine synthesis occurred in the afternoon. Amplitude and mesor of these rhythms were lowest in old rats, an effect generally counteracted by melatonin treatment. The results are compatible with an age-dependent, significant depression of pineal melatonin synthesis during adjuvant-induced arthritis and with decreased amplitude of circadian rhythms in immune-cell proliferation and autonomic activity in lymph nodes and spleen at the clinical phase of the disease. This picture was generally counteracted by melatonin injection, mainly in old rats [137].

A number of studies were carried out to examine the participation of melatonin in altered 24-hour rhythms of serum ACTH, GH, prolactin (PRL), LH, and insulin in rats at the preclinical phase of Freund’s adjuvant arthritis [138]. The data indicated that several early changes in levels and 24-hour rhythms of circulating ACTH, PRL, and LH in FCA-injected rats were sensitive to treatment with melatonin (100 \(\mu g\)). An effect of melatonin treatment on 24-hour variations in hypothalamic 5-HT and DA turnover during the preclinical phase of Freund’s adjuvant arthritis was also apparent [139]. FCA injection suppressed circadian rhythmicity of 5-HT turnover in the anterior hypothalamus, an effect prevented by the previous injection of melatonin. Melatonin decreased the 5-HT turnover rate in the anterior hypothalamus. Melatonin also prevented the changes in 5-HT turnover of medial hypothalamus evoked by Freund’s adjuvant. As far as hypothalamic DA turnover, the preventative effect of melatonin was less clear, as the melatonin sometimes synergized with the mycobacterial adjuvant to modify the normal 24-hour pattern detected in hypothalamic regions [139].

Physiological circulating levels of melatonin at midnight in rats are about 90 \(pg/ml\) [140], whereas the melatonin levels achieved within 15 minutes after systemic administration of a 100-\(\mu g\) dose are about 30 or 200-\(ng/ml\) plasma [141], with a half-life of about 20 minutes [140]. We recently addressed this subject by examining whether the administration of melatonin to pinealectomized rats, in a way that reproduced the plasma values and daily rhythm of endogenous melatonin, could affect immune responses during arthritis development [142]. Pinealectomized rats exhibited a significantly less pronounced inflammatory response, which was restored
to normal by physiological melatonin administration. The physiological doses of melatonin employed effectively counteracted the impaired response of lymph node ODC seen in pinealectomized rats.

It must be thus noted that the pharmacological effect of melatonin on the immune response may not always be beneficial, particularly in young subjects. In autoimmune arthritis developed in mice with type II rat collagen, melatonin administration (1 mg/kg) induced a more severe arthritis. Accordingly, pinealectomy in two strains of mice immunized with rat type II collagen reduced severity of the arthritis, as shown by a slower onset of the disease, a less severe course of the disease (reduced clinical scores), and reduced serum levels of anti-collagen II antibodies [143,144]. Using a 100-μg dose of melatonin, an inflammation-promoting effect could be demonstrated in young rats injected with FCA. In contrast, melatonin administration (10 or 100 μg) to old rats restored the inflammatory response in hind paws of FCA-injected rats to levels found in young rats [137]. Therefore, high levels of melatonin in young animals may stimulate the immune system and cause exacerbation of both autoimmune collagen II and mycobacterial arthritis. Indeed, there are data indicating that rheumatoid arthritis patients have increased nocturnal plasma levels of melatonin and that their synovial macrophages respond to melatonin with increased production of IL-12 and NO [145]. In these patients, inhibition of the antagonism of melatonin synthesis or effect could be therapeutically desirable.

6.5 Conclusions

Temporal organization is an important feature of biological systems, and its main function is to facilitate adaptation of the organism to the environment. The daily variation of biological variables arises from an internal time-keeping system, and the major action of the environment is to synchronize this internal clock to a period of exactly 24 hours. The light–dark cycle, food, ambient temperature, scents, and social cues have been identified as environmental synchronizers or “Zeitgebers” in rats.

This chapter discusses the circadian disruption of hormone release and immune-related mechanisms in the rat adjuvant arthritis model. The experimental manipulation used perturbed the temporal organization by affecting the shape and amplitude of the rhythm. Further experiments are needed to assess whether the changes in amplitude, as well in the timing of 24-hour rhythms discussed herein, can be attributed to an effect on the SCN or to a masking effect on some output(s) of the clock.

Acknowledgments

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Antigenic Recognition by the Brain: The Brain as an Immunocompetent Organ

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7.1 Introduction

Paul Ehrlich postulated more than a century ago that the cells mediating immune function express specific receptors (side chains) for antigen (AG), and that upon AG stimulation the receptors are shed and become antibodies [1]. Today the existence of antigen-specific receptors on B and T lymphocytes and on cells of innate immunity has been proven. The prevailing view is that the immune system is capable of antigen recognition, that it develops an immune response followed by memory, and that it is an autonomous system which provides defense for the host organism [2]. However, recent developments in neuroimmune biology show that the immune system (IS) is part of the neuroimmune supersystem (NISS), which also involves the central nervous system (CNS) and the endocrine system (ES) [3].

Some 25 years ago, Blalock hypothesized that the immune system is capable of chemical sensation [4]. At that time there was no concrete evidence for his proposal. Now we understand a lot about the signaling of the CNS by various immunological events. In this chapter, we briefly discuss how the brain recognizes foreign and altered chemical structures that may occur in the host organism (e.g., infections, trauma, degenerative conditions, cancer) and how it mobilizes the proper mechanisms in the cause of host defense.

7.2 The CNS Has Innate Immune Receptors to Sense Antigens Directly

7.2.1 TLR Expression in the Brain

The brain, neurons, and axons express toll-like receptors (TLRs) known as TLR8 [5]. In the human and mouse brain, mRNA for all TLRs is present, yet it is TLR2 and 4 that are most highly expressed. On microglia, TLR expression is widespread, as
microglia need to be able to respond quickly to both endogenous and exogenous threats. The function of neuronal TLRs in the innate immune response has not yet been identified [6–8].

Apart from viral or bacterial infection of the brain, TLRs binding to antigenic homologous epitopes (homotopes) have little role to play in most neuropathologies. This suggests that they have important roles in physiological regulation. Recent studies indicate that there are many endogenous ligands for TLRs [9–11]. Understanding the role of these receptors in brain injury and pathology without pathogen or infiltrating macrophages is of utmost importance [6].

It has been suggested that TLRs are stress receptors, as they bind to heat shock protein (HSP) mRNA [12] and to heparin sulfate [13]. The currently identified endogenous ligands of TLRs may be divided into two categories: those that alter development, particularly of dendritic cells, and those that result from cellular damage. It is primarily the detection of damage and subsequent activation of inflammatory mediators that link TLRs to most neuropathologies. The pro- or anti-inflammatory response to this damage can be beneficial, detrimental, or even both, depending on the time scale over which the response is activated [6]. For instance, although TLR3 responds to viral infection in a pro-survival manner, it may help West Nile virus to cross into the CNS [14].

It is important to note that both the activation of TLRs and the regulation of inflammation are understood as constituting a fine level of control that can tip an inflammatory event from being beneficial to being detrimental. Evidence is mounting for the role of TLRs in inflammation and regeneration that occurs following spinal cord injury, and further investigations may lead to therapeutic options to optimize both the initial response and ongoing treatment [6].

7.2.2 Innate Immune Defense in the CNS

Innate or natural immunity (NATIM) plays an important role in defending the fetus in utero; it remains active in self-defense and also participates in the physiology of the host for life. This system is ready to act instantaneously at any time, immunization is not necessary, and we never lose our natural immune defense—it is there till our last moment [15,16].

Because the blood–brain barrier (BBB) protects the CNS from invasion by immune/inflammatory cells and even from macromolecules [17], the brain relies heavily on its NATIM system for defense during homeostasis. It appears that all cells in the brain, including neurons and nerves, express innate immune receptors, and are capable of responding to homologous epitopes, which are recognized by innate antigen receptors [6]. Within the CNS, the choroid plexus participates in acute phase responses and is able to produce acute phase proteins (APP) [18]. If, however, this system is incapable of handling the insult, there are mechanisms to admit the cells of adaptive immunity (e.g., macrophages, T and B cells, and other leukocytes) into the CNS [19].

Initially NATIM was considered to be nonspecific immunity. However, recently it has become clear that innate antigen receptors do have specificity and recognize evolutionarily highly preserved homologous epitopes (homotopes; also known as
pathogen-associated molecular patterns or PAMPs [20,21]), which show cross-reactivity and may be present in microorganisms, in altered self-components, and in pathological tissues, including cancer. On this basis, innate immune recognition should be defined as polyspecific, not nonspecific. Because the target antigen of innate immunity is constant, it is possible to use germ-line gene-coded receptors, which are also constant. This allows fully differentiated effector cells to react instantaneously to antigenic challenges, without the necessity of immunization. Homotopes only activate the response, which is then regulated by cytokines, hormones, neurotransmitters, and peptides [22,23].

One may suggest that it is a perfect fit for the CNS to have its own NATIM system. This is an ancient system, known for its capability to respond instantaneously as soon as the target is recognized. So, when a neuron senses an infectious agent (for instance, via its toll-like receptors), recognition is exceedingly fast and the response follows immediately. It has been known for a long time that infection or injury will provoke inflammation, which used to be regarded as a nonspecific defense mechanism of the body. Today it is apparent that the CNS is capable of inducing inflammation by delivering to the target area pro-inflammatory neuropeptides (e.g., substance P [SP], calcitonin gene-related peptide [CGRP], and neurokinins A and B), as well as anti-inflammatory peptides (such as somatostatin [ST] and galanin [GAL]) that inhibit inflammatory reactions. Inflammation attracts leukocytes and accumulates excess nutrients, oxygen, and soluble mediators, such as hormones, neurotransmitters, cytokines, and chemokines [24]. Because of these mechanisms, the CNS is capable of mobilizing the immunological army locally at the site of infection or injury, without delay. Instantaneous recognition coupled with a rapid defense response confers a tremendous biological advantage.

We know that the CNS is sheltered from the invasion of infectious agents and even from macromolecules by the blood–brain barrier [17]. Cells of the adaptive immune system are also kept at bay and are present under physiological situations only at very low numbers. The CNS is known to kill activated killer T cells by Fas ligand, which triggers apoptosis [19]. Again, in this case the CNS relies on innate immune defense, which is inherently there. TLRs are present on all cells of the CNS, which for the most part are ready to act instantly and provide effective defense. In the case of acute illness, the CNS is capable of mounting its own acute phase response, as the choroid plexus produces acute phase proteins [18].

Although our knowledge about the significance of NATIM in physiology and pathology of the CNS is still rudimentary, it is possible to posit that the CNS is an immunocompetent organ, as it is able to specifically recognize a target by its innate receptors and to respond by the mobilization of immune effector mechanisms. As for effector mechanisms, there are many to be considered. The entire immune system is regulated by the CNS, but other mechanisms, such as the induction of apoptosis in T cells and maintenance of a defense barrier, are also involved. The ability to produce the entire repertoire of biological mediators (autonomy) results in tremendous regulatory potential. The nerve growth factor family, as well as members of the TNF cytokine family, is cytotoxic mediators [25]. The CNS is defended by NATIM for life. This form of immunity is never lost and provides reliable, instantaneous defense.
However, if the built-in defense mechanisms of the CNS fail, there are mechanisms to permit involvement of the adaptive immune system. These changes are initiated by adhesion molecules expressed by capillary endothelial cells in conjunction with cytokines. Both leukocytes and endothelial cells express adhesion molecules, which slow down and stop circulating leukocytes and eventually trigger diapedesis of the leukocytes. At sites of trauma, the BBB may be damaged, and hence blood-borne cells will gain access to the injured tissue very quickly [19].

### 7.2.3 Cells and Mediators of NATIM

The cells that play important roles in NATIM are: Neurons, glia cells, monocyte/macrophages, natural killer (NK) cells, NKT cells, marginal zone (CD5+) B lymphocytes making natural antibodies, neutrophil−, eosinophil – and basophil granulocytes, platelets, mast cells, dendritic cells, Langerhans cells, Kupffer cells in the liver, reticulo-endothelial system (RES) cells, epithelial cells. Major organs involved are the hypothalamus, pituitary, adrenals, bone marrow, and liver; all of these organs are activated during acute illness [22].

Major cytokines are IL-1β, IL-6, TNFα, GM-CSF, IL-1Ra, IL-2 through -5, –IL-10, IL-12 through -18, TGFβ3, and interferon (IFN). Other serum components are natural antibodies, defensins, complement, acute phase proteins, fibrinogen and other clotting factors, kallikrein, bradykinin, and the like. Injured or sick cells and tissues signal the immune system and the brain by the release of cytokines (CTKs) and chemokines (CMKs) [23].

### 7.2.4 Other Organs/Cells That Express TLR

All leukocytes express TLRs [26]. TLRs are expressed in the pituitary gland [27], in the adrenal gland [28], in the liver [29], in mucosal epithelial cells [30], in endothelial cells [31], in vascular smooth muscle [32], and also in the cornea [33]. These facts suggest that the entire body, including the CNS, endocrine organs, the liver, epithelium and endothelium, and possibly even more, participate in innate immune host defense. The CNS is capable of directly sensing infectious agents through TLRs, and can react instantaneously by causing inflammation, the final effector response of all immune compartments. Similarly, the pituitary produces proopiomelanocortin (POMC) in response to LPS (TLR4 is involved), mucosal epithelial TLRs participate in inflammation and respond to pathogens, corneal TLRs were found to fight infection, and endothelial TLRs were observed to play important roles in homeostasis of the heart. Therefore, in addition to participating in NATIM, TLRs fulfill important physiological functions. This is true for cytokines and also for cellular elements of the immune system, whether natural or adaptive immune cells are considered.

### 7.2.5 Neuroimmune Regulation of NATIM

Both the bone marrow and the thymus are pituitary dependent. Growth and lactogenic hormones (GLHs) support the maturation of lymphocytes and leukocytes in
both organs. The hypothalamus–pituitary–adrenal axis antagonizes GLHs, and suppresses adaptive immunity and inflammation. Catecholamines also suppress adaptive immunity through their beta-adrenergic receptors, and stimulate NATIM. During homeostasis, NATIM and ADIM are well balanced. Some immune functions, such as surface receptor expression, are promoted by glucocorticoids (GCs; part of NATIM). Physiological levels of GCs were found to be necessary for normal immune function. Other steroids, thyroid hormones, and vitamin D also modulate immune function, by either enhancement or suppression. Neuropeptides (NPEPs) play a major role in regulating neurogenic inflammation, though they also modulate immunity. NPEPs also have a role in feedback signaling to the CNS, along with cytokines and chemokines [18,23].

Glucocorticoids and beta-2 adrenergic agonists induce interleukin-10-secreting regulatory/suppressor T cells (Tsrs) in vitro from peripheral blood leukocytes of allergic patients [34]. It has long been established that elevated levels of glucocorticoids suppress adaptive immunity [35]. More recently, it became apparent that physiological levels of glucocorticoids are needed for T lymphocyte development and function [36]. Elevated levels of glucocorticoids and catecholamines stimulate Tsr development and also amplify NATIM during acute illness [16].

### 7.2.6 Acute Illness

During acute febrile illness (in which an acute phase response or APR is mounted), the hypothalamus is activated by IL-1, -6, and TNF-α to secrete corticotropin-releasing hormone (CRH) and vasopressin (VP), which act on the pituitary gland and indeed on the entire organism. Growth and lactogenic hormones are suppressed, the thymus is involuted, and the entire ADIM system is suppressed. The HPA axis is activated and regulatory/suppressor T cells are stimulated by GCs and catecholamines (CATs). Because Tsr is amplified by DC and CATs, we suggest that it has the characteristics of innate immune cells. The bone marrow is stimulated by IL-6, colony-stimulating factors (CSFs), insulin (INS), and leptin (LEP). The liver is induced to produce acute phase proteins (APP) by IL-6, GCs, and CATs. The cytokines (primarily IL-1) induce fever, whereas alpha-melanocyte stimulating hormone (αMSH) acts as a suppressor of fever. Catabolism and lipolysis (brought about by TNF and leptin) prevail in the body when the CNS, pituitary, leukocytes, and bone marrow are stimulated. Insulin resistance and hyperglycemia (induced by IL-1, TNF, and GCs) occur in a response aimed at nourishing the defense system of the body. The GLH–IGF (insulin-like growth factor) axis is replaced by INS, glucagon, and leptin. Cholinergic agents are anti-inflammatory during APR [16,37,38].

The APR is an excellent example of the regulatory power of the hypothalamus in emergency situations, in which a rapid response can be life-saving. The profound neuroendocrine and metabolic response generated by the neuroimmune system in response to infection (or trauma) suppresses ADIM, which needs a long time to act, and rapidly amplifies NATIM, which is ready to act instantaneously against the infecting (causative) agent. This defense strategy usually works very well; most of us have had febrile illnesses and recovered numerous times during our lifetimes.
7.3 Sensing Adaptive Immune Reactions by the CNS

In the adaptive immune system, an antigen is recognized first by phagocytic cells, such as a macrophage, dendritic cell, or the like. The specific effector cells of ADIM are T and B lymphocytes, which bear immunoglobulin antigen receptors on their surfaces. These receptors have exquisite specificity for the antigenic determinants (epitopes) they recognize. Lymphocytes mediate the highly specific adaptive immune response. After phagocytosis of exogenous antigens by antigen-presenting cells (APCs), the antigen is digested (“processed”) and short peptides are presented sequentially by surface major histocompatibility (MHC) molecules to CD4+ T cells. T helper type 2 (Th2) cells develop via stimulation by epitopes presented in the context of surface MHC-II. B lymphocytes also recognize macromolecular antigens via surface immunoglobulin receptors (conformational recognition), pinocytose the antigens, and process the antigens and present epitopes via their MHC-II to Th2+ helper cells. Once activated, the helper cells secrete cytokines (IL-4, -6, -8, etc.) and stimulate the presenting B lymphocytes for antibody formation. In turn, Th1 cells induce cell-mediated immunity by recognizing endogenous antigens, which may be presented by any nucleated cell after processing and presentation of the epitopes via their surface MHC-I molecules. IL-2 and IFN are major cytokines of this reaction. Effector CD4+ cells, which deliver delayed-type hypersensitivity reactions, and CD8+ killer T cells are induced. CD4+ cells induce an inflammatory response at sites of antigen depots, whereas killer T cells recognize cells with abnormal MHC-I (e.g., virus-infected or cancer cells). Such abnormal cells trigger killer T cells for cytotoxicity. The adaptive immune system is clonal and the specific clones proliferate only when stimulated by antigen, a process that takes approximately 1 week in the primary response and in which most of the antibodies produced are immunoglobulin (Ig)M. After repeated immunization, secondary responses develop, which are boosted by memory cells and hence develop faster (within 3–4 days) and produce predominantly IgG antibodies [2].

7.3.1 Cytokine Feedback Signals to the Hypothalamus

After injection of rats with various antigens (e.g., lipopolysaccharide, bovine serum albumin, tetanus anatoxin), C-fos gene and protein expression were induced in neurons of the paraventricular nucleus (PVN) in the hypothalamus. These experiments indicate that after ordinary immunization, there is activation of neurons in the PVN, which is the center of neural immunoregulation. This result proves that the CNS receives signals as soon as an immune reaction is initiated. This was true for both T-cell-dependent and T-independent antigens [39].

By now there is voluminous evidence for the existence of feedback pathways between the immune system and the brain. The idea of immune-derived feedback by cytokines was pioneered by Besedovsky and co-workers, and it has subsequently been proven by many laboratories [40].

The following cytokines were found to serve as feedback signals from the immune system to the brain: IL-1, TNFα, different types of interferons, IL-2, IL-6,
IL-11, IL-12, leukemia inhibitory factor, granulocyte macrophage colony-stimulating factor, oncostatin, and stem cell factor. These were found to affect the HPA axis and the release of other pituitary hormones [40]. These CTKs represent macrophages, T cells, NK cells, mast cells, bone marrow cells, and stem cells, which are members of both the adaptive and innate immune systems. CTKs, which are produced after cell activation during immune reactions or other (e.g., bone marrow) functions, signal the brain and endocrine organs directly or via sensory nerves.

There is compelling evidence indicating that cytokines fulfill physiological roles not only in the immune system, but also in the CNS and in other tissues and organs. Therefore, shared CTKs allow organ- and tissue-specific regulation. CTKs may also inform the brain about immune activity, as well as about other physiological processes (e.g., GM-CSF indicates granulocyte/macrophage production) [25,41].

The ascending and descending signaling pathways elicited by IL-1β were investigated. The central network consists of ascending pathways that are triggered after the transduction of IL-1β signals across the blood–brain barrier. Brainstem neurons, primarily located in the area postrema, nucleus tractus solitarius, and ventrolateral medulla, are the first central neurons recruited, and by direct and indirect pathways converge on the paraventricular nucleus to initiate HPA axis responses. Descending neuroimmune pathways from the paraventricular nucleus also transmit IL-1β signals down to the brainstem cell groups and to thoracic spinal cord preganglionic neurons, and via sympathetic projections innervate immune end organs such as the thymus and spleen. The coordination of these neural networks in response to a systemic immune challenge is important for the regulation of peripheral immune responses by the brain [42].

### 7.3.2 Neuroimmune Regulation of ADIM

Our initial observations on the immunoregulatory role of the pituitary gland revealed that hypophysectomized (Hypox) young rats (100 g body weight) did not respond to primary immunization with various antigens; the thymus, spleen, and bone marrow of these rats showed atrophy and lack of DNA and RNA synthesis. Humoral, cell-mediated, and autoimmune reactions were inhibited. Replacement doses of prolactin (PRL), growth hormone (GH), and also placental lactogen reversed these deficiencies [43–49]. These findings have been confirmed by a number of laboratories. However, it was also observed that PRL gene and receptor knockout mice remained immunocompetent [50,51]. The explanation for these results is that GH was present, and was equally capable of maintaining immunocompetence, in such knockout animals. Additional observations also support the immunoregulatory role of GLHs. For example, pituitary dwarf individuals are immunocompetent, as their PRL is normal [52]. Pit-1-deficient animals and humans have residual GLHs, which explains their survival [53,54]. Newborn mice treated with PRL antibodies (or with ergoline) became retarded and showed developmental abnormalities, and about 30% did not survive [55]. The dopaminergic agent bromocriptine inhibits PRL secretion and is immunosuppressive [56]. The secondary antibody response is partially pituitary dependent [47]. Long-surviving Hypox rats have residual serum PRL (~40% of normal). If this
PRL is neutralized by antibodies, the rats develop rapid wasting, immunodeficiency, and bone marrow failure and die within 6 weeks [57]. Congenital lack of the pituitary gland is lethal [58]. Lymphocytes are capable of synthesizing GLHs. The lymphocyte PRL gene is associated with the pituitary-independent placental promoter [59,60]. T lymphocytes secrete PRL into the rheumatoid fluid, which stimulates inflammation [61]. Type I cytokines and GLHs share the JAK-STAT signal transduction pathway [62]. The differentiation of innate immune B lymphocyte is regulated by estradiol, and the differentiation of adaptive immune B lymphocytes is regulated by PRL [63]. In this context, it is important to keep in mind that both the immune and neuroendocrine systems are redundant systems and in case of the destruction of one pathway several others may be available to substitute for the function lost.

7.4 Sensing and Regulating Inflammation: Mast Cells as Sensory and Neuroeffector Cells

Tissue mast cells have long been regarded as inflammatory cells that store numerous inflammatory mediators and discharge them in response to various noxious stimuli, thereby causing inflammation [64]. More recently, it has become clear that mast cells are integrated into the immune system by binding IgE antibodies to their surface Fc-epsilon receptors (Fc,R). After the specific antigen (allergen) meets its antibodies on the surface of mast cells, the mast cells discharge their contents and a rapidly developing inflammatory response follows (immediate hypersensitivity) [65]. At present, IgE-mediated hypersensitivity is accepted in mainstream immunology as the cause of allergy, and major efforts are being undertaken to inhibit the IgE response on the assumption that without the antibody, allergy will not develop. However, it is well known that asthma may also be of IgE-mediated (allergic) origin, though there is a nonimmune, “idiopathic” form of this disease [66].

7.4.1 Mast Cells as Sensory Cells

Mast cells are innervated (Figure 7.1) [67,68] and mediate neurogenic inflammation, which is a major effector arm of the neuroimmune supersystem [3,24]. Therefore, mast cells serve as nociceptors, immunologically specific receptors capable of delivering information to the CNS about approximately 10^11 epitopes. Mast cells also recognize complement split products (C3a, C5a; anaphylatoxins) [2] and inform the brain about complement (C’) fixation, in addition to causing inflammation in response to C’ activation. Bradykinin is an inflammatory mediator that signals the brain about inflammatory events via sensory nerves [24,69].

7.4.2 Mast Cells as Neuroeffector Cells: Neurogenic Inflammation

The sensory nerves that innervate mast cells come from the dorsal route of the spinal cord, but sensory fibers are also present in the vagus nerve. The neuropeptides, substance P, calcitonin gene-related peptide, and neurokinins A and B (tachykinins)
are able to induce mast-cell discharge, and therefore they are pro-inflammatory. In contrast, somatostatin and galanin are anti-inflammatory mediators [24]. These mediators enable sensory nerves to induce and also to inhibit inflammation. The CNS ensures that the correct amount and type of mediators are released into the target area where a pathological process is to be controlled.

### 7.5 Some Functions of the Sensory Nerve Mast Cell Pathway

There is evidence to suggest that the sensory nerve mast cell (SNMC) pathway regulates blood flow and participates in gastrointestinal physiology [67,68]. Sensory nerves may induce or down-regulate inflammation by regulating the discharge of mast cells. Under normal conditions, inflammation is the ultimate phase of host defense reactions exerted by the CNS, NATIM, and ADIM systems. By strict regulation of the inflammatory process, the CNS ensures that this potentially harmful reaction will provide protection for the host while staying within limits compatible with survival. Maladjustment of the SNMC regulatory pathway has the following pathological consequences.

#### 7.5.1 SNMC Malfunction Causes Diseases

The current dogma is that allergy is due to excess IgE antibodies, which discharge mast cells after contact with minute amounts of antigen (allergen) and create a powerful

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**Figure 7.1 Neurogenic inflammation.** A schema outlining our concept that mast cells may behave as sensory receptors for antigens. As a result of passive sensitization by specific antibodies (IgE or IgG), upon subsequent interactions with an antigen, mast cells will release mediators that may affect nerves in the local environment. Information may pass back to the nerve cell body, or affect, by axon reflex, local tissue targets. Through interneuronal contacts, information would be expected to go to the spinal cord and/or to the central nervous system. Alternatively, the CNS might act by an efferent system to modulate mast cell function.

*Source: From Bienenstock, J.*
inflammatory reaction, which could be life-threatening. However, allergy cannot be transferred by IgE antibodies [70], and serum IgE titers and skin reactions do not always correlate with the severity of allergy [71,72]. In addition, asthma has two distinct etiological types: allergic asthma and idiopathic asthma [66]. These facts are hardly compatible with the prevailing view that allergy and asthma are caused by IgE antibodies. Given the fact that mast cells and inflammation are regulated by the CNS, we suggest that allergy is the result of excessive up-regulation of mast-cell sensitivity to the allergen by the nervous system. Bronchial hypersensitivity has the same etiology. In the case of idiopathic asthma, mast cells function as nociceptors and respond to nocuous stimulation by triggering an exaggerated inflammatory reaction.

Hyperactive SNMC pathways are involved in arthritis [73], airway inflammation [74], and inflammatory conditions in the heart [75]. Bradykinin induces synovial inflammation and also signals the hypothalamus [69,76]. Vagal sensory efferents respond to pro-inflammatory cytokines (e.g., IL-1β) in visceral organs and provide inflammatory feedback signals to the hypothalamus [77]. Migraine headache is caused by a maladjusted SNMC pathway [78].

### 7.5.1.1 Alzheimer's Disease

In 2006, it was shown that the Asp299Gly mutation in TLR4 caused a decreased inflammatory response and correlated with protection from late-onset Alzheimer's disease (AD) [79]. The involvement of TLRs in AD is controversial. The innate immune system is important in both the periphery and the CNS in combating AD. Although TLR2 appears to be beneficial, the role of TLR4 is more controversial. A consistent feature of neuropathological events is the activation of microglia by TLRs. Activated microglia protect against invading pathogens and spinal cord injury; decreased activation may lead to cancer and inappropriate activation to neuropathic pain. TLRs are involved in both ischemia and reperfusion during stroke. It has also been suggested that chronic TLR malfunction can lead to AD and that TLRs respond to brain lesions [6].

Although the brain is often considered to be an immunologically privileged site, many of the pathological events are caused by invading cells. Such cells express TLRs, which manage inflammation in the CNS and stimulate the production of chemokines that regulate the invasion of cells into the CNS. The role of TLRs in neuropathology has not been fully established, and further investigation is necessary for better understanding. This receptor family offers therapeutic potential in the treatment of such conditions as neuropathic pain, Alzheimer’s disease, spinal cord injury, and multiple sclerosis. A more complete understanding of their role is essential for developing this potential [6].

### 7.6 Conclusions

The expression of TLRs by neurons proves that the brain is able to sense infections and other pathological conditions directly, using innate immune receptors.
Preliminary results indicate that TLRs are likely to be involved in inflammation and in physiological regulation as well. TLRs may play a role in neuropathic pain, Alzheimer’s disease, other inflammatory conditions in the brain, spinal cord injury, and multiple sclerosis.

Adaptive immune responses are sensed by the brain via cytokine feedback signals. The CNS exerts a regulatory influence either through innervation or by hormones and other soluble mediators, such as catecholamines, acetylcholine, tachykinins, somatostatin, galanin, cytokines, and chemokines.

Mast cells are innervated and are regulated by sensory neuropeptides (e.g., tachykinins, somatostatin, galanin). Here the CNS is informed by mediators released by mast cells after the mast cells are activated by antigen via their membrane-bound antibodies (IgE and IgG). These antibodies recognize about $10^{11}$ epitopes. Bradykinin and complement components C3a and C5a signal the brain via mast cells. Malfunctions of the sensory nerve mast cell regulatory pathway may cause allergy, asthma, arthritis, airway inflammation, and migraine headache.

The acute phase response is an emergency defense reaction mediated by the rapid stimulation of NATIM. APR is initiated by IL-1β, -6, TNF-α and CSF. When hypothalamic CRH and VP activate the HPA axis, GC and CATs then amplify NATIM and stimulate Tsrs, which have the characteristics of innate immune cells. GLHs are decreased, the thymus becomes atrophic, and ADIM suppressed by Tsrs.

It is now clear that the nervous, endocrine, and immune systems form a neuroimmune supersystem, which is characterized by continuous interaction and mutual reliance on each other and which coordinates and regulates all physiological and pathological events in higher organisms for their entire lifetime [3].

References


Section E

Pathophysiology

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8 Neurogenic Inflammation and the “Inflammatory Reflex”: Two Pathways of Immunoregulation by the Nervous System

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8.1 Neurogenic Inflammation: A Spinal Cord Reflex to Initiate Immune Response

Inflammation is a process of interactions among soluble factors and different cells that can arise in any tissue in response to traumatic, infectious, postischemic, toxic, or autoimmune injury. It leads to an abnormal condition of redness, swelling, heat, and pain localized to the inflamed tissue. The primary purpose of inflammation is to eliminate the pathogenic insult and to remove injured tissue components in order to restore normal architecture or to create a scar.

There are three steps in inflammation:

1. **Initiation** of mechanisms to find and remove foreign and injured material; this is stimulated by recognition factors of injury. This includes vasodilation and increased capillary permeability due to alterations in the vascular endothelium, which leads to increased blood flow (hyperemia) that causes redness (erythema) and entry of fluid into the tissue (edema). This phase of the inflammatory response is neurogenic and is mediated by a spinal reflex. It can be demonstrated by scratching the skin with a fingernail. The “weal and flare reaction” that occurs is composed of (a) initial blanching of the skin due to vasoconstriction; (b) the subsequent rapid appearance of a thin red line when the capillaries dilate; (c) a flush in the immediate area, generally within a minute, as the arterioles dilate; and (d) a wheal, or swollen area, that appears within a few minutes as fluid leaks from the capillaries. It is usually terminated after several tens of minutes.

2. **Amplification** of response through activation of soluble and cellular inflammatory mediators. This includes the acute cellular response that takes place over the next few hours. The hallmark of this phase is the appearance of neutrophils in the tissues. These cells attach themselves to endothelial cells within the blood vessels (margination) and cross into the surrounding tissue (diapedesis). If a vessel is damaged, fibrinogen and fibronectin are
deposited at the site of injury, platelets aggregate and become activated, and the red cells stack together to help stop bleeding. The dead and dying cells contribute to pus formation.

3. **Termination** of inflammatory response by specific inhibitors. Over the next days, resolution may occur, meaning that the inflammation will stop because of a counter-regulatory anti-inflammatory response leading to release of the powerful anti-inflammatory cytokine IL-10 and transforming growth factor (TGF)-β. Furthermore, repair processes start and various growth factors are produced. If it is not possible to return the tissue to its original form, scarring is generated by fibroblasts, collagen, and new endothelial cells.

However, when the ability to remove foreign materials is impaired, or regulation is altered, inflammation may become harmful to the host. In this situation the innate immune response may cause cell and tissue damage and hence multiple organ failure, which is known as sepsis. Inflammation may be excessive and totally inappropriate (e.g., in allergy, autoimmune diseases, sepsis), and sometimes the inflammatory response cannot terminate and becomes chronic [1–3].

A number of inflammatory stimuli (toxins, antigens, bacterial and chemical material, etc.) have been identified. These stimuli can interact with cell receptors created after a sensitizing exposure to trigger an inflammatory cascade that induces host defense. In this scenario, neurogenic inflammation occurs: this is defined as a process by which inflammation is triggered by the nervous system. This means that nerve cells recognize a stimulus and initiate the inflammatory response independent from the immune system. This is called the inflammatory spinal reflex [4–6] (Figure 8.1). Further progress of the response depends on the type of the inflammatory stimulus. For example, chemical toxins may induce inflammation only by neuronal effects. However, bacterial materials may in parallel initiate an immunological inflammation. Alternatively, neurogenic inflammation may evolve into immunological inflammation. Thus, a close relationship exists between somatic and autonomic nerves and inflammatory cells, especially mast cells, during the course of inflammation [7].

Most of the evidence for neurogenic inflammation has been derived from studies of fine unmyelinated (C−) or myelinated (Aδ−) fibers, which derive from the dorsal root ganglia and can be activated by various chemical or biochemical toxins. Stimulation of these fibers leads to the release of various neuropeptides from peripheral nerve terminals.

Neuropeptides are a group of small peptides with 4 to more than 40 amino acids. More than 20 neuropeptides have been identified. However, regarding the function of a neuropeptide, organ- and species-specific effects must be considered. The most recognized neuropeptides are substance P (SP), neurokinin (NK)-A, neurotensin, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), neuropeptide Y, somatostatin (SOM), β-endorphin, enkephalin, galanin, dynorphin, atrial natriuretic peptide, melanocyte-stimulating hormone (MSH), parathyroid hormone-related protein, corticotropin-releasing hormone (CRH), and urocortin [7]. Under physiologic conditions, immune, tissue, and neural cells are all capable of producing and releasing neuropeptides.

In neurogenic inflammation, the release of SP, CGRP, NK-A, NK-B, and somatostatin from nerve endings leads to extravasation from postcapillary venules and
increased blood flow due to dilation of arterioles, which causes chemotaxis of neutrophils and adherence to the vascular wall [7] (Figure 8.1).

Perhaps the most potent mediator of neurogenic inflammation is SP. Moreover, SP is the most abundant neuropeptide in the CNS, being widely distributed in the

**Figure 8.1** This diagram demonstrates the pathways through which the immune and nervous systems act together to eliminate an inflammatory stimulus. In this situation the type of response—neurogenic or immunological—depends on the type of the signal. Chemical toxins predominantly induce a neurogenic inflammation via a spinal reflex. The subsequent release of neuropeptides induces vasodilation and capillary leakage leading to the characteristic symptoms of redness, swelling, and pain. In contrast, bacterial material and tissue debris induce an immunological inflammation with the local release of cytokines. However, these cytokines may affect somatosensory nerve endings and transmit the information about inflammation into the brain, where it will be processed and a brain-mediated anti-inflammatory response may be initiated. In this scenario, the HPA axis, the SNS, and the efferent vagus will be activated. This leads to the release of glucocorticoids, catecholamines, and acetylcholine. These very potent anti-inflammatory mediators induce the production of anti-inflammatory cytokines in both immune and nonimmune cells. The goal of this reaction is to confine inflammation locally and to prevent spillover of cytokines into the general circulation. However, if this mechanism does not succeed, a systemic inflammatory syndrome may occur with cytokines in the blood. These blood cytokines may directly access the brain via the circumventricular region or the area postrema. This informs the brain about a situation that is more severe than a local inflammation, and may lead to an alarm situation that results in amplification of the brain-mediated anti-inflammatory response syndrome.
central, peripheral, and enteric nervous systems of many species. It belongs to a family of bioactive peptides, the tachykinins, a group that also includes NK-A and NK-B. SP acts in the CNS as a neurotransmitter/neuromodulator affecting behavior and the neurochemical response to stress—both psychological and physical stress. It coordinates the response to stress by interacting with the HPA axis and the SNS. In the dorsal horn of the spinal cord, SP is involved in sensory, and most notably nociceptive, pathways. In the periphery, SP has been identified in C-type sensory nerve endings and autonomic afferents throughout the body [8]. Inoculation of the skin with SP induces vasodilatation and increases vascular permeability, which, with its chemoattractant properties, facilitates traffic to sites of inflammation and contributes to urticaria. CGRP is less potent, but it can potentiate the effects of SP and alone causes marked vasodilation and hyperemia in the skin [7]. SP, as well as histamine, causes the formation of focal reversible endothelial gaps between endothelial cells of the vasculature, which is responsible for the increase in vascular permeability [9]. SP, as well as other sensory neuropeptides, may also interact with receptors on mast cells, which degranulate and release several inflammatory mediators, such as histamine; this reaction can itself cause urticaria and may further enhance the neurogenic inflammatory response [10]. Histamine can also bind to a histamine receptor on the sensory nerve, producing a prodromic nerve impulse up the sensory nerve and an antidromic impulse down another axon, resulting in a further release of SP and/or other sensory neuropeptides and further inflammation [11]. Thus, neurogenic inflammation may be evident at a site distant from the original exposure.

In addition to its role in neurogenic inflammation, SP is involved in immunological inflammation as well. Lymphocytes and macrophages have receptors for SP, and these cells can be stimulated by SP to produce cytokines [7,12]. Hence, macrophages stimulated by SP produce the inflammatory mediators prostaglandin (PG)-E2, thromboxane B2, and superoxide ions [12]. It is of interest that SP down-regulates the synthesis of the anti-inflammatory cytokine TGF-β [13]. These results suggest a mechanism whereby SP may also act as a pro-inflammatory mediator by limiting the production of TGF-β. Moreover, it was shown that treatment with SP alone did not enhance IL-10 secretion in either freshly isolated or cultured cord blood monocytes (FICBM), whereas treatment with SP in combination with lipopolysaccharide (LPS) leads to a synergistic interaction in up-regulation of IL-10 secretion. Fragments of SP (SP1-4 and SP5-11), whether in the presence or absence of LPS, show little effects on IL-10 secretion. SP reverses the inhibitory effect of interferon (IFN)-γ on LPS-induced IL-10 secretion by FICBM. In addition, SP antagonists and anti-SP polyclonal antibodies blocked the SP effect on IL-10 secretion, indicating that these effects are specific and mediated by the SP receptor [14]. Because SP is the classic mediator of neurogenic inflammation, and because SP can also stimulate immune cells to produce and release classic pro-inflammatory cytokines (TNF-α, IL-1, and IL-6), SP may serve as a key mediator in the transition of neurogenic to immunological inflammation. Thus, the route of communication between the immune and nervous systems is receptor and mediator sharing. Neural cells express cytokine receptors and produce neurohormones, neurotransmitters, and neuropeptides, whereas immune cells express receptors for neurohormones, neurotransmitters,
and neuropeptides and produce cytokines. In this context, neurogenic inflammation seems to be less than fully independent of the immune system. Moreover, most cells expressing receptors for neuropeptides also generate the neuropeptide-degrading peptidases neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE), thereby terminating the inflammatory stimulus (see below). Similarly, cells synthesizing receptors for the neurotransmitters acetylcholine and noradrenaline also generate enzymes that control the effects of these molecules. Thus, a close interaction between neuromediators, target cell receptors, and neuropeptide-degrading enzymes is critical for controlling neurogenic and immunological inflammation [15].

Finally, recent findings suggest that classic autonomic neurotransmitters, cytokines as well as neuropeptides, can also be generated by nonneuronal and nonimmune cells such as keratinocytes, astrocytes, endothelial cells, alveolar cells, and the like. Interestingly, there is histological evidence of the presence of cytokines, especially IL-6, within both sensory and autonomic nerves. The release of the pro-inflammatory cytokines with nervous discharge provides a powerful stimulus for inflammatory immune cells in the areas affected to amplify immune response, and indicates a further linkage between neurogenic and immunological stimulation [7,16]. This demonstrates the complex interaction among tissue, neural, and immune cells in initiation, amplification, and termination of an inflammatory signal (Figure 8.1).

Consequently, as already mentioned, the distinction between neurogenic and immunological inflammation seems to be limited to the catalysts, because the two types cross over after the release of mediators. For instance, it seems that chemical toxins (e.g., cigarette smoke) may stimulate chemosensitive sensory nerve endings, whereas bacterial material (e.g., LPS) may activate immune cells (especially macrophages), and mechanical injury or ischemia may at first affect local tissue cells (Figure 8.1). However, crossovers will follow.

Furthermore, the clinical appearance of neurogenic and immunological inflammation is not identical in all cases. In the upper airways and skin, the initial complaint associated with exposure to chemical irritants is burning or pain, whereas itching is the initial complaint associated with immune-mediated exposures. It may be that there is individual variability in the degree to which crossover is clinically manifested [6,17].

Progress has been made in understanding the regulation of neurogenic inflammation. Recent studies indicate that NEP and ACE play an important role in down-regulation of neurogenic inflammation. Although ACE is capable of degrading SP, NEP additionally cleaves the neuropeptides NK-A, NK-B, VIP, PACAP, atrial natriuretic peptide, and endothelins. Both NEP and ACE have been identified in different tissue cells (skin, lung, mucosa, CNS, etc.). In vivo studies using NEP knockout mice demonstrated a significant increase of plasma extravasation and cutaneous inflammation. Maximal inflammation occurred 6 hours after challenge in a model of experimentally induced contact dermatitis, which confirms the anti-inflammatory role of NEP [17,18]. Similar effects on cutaneous inflammation (contact hypersensitivity) were observed for ACE. These findings imply that up-regulation of NEP is a potential therapeutic approach to limit the effects of neurogenic inflammation. Down-regulation of NEP and ACE may result in an uncontrolled stimulation of neuropeptides and lead to chronic inflammation [15].
Moreover, vagus nerves supply most of the sensory fibers to different organs, for example the airways. Inappropriate activation of these nerves can lead to many of the symptoms of allergic and chronic obstructive pulmonary disease, such as coughing, mucus hypersecretion, and bronchoconstriction [17]. The afferent sensory fibers, which contain neuropeptides—in particular CGRP, SP, and NK-A—have receptors sensitive to bradykinin, hypertonic saline, low chloride, cigarette smoke, and ozone. The sensory nerve activity may be enhanced during airway inflammation (e.g., bronchitis) so that protective central and local reflexes become exacerbated and deleterious, and may contribute to the pathophysiology and symptomatology of airway inflammatory diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [17,19]. Similarly, inappropriate activation of trigeminal afferent nerves, comparable to the vagal afferent nerves innervating the lower airways, leads to many of the symptoms associated with allergic rhinitis (including sneezing, mucus secretion, vascular engorgement, and plasma exudation). The mechanisms involved in the increased responsiveness of sensory nerves under inflammatory conditions are not completely known. However, there is evidence that interactions of inflammatory mediators (e.g., PG, bradykinin) with sensory and autonomic nerves lead to an increase in neurotransmitter release [19].

Neurogenic inflammation and its interference with immunological inflammation, as well as the impairment of control mechanisms due to psychological stress, are co-factors in the development of chronic inflammatory diseases such as asthma, rhinitis, rheumatoid arthritis, and migraine headache. It is interesting that organs with a high density of neuropeptide receptors, such as the intestines and the lungs, have been proposed to be more susceptible to perturbations from inflammation [20]. Indeed, nervous innervation of an organ is a requirement for establishing certain inflammatory reactions experimentally [21]. For example, Levine et al. found, in a rat model, that joints developed more severe arthritis following injection of Freund’s adjuvant into the footpad when they were more densely innervated by SP-containing primary afferent neurons. Infusion of SP into the knee increased the severity of arthritis, whereas the injection of SP receptor antagonist did not. These results suggest a significant physiological difference between joints that develop mild and severe arthritis, and indicate that the release of intraneuronal SP contributes to the severity of arthritis. Moreover, if the nerve innervating a joint was cut, arthritis could not be induced in the denervated joint [22]. This is reminiscent of clinical cases in which rheumatoid arthritis resolves on the side affected by a cerebral ischemia and following hemiplegia [22].

Another disorder that increases significantly over time is migraine headaches. On the basis of pharmaceutical responses of migraine and animal experiments, it is supposed that neurogenic inflammation plays a role in the pathophysiology of migraine headaches. It was demonstrated in a rat model that stimulation of trigeminal sensory fibers leads to changes consistent with those of migraine, and that these changes could be blocked by the serotonin antagonists; these results suggest that neurogenic inflammation mediates migraine [5].

The activation of sensory neurons as a key mechanism of neurogenic inflammation is also under the control of supraspinal neurons and descending spinal tracts, which may down-regulate the reaction. This is why “spinal” animals with spinal
cords transected proximally have exaggerated inflammatory responses [23]. Furthermore, there is convincing evidence that stress can activate mast cells to degranulate and support neurogenic inflammation. Upon emotional excitement, autonomic nerves convey these stimuli to primary afferent sensory nerves that transmit the impulses antidromically, resulting in the release of various neuropeptides from sensory nerve endings and degranulation of mast cells [24]. This is the presumed basis of psychogenic urticaria. That the brain can cause mast-cell degranulation is also evident from experimental studies which demonstrated that acute psychological stress in rats (induced by immobilization) leads to mast-cell degranulation in the dura, bladder, and intestine [7]. Furthermore, isolation stress has been shown to cause mast-cell degranulation in the skin. This was mediated by the inflammatory neuropeptides SP, corticotrophin releasing factor (CRF), and neurotensin—mediators that are known to induce neurogenic inflammation [25].

Moreover, many skin disorders, such as atopic dermatitis and psoriasis, worsen during stress and are associated with increased numbers and activation of mast cells, which release vasoactive, nociceptive, and pro-inflammatory mediators [26]. Furthermore, migraine headache is often precipitated by stress. Kandere-Grzybowska et al. demonstrated, in various experimental studies with knockout mice, that acute restraint stress resulted in increased dura mast-cell activation through the activation of NK-1 receptors by neuropeptides. The fact that SP /–/ mice had intact vascular permeability response to stress indicates that some other NK-1 receptor agonist may substitute for SP [27].

The number of activated mast cells is also increased in the adventitia of coronary segments with plaque rupture and in spastic atherosclerotic coronary segments. Laine et al. identified and quantified contacts between mast cells and nerves in the adventitia of normal and atherosclerotic coronary segments. The authors concluded that neurogenic stimulation of mast cells in the adventitia of coronary arteries may induce the release of vasoactive compounds, such as histamine and leukotrienes, which can contribute to the complex neurohormonal response that leads to abnormal coronary vasoconstriction [28].

In summary, there is cumulative evidence that mast cells, which connect neurogenic and immunological inflammation, can be activated by psychological stress. In this context, stress can influence a variety of inflammatory diseases in the skin and the joints, as well as in the cardiopulmonary, urinary, and nervous systems. Hence, we know that psychological factors can precipitate or increase the morbidity of diseases mediated by neurogenic inflammation [25].

### 8.2 The “Inflammatory Reflex”: A Mechanism to Counter-Regulate Immunological Inflammation

In the scenario of immunological inflammation, microbial signals, as well as host cell products that are altered (e.g., fragmented matrix proteins or oxidized lipoproteins), abnormally released (e.g., heat shock proteins), or released in abnormally
large amounts, interact with specific cell receptors and induce the production of various inflammatory mediators. These mediators include cytokines; products of the plasma enzyme systems (complement, the coagulation system, kinin, and fibrinolytic pathways); lipid mediators (prostaglandins and leukotrienes) released from tissue cells or macrophages; and vasoactive mediators released from mast cells, basophils, and platelets. These inflammatory mediators control different types of inflammatory reactions. Fast-acting mediators, such as vasoactive amines and the products of the kinin system, modulate the immediate response. Later, newly synthesized mediators such as leukotrienes become involved in the accumulation and activation of other cells. Though of obvious benefit and even life saving on occasion, inflammation is a two-edged sword, with undesirable consequences such as systemic shock and circulatory collapse, wasting if prolonged, and local tissue injury in many organs [2,3,29].

Therefore, a crucial commitment in immunological inflammation is to convert the response from the antibacterial, tissue-damaging mode to a mode that promotes tissue repair and epithelial closure. This transformation begins as complement, neutrophils, and macrophages kill microbes, and macrophages secrete more serine protease inhibitor (SLPI), a serine protease inhibitor expressed late after exposure to microbial products or cytokines. SLPI has anti-inflammatory and wound-healing effects. Furthermore, SLPI binds and synergizes with proepithelin, a cytokine that promotes epithelial growth and suppresses neutrophil activation, protecting it from proteolytic conversion into pro-inflammatory epithelins. Furthermore, macrophages ingest apoptotic neutrophils and degrade their residual stores of elastase. The ingestion of apoptotic neutrophils induces in macrophages production of the anti-inflammatory and tissue-repair cytokine TGF-β [3,30–32]. To control the potentially harmful pro-inflammatory response, the immune system releases further anti-inflammatory mediators such as IL-10, IL-1 receptor antagonist (ra), and soluble TNF receptors, which either inhibit the production of (e.g., IL-10), neutralize (e.g., soluble TNF-α receptors 1 (p55) and 2 (p75)), or competitively antagonize (IL-1ra) pro-inflammatory mediators [31,33,34]. Interestingly, TNF-α, IL-1β, and PG by themselves are powerful inducers of the compensatory anti-inflammatory response syndrome (CARS).

In addition to the auto-regulatory pathways of the immune cells, the delicate balance between pro- and anti-inflammatory responses is controlled by brain-dependent “central” mechanisms. However, in contrast to neurogenic inflammation, where inflammation is mediated by a spinal reflex, the counter-regulatory inflammatory reflex involves the brain. Under these circumstances, inflammatory stimuli activate sensory pathways that relay information to the hypothalamus and brainstem and lead to activation of a brain-mediated anti-inflammatory response that is fast and subconscious (Figure 8.1). This should prevent spillage of inflammatory products into the circulation. Tracey described the neural control of acute local inflammation as reflexive, directly interconnected, and controllable [29,35].

The inflammation-sensing pathways can activate responses even when the inflammatory agents are present in tissues in quantities that are not high enough to reach the brain through the bloodstream. This type of response involves the expression of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 in the periphery. The mechanisms by which local inflammation can affect brain function have been
the subject of much debate. Recently published data suggest that the vagus nerve is important for immune-to-brain communication. The dorsal vagus complex can respond to increased levels of local and circulating pro-inflammatory cytokines such as TNF-\(\alpha\). Peripheral stimulation of the *afferent* vagus nerve significantly attenuated the development of LPS-induced hypotension (shock) in rats exposed to lethal doses of endotoxin. Experimental activation of the cholinergic anti-inflammatory pathway by direct electrical stimulation of the *efferent* vagus nerve inhibited the synthesis of TNF in liver, spleen, and heart, and attenuated serum concentrations of TNF during endotoxemia. Vagotomy significantly exacerbates TNF responses to inflammatory stimuli and sensitizes animals to the lethal effects of endotoxin [35,36].

It is not completely clear how the vagus nerve detects low doses of endotoxin or inflammatory agents, but neurons in the vagus nerve express IL-1 receptor mRNA and discrete IL-1 binding sites have been identified on glomus cells. Electrophysiological studies indicate that vagus nerve signals can also be activated by TNF, other cytokines, mechanoreceptors, chemoreceptors, temperature sensors, and osmolarity sensors. This presumption allows us to draw an analogy to neurogenic inflammation. However, in the context of immunological inflammation the cholinergic anti-inflammatory reflex inhibits inflammation, whereas in neurogenic inflammation the axonal reflex initiates inflammation (Figure 8.1). The sensory input from the inflammation site into the CNS is organized somatotopically, such that information from a discrete peripheral site is localized precisely in the ascending fiber pathways and brain. Thus, the inflammation-derived sensory input can be processed differentially in the brain, depending on the location of the inflammatory site and the nature of the sensory signal [29].

The vagus-mediated inflammatory reflex is described as localized, rapid, and discrete; but it can also induce a systemic anti-inflammatory response if necessary. This can occur because vagus nerve activity is relayed to the medullary reticular formation, to the locus coeruleus, and to the hypothalamus. Furthermore, sympathetic nerve fibers can also be affected, leading to an increased release of catecholamines. Monocytes/macrophages and other immune cells bear functional adrenoreceptors, and norepinephrine (NE) fulfills criteria for neurotransmission with cells of the immune system as targets [37,38]. Through stimulation of these receptors, locally released NE, or circulating catecholamines such as epinephrine, affect lymphocyte traffic, circulation, and proliferation, and modulate cytokine production and the functional activity of different lymphoid cells. In addition, recent evidence suggests that NE and epinephrine, through stimulation of the \(\beta_2\)-adrenoreceptor-cAMP-protein kinase A pathway, inhibit the production of type 1/proinflammatory cytokines, such as IL-12, TNF-\(\alpha\), and INF-\(\gamma\), by antigen-presenting cells and T helper (Th) 1 cells, whereas they stimulate the production of type 2/anti-inflammatory cytokines such as IL-10 and TGF-\(\beta\). Through this systemic mechanism, endogenous catecholamines may cause a selective suppression of Th1 responses and cellular immunity, and a Th2 shift toward dominance of humoral immunity. However, in certain local responses, and under certain conditions, catecholamines may actually boost regional immune responses, through induction of IL-1, TNF-\(\alpha\), and primarily IL-8 production. Thus, activation of the SNS during an immune response might be aimed at localizing the inflammatory response, through induction of neutrophil accumulation and stimulation...
of more specific humoral immune responses, although systemically it may suppress Th1 responses and thus protect the organism from the detrimental effects of pro-inflammatory cytokines and other products of activated macrophages [39].

The anti-inflammatory effects of the sympathetic and parasympathetic nervous systems seem to be synergistic in this setting. Classical teaching stresses that actions of the sympathetic and parasympathetic nervous systems are usually in opposition, but in many situations the two systems do function synergistically. The combined action of these neural systems is significantly anti-inflammatory, and is positioned anatomically to constrain local inflammation by preventing spillover of potentially lethal toxins into the circulation through both local (neural) and systemic (humoral) anti-inflammatory mechanisms [29,40,41] (Figure 8.1).

However, when inflammation cannot be confined locally, a transfer of cytokines from tissue into blood may occur, generating a systemic inflammatory response syndrome that involves the whole organism in the inflammatory process. In this scenario, cytokines may activate the inhibitory neuroimmune pathways directly, via the bloodstream, in order to modulate and dampen the immune system and to prevent more severe and excessive catabolic effects—including the ultimate deleterious consequence, septic shock and death. Pro-inflammatory cytokines can gain access to brain centers that are devoid of blood–brain barrier in the circumventricular region or the area postrema. Along these lines, clinical and experimental studies suggest that cytokines in the blood can activate the HPA axis as well as the autonomic nervous system, and provide a shortcut by which immune recognition of an infectious challenge in the blood rapidly induces the secretion of neuroimmune modulators such as glucocorticoids, adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (MSH), catecholamines, and acetylcholine. This pathway may signal a more severe situation compared to cytokines in tissue [42].

The question is, however, what happens if the inhibitory neuroimmune pathways are activated without a significant systemic inflammation? This can result from production of cytokines in the brain following infection, injury, or ischemia [41]. The main sources of brain cytokines are endothelial cells, invading immune cells, astrocytes, and microglia. These cells can produce cytokines such as IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF-α in response to hypoxia, endotoxin, and cell detritus [43–48]. Moreover, marked increases of TNF-α were localized immunocytochemically to neurons of injured cerebral cortex. Woodroofe et al. demonstrated increased levels of IL-1 and IL-6 in the rat brain parenchyma following mechanical injury by microdialysis [43]. These brain-derived cytokines can directly activate inhibitory neuroimmune pathways and cause a brain-mediated immunodepression.

We demonstrated that sterile cerebral inflammation resulting from intracerebroventricular or intra-hypothalamic infusion of rat recombinant IL-1β, but not TNF-α, particularly diminished the endotoxin-induced TNF-α, but increased the IL-10 concentration in stimulated whole-blood cultures. Blocking the HPA axis by hypophysectomy (HPX) led to complete recovery of the diminished TNF-α concentration and temporarily inhibited the IL-10 increase. Blocking the SNS transmission, by application of the β2-adrenoreceptor antagonist propranolol, not only inhibited the increase but further down-regulated the endotoxin-induced IL-10 concentration in
the supernatants of whole-blood cell cultures, whereas the TNF-α decrease was only partially prevented. Interestingly, HPX and propranolol also diminished the cell invasion into the cerebrospinal fluid (CSF) [49].

Moreover, we could show that intra-cerebroventricular and intra-hypothalamic infusion of IL-1β (but not TNF-α) dramatically increased neutrophil counts, whereas lymphocytes dropped. Blocking the HPA axis by HPX abolished the neutrophilia, although the lymphopenia remained unchanged. Furthermore, application of propranolol prevented the decrease of lymphocytes and diminished the neutrophilia. All parameters were normalized within 48 hours after termination of infusion [50].

Additionally, we were able to show in a rat model that increased intracranial pressure leading to sympathetic activation rapidly induces an impressive systemic IL-10 release. This effect could be blocked by the β-adrenoreceptor antagonist propranolol [41]. Elenkov et al. demonstrated that NE and epinephrine suppressed IL-12 production in a dose-dependent fashion. However, at physiological concentrations, both catecholamines dose-dependently increased the production of IL-10. The effects of either catecholamine on IL-12 or IL-10 secretion were blocked completely by propranolol [51]. These findings also support the hypothesis that the central nervous system may regulate IL-12 and IL-10 secretion and, hence, the Th1/Th2 balance, via the SNS.

Through our clinical investigations, we could also show that brain-injured patients have high IL-10 plasma levels immediately after the acute event. This correlated with signs of increased intracranial pressure and sympathetic activation, as well as with an increased risk of infectious complications [41,52]. We made similar observations of patients after neurosurgical procedures, where we could show that a local inflammation in the CSF with high levels of IL-6 and IL-8 was associated with a monocytic deactivation, with a decreased expression on human leukocyte antigen, serotype DR (HLA-DR) molecules and an increased risk of developing infectious complications [53,54].

8.3 Conclusions

In summary, catecholamines and acetylcholine may control local and systemic immune response during all phases of inflammation. In detail, afferent somatosensory nerves, including the vagus, are able to recognize various signals of things that could threaten the organism. The resulting information may initiate an inflammatory response (neurogenic inflammation) via a spinal reflex. Furthermore, depending on the signal type, an additional immunological inflammation may occur. The released pro-inflammatory cytokines may again affect somatosensory nerves and signal the brain that a local inflammatory reaction has occurred. This may activate inhibitory neuroimmune pathways such as the HPA axis, the SNS, and the vagus to support a local anti-inflammatory response. However, if inflammation cannot be confined locally, cytokines may spill over into the blood and induce a SIRS and septic shock. In this situation, blood cytokines may directly affect the brain and induce a central CARS in order to restore homeostasis. In addition, the delicate balance between pro- and anti-inflammatory responses is influenced by the mind: psychological stress can shift the immune system to one side with consequences for the immune status. Thus,
if the inflammatory response dominates, chronic inflammatory diseases (e.g., psoriasis, asthma) may be supported; if the anti-inflammatory response is outbalanced, subsequent immunodepression with risk of infectious complication may occur.

References


9

Role of Tachykinins in Asthma and Allergic Disease

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9.1 Introduction

The inflammation that results from the release of substances from primary sensory nerve terminals is called neurogenic inflammation. More than 100 years ago, the first observations were made that activation of dorsal root ganglia neurons results in vasodilation. Since then, abundant evidence has been accumulated to suggest that activation of peripheral terminals of sensory neurons by local depolarization, axonal reflexes, or dorsal root reflexes releases bioactive substances. These substances act on target cells in the periphery, such as mast cells, immune cells, and vascular smooth muscle cells, to produce inflammation (redness and warmth, swelling, and hypersensitivity) [1].

Small-diameter sensory neurons that are sensitive to capsaicin are of major importance in the generation of neurogenic inflammation. The neuropeptides substance P and calcitonin gene-related peptide (CGRP) are considered to be the major initiators of neurogenic inflammation [2,3]. Substance P and neurokinin A are members of the tachykinin peptide family and are potent vasodilators and contractile agents of smooth muscle [4]. In studies on rodent airways, substance P and neurokinin A have been implicated as the neurotransmitters mediating the excitatory action of the nonadrenergic/noncholinergic (NANC) nervous system. These noncholinergic excitatory nerves can be activated by mechanical and chemical stimuli, generating antidromic impulses and a local axon reflex that leads to noncholinergic bronchoconstriction and neurogenic inflammation [5,6].

Substance P and neurokinin A have various effects that could contribute to the changes observed in the airways of patients with (allergic) asthma. These include smooth muscle contraction, submucosal gland secretion, vasodilation, increase in vascular permeability, stimulation of cholinergic nerves, stimulation of mast cells, stimulation of B and T lymphocytes, stimulation of macrophages, chemoattraction of eosinophils and neutrophils, and the vascular adhesion of neutrophils. This chapter discusses the role of tachykinins and their receptors in allergic airway inflammation.
9.2 Localization and Production of Tachykinins in the Airways

The tachykinin peptide family is one of the largest peptide families described in the animal organism. They have been isolated from invertebrate, protochordate, and vertebrate tissues. Tachykinins, defined as peptides having the characteristic C-terminal pentapeptide F–X–G–L–M–NH₂, are identified as aromatic when X is an aromatic amino acid residue (F or Y) or aliphatic when X is an aliphatic amino acid residue (V or I). All tachykinins are amidated at the C-terminus, and this is crucial for their biological activity [4]. The four major mammalian tachykinins are substance P, neuropeptide A, neuropeptide B, and the recently discovered hemokinin 1 [7].

Mammalian tachykinins are derived from three preprotachykinin genes that, according to the Human Genome Organization (HUGO) Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/) are known as TAC1, TAC3, and TAC4. The TAC1 gene (previously known as the PPT-I or PPT-A gene) codes for substance P, in addition to neuropeptide A and its extended forms [8–10]. The TAC3 gene (also known as PPT-II or PPT-B) encodes the sequence for neuropeptide B [11]. Hemokinin 1 is coded by the TAC4 gene (or PPT-C) [12,13].

Tachykinins (substance P, neuropeptide A, and neuropeptide B) have previously been considered a neuropeptide group because of their widespread distribution in the central and peripheral nervous systems (capsaicin-sensitive primary afferent neurons and capsaicin-insensitive intrinsic neurons). This terminology is no longer used, because their presence in a variety of nonneuronal structures has been demonstrated repeatedly [4,14]. Furthermore, mRNA expression studies suggest that hemokinin 1 has a unique distribution outside neuronal tissues [15].

In the airways, a distinct subpopulation of primary afferent nerves that are characterized by their sensitivity to capsaicin are considered to be the principal source of substance P and neuropeptide A [16,17]. Although these tachykinins can also be expressed in capsaicin-resistant neurons [18,19], capsaicin pretreatment of experimental animals caused an almost total depletion of substance P and neuropeptide A immunoreactivity [17,20]. Radioimmunoassay and immunohistochemistry demonstrated substance P- and neuropeptide A-positive nerves beneath and within the epithelium, around blood vessels and submucosal glands, and within the bronchial smooth muscle layer [17,21]. In guinea pigs, airway sensory nerves containing tachykinins are easily located, but in human airways, tachykinergic innervation is sparse. In the bronchial tree, tachykinergic sensory innervation originates from the (right) vagus nerve, whereas in the lung the fibers are of both vagal parasympathetic and thoracic spinal origin [17]. Additional, nonneuronal sources of tachykinins in the airways have been reported. Chu and colleagues demonstrated staining for substance P in the airway epithelium [22], and Maghni and co-workers reported the presence of substance P in airway smooth muscle cells [23]. There is also evidence for the production of substance P by eosinophils, monocytes and macrophages, lymphocytes, and dendritic cells [24–27]. Immunoreactivity for neuropeptide B has not yet been found in the airways. Polymerase chain reaction (PCR) techniques demonstrated transcripts
of the mouse and the human TAC4 gene in lung tissue [28,29], which may result in formation of hemokinin 1 and thus represent another source of tachykinins in the airways.

### 9.2.1 Airway Tachykinin Receptors

The biological activity of tachykinins depends on their interaction with three specific receptors: the tachykinin NK₁, NK₂, and NK₃ receptors [30]. These receptors belong to the rhodopsin-like G-protein-coupled receptor family. This group of proteins shares the same structural motif: a bundle of seven hydrophobic transmembrane domains with three extracellular loops, three intracellular loops, an extracellular amino terminus, and a cytoplasmic carboxyl terminus [31]. The human tachykinin NK₁ and NK₂ receptors are proteins of 407 and 398 amino acids, respectively. The human tachykinin NK₃ receptor is N-terminally extended and has 465 residues [14].

The tachykinin receptor displaying the highest affinity for substance P was named the tachykinin NK₁ receptor. The receptor showing the highest affinity for neurokinin A was termed the tachykinin NK₂ receptor, and the receptor with the highest affinity for neurokinin B was called the tachykinin NK₃ receptor [32,33]. Hemokinin 1 and its elongated forms act as tachykinin NK₁ receptor-preferring agonists [13]. It should be emphasized that all tachykinins can act as full agonists on the three different receptors, but with lower affinities than on the preferred receptor [14].

The three tachykinin receptors are heterogeneously distributed within each species. In addition, there are marked species-related differences in their pattern of expression. However, they are distributed in the central and peripheral nervous systems of several species. Their presence can also be demonstrated, functionally or histochemically, in peripheral tissues such as the gastrointestinal tract, urogenital tract, immune system, cardiovascular system, skin, and skeletal muscle [4].

An overwhelming amount of functional data indirectly demonstrates the broad expression of tachykinin NK₁ and NK₂ receptors in the airways. Also, functional evidence exists for the presence of tachykinin NK₃ receptors [34]. Messenger RNA for the tachykinin NK₁ and NK₃ receptors has been found in human pulmonary veins, arteries, and bronchi; mRNA for the tachykinin NK₂ receptor was abundantly expressed in human bronchi, although only a low expression of this receptor was found in human pulmonary veins and arteries [35]. In a study on surgical specimens, using specific antibodies, tachykinin NK₁ and NK₂ receptor proteins were detected in human bronchial glands, bronchial vessels, and bronchial smooth muscle. Tachykinin NK₁ receptors were occasionally found in nerves and tachykinin NK₂ receptors in inflammatory cells, such as T lymphocytes, macrophages, and mast cells [36]. A study on endobronchial biopsies revealed immunoreactivity for the tachykinin NK₁ receptor in the epithelium and the submucosa. Goblet cells appeared to be the cells with the strongest staining. In the submucosa, staining was localized to the endothelial cells of the blood vessels, the surfaces of inflammatory cells, and some smooth muscle cells [22]. Immunohistochemistry on human pulmonary vessels, from tissue obtained at lobectomy, revealed positive staining for tachykinin NK₁ receptors.
on the endothelium of both veins and arteries [37]. Inflammatory cells such as macrophages, lymphocytes, neutrophils, dendritic cells, and mast cells also express functional tachykinin NK$_1$ receptors [24,26,27,38,39].

### 9.3 Role of Tachykinins in Respiratory Pathophysiology

Tachykinins elicit a broad spectrum of biological activity (in the cardiovascular, gastrointestinal, urogenital, immune, and nervous systems), which may vary in different species and even in the various strains of single species [4]. This strongly supports the concept of a general, important functional significance of these peptides. However, mice with a disrupted preprotachykinin I gene (TAC1 gene, encoding for substance P and neurokinin A and its extended forms) or a disrupted tachykinin NK$_1$ or NK$_3$ receptor gene (the TACR1 and TACR3 genes) remain in good health and fertile, which demonstrates that neither the tachykinins substance P and neurokinin A, nor the tachykinin NK$_1$ and NK$_3$ receptors, are essential for life and health, at least in mice.

The adequate stimuli for tachykinin release from the sensory nerves in the airways are of chemical nature (especially chemicals that are produced during inflammation and tissue damage). Given this fact, it can be suggested that tachykinin release occurs only in response to pathological, rather than physiological, conditions. However, because an important interaction exists between the airways and the external environment, this can also be interpreted as a mechanism to detect potential hazardous stimuli and to react physiologically to such stimuli.

#### 9.3.1 Airway Smooth Muscle Tone and Airway Responsiveness

The bronchoconstrictor effect of exogenous substance P was first reported in 1977 in guinea pigs and cats. Since then, substance P has been shown to cause dose-dependent bronchoconstriction in various animal species, both in vitro and in vivo [40–42]. Exogenous tachykinins are also contractors of human airways. This too has been demonstrated both in vitro and in vivo. Substance P contracts human bronchi and bronchioles, but is less potent than histamine or acetylcholine in doing so [41,43]. Neurokinin A is a more potent constrictor and is, on a molar basis, 2–3 orders of magnitude more potent than histamine or acetylcholine. Neurokinin B does not exert a contractile action on human airways [44]. The tachykininergic contraction was demonstrated to become more important in more distal airways [45]. In vivo, inhalation or intravenous infusion of tachykinins also influences bronchomotor tone in humans, although the observed effects are rather small. Intravenously administered substance P had demonstrable effects on the vascular smooth muscle and control of ventilation, but showed little effect on airway function [46]. Another study reported a bronchodilating effect of intravenous substance P while neurokinin A-induced bronchoconstriction [47]. Inhalation of substance P or neurokinin A did not influence airway conductance in healthy individuals [48], unless the activity of the degrading enzyme neutral endopeptidase was blocked by thiorphan [49].
The role of tachykinins in the regulation of airway function has been under even more intense investigation since the demonstration that the atropine-resistant contraction (about 60% of the contractions that can be induced by electrical field stimulation [EFS]) in guinea pig airways was ascribable to transmitters released from substance P-containing afferent neurons [50]. Such a noncholinergic bronchoconstriction has not been consistently demonstrated in human airways. Human bronchi respond to EFS with a fast, cholinergic contraction followed by a slow relaxation. However, in a few bronchial preparations, a small, noncholinergic contraction was seen upon EFS [51]. The fact that capsaicin, which mediates tachykinin release, contracts human bronchi has led to the hypothesis that similar noncholinergic excitatory nerves exist in human airways [52]. This was further supported by the observation that up to 45% of isolated human bronchi contracted spontaneously when peptidase inhibitors were added to the organ bath [53]. Moreover, tachykinin receptor antagonists blocked the capsaicin-induced contraction [54]. However, other studies did not confirm the latter finding [55]. Inhalation of capsaicin-induced bronchoconstriction in normal volunteers, but this response was considered a vagally mediated cholinergic reflex [56]. At present, it is unclear whether excitatory noncholinergic, nonadrenergic mechanisms exist in human airways.

In guinea pig airways, tachykinin NK₂ receptors (and to a lesser extent tachykinin NK₁ receptors) have been shown to be involved in the bronchoconstrictor response to exogenous tachykinins, capsaicin, and EFS [57–59]. Strain differences were observed in rats: more particularly, in BDE rats, contraction is mediated via tachykinin NK₂ receptors, whereas in Fisher 344 rats, contraction is mediated via tachykinin NK₁ receptors [60,61]. In humans, it has long been thought that only tachykinin NK₂ receptors were involved in the direct contraction of isolated bronchi [62]. However, in small-diameter bronchi, tachykinins also cause contraction via stimulation of tachykinin NK₁ receptors [63]. In medium-sized bronchi, tachykininergic contraction is partially mediated through tachykinin NK₁ receptors [64].

Tachykinin-induced contractions of guinea pig airways and isolated human bronchi are direct effects, as antihistamines and muscarinic receptor antagonists do not influence them [51]. In human medium-sized bronchi, activation of the tachykinin NK₁ receptor on the smooth muscle caused inositoltriphosphate formation and rise of intracellular calcium levels and subsequent contraction of the smooth muscle cells [64]. However, in small-diameter bronchi, contraction appears to be mediated through the formation of prostanoids, which represents an indirect mechanism [63].

Another possible indirect mechanism by which tachykinins could mediate contraction is the facilitation of cholinergic contraction. Substance P facilitated release of acetylcholine from postganglionic cholinergic nerves in guinea pig [65], rabbit [66], and human airways [67,68], probably through the tachykinin NK₁ receptor. In guinea pig trachea, tachykinin NK₂ receptors were also found to be involved in the facilitation of acetylcholine release [69]. Substance P is also known to induce degranulation of human and rat mast cells, with subsequent release of histamine and serotonin, which in turn could cause contraction [70,71]. Both a receptor-dependent and a receptor-independent mechanism have been reported for mast-cell activation induced by substance P. Higher (micromolar) concentrations caused a direct activation
of G proteins [72]. Lower concentrations caused mast-cell activation by interaction with tachykinin receptors. The histamine release from a murine mast-cell line is mediated by tachykinin NK₂ receptors [73], whereas the mast-cell degranulation induced by substance P in Fisher 344 rat airways involved tachykinin NK₁ receptors [61,74].

9.3.2 Cough

An afferent (sensory) arm and an efferent arm that synapses in the brainstem areas, including the nucleus tractus solitarius, make up the reflex arch that mediates the cough reflex. Mediators and mechanisms of the sensory arm of this reflex pathway are not completely understood, though pharmacological evidence exists for the involvement of substance P [75,76]. The role of tachykinins in the regulation of the cough response appears to be complex, as antagonists for the three tachykinin receptors have antitussive activity in preclinical models [77–79]. In guinea pigs, substance P locally applied into the airways has been shown to produce or potentiate cough produced by other stimuli [79]. These findings support a role for substance P at a peripheral site of action, but central sites of action have also been reported [81].

9.3.3 Secretion of Mucus, Water, and Electrolytes

Relatively strong evidence exists that tachykinins released from sensory nerve fibers are physiological (and pathological) regulators of secretion in the upper and lower airways. A local release of tachykinins in the nasal mucosa could play a role in the defensive response to irritants. Intranasally administered capsaicin-induced sneezing, pain, and nasal secretion in both experimental animals and humans [82]. Similarly, local tachykinin administration evoked secretion. In the lower tracheobronchial tree, seromucous glands and goblet cells produce mucus. Both sources are under neural control. Depending upon species and airway level, innervation comprises parasympathetic, sympathetic, and “sensory-efferent” pathways [83]. The transmitters of the latter pathway are substance P and neurokinin A, which act via tachykinin NK₁ receptors [84,85]. Recently, an involvement of tachykinin NK₃ receptors in porcine airways has been suggested [85]. However, compared to cholinergic control, the excitatory nonadrenergic/noncholinergic (eNANC) neural control of human airway secretion appears to be a minor component [83]. Mucus secretion is closely coupled to liquid secretion to ensure optimal ciliary transport and presence of adequate concentrations of antibacterial substances on the airway surface. Substance P has also been shown to be involved in this process in porcine airways [86].

9.3.4 Alveolar Epithelial and Microvascular Permeability

The involvement of sensory nerves in producing increased vascular permeability and plasma extravasation was first described in skin and conjunctiva and termed neurogenic inflammation [87]. Neurogenic inflammation has also been described in the
airways. Plasma extravasation in rat and guinea pig airways could be induced by a variety of c-fiber stimuli and was abolished by capsaicin pretreatment and inhibited by a substance P antagonist, whereas exogenously administered substance P mimicked the increased vascular permeability induced by either capsaicin or electrical nerve stimulation. This led to the conclusion that substance P released from the sensory nerves mediates neurogenic inflammation in the airways [88–90]. Plasma extravasation, induced by cold air, hypertonic saline, or isocapnic hyperpnea, also depends on stimulation of sensory fibers and is inhibited by tachykinin receptor antagonists [91,92]. Once plasma extravasation occurs, leukocytes initiate a process that results in slowing down their velocity, rolling onto and adhering to the venular endothelium. The involvement of the tachykinin NK₁ receptor in all these phenomena has been demonstrated by the use of specific antagonists [91,93,94]. It is not known how much of the effect is due to a tachykinin NK₁ receptor-mediated effect on leukocytes and endothelial cells nor how much is merely the consequence of inhibition of tachykinin NK₁ receptor-mediated plasma extravasation. Tachykinin NK₂ receptors also mediate part of the neurogenic plasma extravasation in the secondary bronchi and intraparenchymal airways of the guinea pig [91,95].

In rat trachea, the presence of tachykinin NK₁ receptors on the endothelial cells of the postcapillary venules has been demonstrated with specific antibodies. Upon ligand binding, these receptors are internalized in endosomes. Furthermore, vagal nerve stimulation increased the number of tachykinin NK₁ receptor immunoreactive endosomes. These findings indicate a direct effect of the released substance P on the tachykinin NK₁ receptors of the endothelium [96]. Further evidence for a direct effect on the endothelium is the fact that intravenously administered substance P caused the formation of intercellular gaps between endothelial cells in postcapillary and collecting venules in rat trachea [97,98].

Increased microvascular permeability is not the only mechanism responsible for plasma exudation and edema formation. Electrical stimulation of the vagal nerve caused an increase in negativity of interstitial fluid pressure in rat trachea. Such increases in negativity of the interstitial fluid pressure will promote fluid filtration and thus plasma extravasation [99]. As this phenomenon can be abolished by pretreatment with mast-cell degranulating agents, an indirect pathway via mast cells can be inferred. Also, other experiments revealed a role for mast cells and serotonin release in tachykinin-induced plasma exudation in the respiratory tract. In rabbit airways, involvement of 5-HT receptors and arachidonic acid derivatives has been suggested [100,101]. In Fisher 344, but not in BDE, rats, such an indirect mechanism involving tachykinin NK₁ receptor-mediated mast-cell activation, serotonin (5-HT) release, and stimulation of 5-HT₂ receptors has also been reported [102].

Direct evidence for neurogenic plasma extravasation in human airways could not be demonstrated for a long time, because of difficulties in the assessment [3]. However, microvascular leakage can now be measured in human airways with a dual-induction model. First, substance P is inhaled and then sputum is induced by inhalation of hypertonic saline solution. With this method, it was demonstrated that inhalation of substance P induced significant increases in the levels of α2-macroglobulin, ceruloplasmin, and albumin in induced sputum [103].
9.3.5 **Cell Growth and Proliferation of Mesenchymal Cells**

Tachykinins stimulate the proliferation of a number of cell types, including fibroblasts [104], smooth muscle cells [105], epithelial cells [106], and endothelial cells [107]. This could have important implications for wound healing and tissue repair. The substance P-induced proliferation of human fibroblasts is mediated by interaction with tachykinin NK1 receptors [108, 109]. The proliferation of rabbit airway smooth muscle cells also involves tachykinin NK1 receptors [105]. The proliferation of smooth muscle cells in response to tachykinins is coupled to inositoltriphosphate formation and DNA synthesis. In the tracheal epithelium of guinea pigs, it was shown that, in addition to tachykinin NK1 and NK2 receptors, CGRP receptors are involved in the proliferative response [110]. Endothelial-cell proliferation induced by tachykinins is a likely mechanism in the neovascularization observed *in vivo* in response to peptides of this family [111].

9.3.6 **Immunomodulation**

Tachykinins are known to have a wide variety of modulatory effects on inflammatory/immune cell functions. This topic has been reviewed elsewhere [112, 113].

9.4 **The Role of Tachykinins in Animal Models of Asthma**

Tachykinins have been found to be involved in antigen-induced bronchoconstriction, airway inflammation, and enhanced bronchial responsiveness in various animal models. A combination of a tachykinin NK1 receptor antagonist, CP-96,345 [114], and a tachykinin NK2 receptor antagonist, SR-48968 [115], inhibited bronchoconstriction produced by ovalbumin challenge in sensitized guinea pigs [116]. The NK1 receptor antagonist CP-96,345 was also able to limit antigen-induced plasma extravasation [117]. By using NK1/NK2 receptor blockade, the involvement of endogenous tachykinins in antigen-induced bronchial hyperresponsiveness was demonstrated [118].

The relative contribution of the NK1 and NK2 receptors in antigen-induced airway changes has been studied in guinea pigs and rats. In conscious, unrestrained guinea pigs, the NK1 receptor is involved in both the development of antigen-induced airway hyperresponsiveness to histamine and the antigen-induced infiltration of eosinophils, neutrophils, and lymphocytes [119]. In contrast, the NK2 receptor is involved in the development of the antigen-induced late reaction [120]. The effect of allergen challenge has also been studied in the Brown Norway (BN) rat model [121]. Substance P was found to increase 2.4-fold in bronchoalveolar lavage fluid (BAL) after challenge with ovalbumin. The tachykinin NK1 receptor antagonist CP-99,994 and the tachykinin NK2 receptor antagonist SR-48968 were not able to reduce the early airway response to ovalbumin, but both antagonists reduced the ovalbumin-induced late airway responses. An interesting finding in this study was that the NK2 tachykinin receptor antagonist decreased the number of eosinophils in BAL fluid and decreased the expression of both Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines in BAL cells.
In a mouse model of asthma, we observed no influence of the tachykinin NK1 receptor on the development of allergic airway inflammation. However, allergen-induced goblet cell hyperplasia, one of the features of airway remodeling, was decreased in mice lacking the tachykinin NK1 receptor [122].

So, from animal studies, it seems that both the tachykinin NK1 and NK2 receptors are involved in antigen-induced airway effects. In addition, it is possible that tachykinin NK3 receptors are involved in this process: administration of the tachykinin NK3 receptor antagonist SR-142801 via aerosol caused a significant reduction in neutrophil and eosinophil influx in the airways of ovalbumin-sensitized and -challenged mice [123].

In animal models, tachykinins and their receptors have been shown to be involved in airway responses to nonspecific stimuli. Both the NK1 and the NK2 tachykinin receptors have been found to be involved in airway contraction induced by cold air [124,125], hyperventilation, and cigarette smoke [126]; in plasma extravasation induced by hypertonic saline [127,128]; and in airway hyperresponsiveness induced by viruses [129], IL-5, and nerve growth factor [130,131]. In addition, the tachykinin NK3 receptor was found to be involved in citric acid-induced cough and enhanced bronchial responsiveness [77]. From studies performed in tachykinin NK1 receptor knockout mice, it is becoming apparent that the tachykinin NK1 receptor plays a role in cigarette smoke-induced airway inflammation [132].

9.5 Conclusions

The tachykinins substance P and neurokinin A are present in human airways, in sensory nerves and immune cells. Tachykinins can be recovered from the airways after inhalation of ozone, cigarette smoke, or allergens. They interact in the airways with tachykinin NK1, NK2, and NK3 receptors to cause bronchoconstriction, plasma protein extravasation, and mucus secretion, and to attract and activate immune cells. In preclinical studies, they have been implicated in the pathophysiology of asthma, including allergen- and cigarette-smoke-induced airway inflammation and bronchial hyperresponsiveness. Clinical studies with potent tachykinin receptor antagonists (such as the recently described dual NK1/NK2 or triple NK1/NK2/NK3 tachykinin antagonists [133,134]) should permit a definite judgment on their importance in allergic airway diseases.

References


10 Modulation of Immune Response by Head Injury

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10.1 Traumatic Brain Injury: Epidemiology

Traumatic brain injury (TBI) remains a social burden worldwide, leading to long-lasting disabilities and death. In the United States, approximately 2 million injuries are recorded annually, causing 56,000 deaths, leaving 18,000 survivors with permanent neurological deficit, and incurring an estimated cost of US$56 billion to the community [1]. Despite a smaller population in Australia, more than 20,000 hospitalizations involving TBI were recorded in 2004–2005, resulting in 26,000 episodes of inpatient care with approximate direct costs of $184 million [2]. Despite increased efforts to improve prevention, TBI will remain a worldwide health issue in both developed and developing countries. The World Health Organization estimates that by 2020, road traffic accidents as the main cause of TBI will rank third as a burden of disease and disability, following heart infarct and major depression.

Sadly, to date there are no specific therapies for the treatment of severe brain injuries, despite advances in neuroimaging, neurosurgery, and critical care management that have improved diagnosis, intracranial pressure monitoring, and patient outcome. The major challenge for clinicians is to reverse the deleterious effects of brain edema, which is still the main cause of mortality, at approximately 30% in severe TBI patients [3].

The recently completed IMPACT study, which analyzed the raw data of more than 8,000 TBI patients recruited in 10 randomized clinical trials since 1984, reported that TBI patients with the worst outcomes (low Glasgow Outcome Scale [GOS]) suffer from severe diffuse brain injury, which is more frequently associated with secondary hypoxia and hypotension [1]. A multicenter clinical trial conducted in Australia and New Zealand found that 50% of patients with moderate/severe TBI had an
unfavourable outcome at 12 months follow-up, with a mortality rate of 35%. Furthermore, 70% of severe TBI patients presented with evidence of diffuse injury [4,5]. Perhaps the failure to translate preclinical therapies into the clinical setting is due to the reliance on successful animal studies, most of which used models of focal TBI and thus failed to mimic the diffuse injuries seen more commonly in clinical practice. It is clear that future studies designed to improve outcomes and understand the mechanisms of brain protection must examine more extensively the mechanisms of diffuse TBI.

10.2 Primary and Secondary Brain Injury

The pathophysiology of TBI can be divided into two temporal phases. Primary TBI is induced at the time of the incident and is classified as focal or diffuse brain injury depending on patterns of tissue damage. Secondary brain injury is defined as the delayed degeneration of viable tissue surrounding the initial damage and is the result of physiological, cellular, and molecular alterations. It is well established that secondary brain injury is the major cause of both mortality and physical and cognitive impairments.

In clinical practice, classification of primary brain injury is determined by neuroimaging scans that reveal specific types of damage, including skull fractures, tissue lacerations, subdural or epidural hemorrhages, and contusions, which constitute the hallmarks of focal TBI. Alternatively, widespread swelling of and axonal damage at the white matter and additional rupture of the cerebrovasculature presenting as petechial hemorrhages are characteristic features of diffuse TBI [6]. Despite this clear definition of two types of brain damage, in reality each patient presents with a unique form of TBI in which a distribution of focal and diffuse injuries often coexists. In the past decade, more emphasis has been placed on discerning the morphological and molecular pathways specific to focal and diffuse brain trauma, as it has become evident from human and animal studies that they differ significantly. Neurotrauma research pursued over the past 20 years has focused mainly on focal (rather than diffuse) TBI, and a variety of cerebral-contusion animal models have been developed to explore the pathological sequelae leading to secondary brain damage. However, the pathophysiology of diffuse brain injury remains less understood and more difficult to reproduce in the laboratory using animal models.

Among the systemic events occurring after severe head trauma, respiratory distress or hypoxia and drop in systemic blood pressure (hypotension) further compromise the injured brain by decreasing cerebral perfusion pressure and by generating ischemic injuries. These secondary insults are mostly concomitant to the extracranial injuries, such as lung trauma or obstructed airway, often observed in severe multitrauma patients. Reduced oxygen delivery to the injured brain further alters the homeostasis of the parenchymal environment, causing cerebral hypoxic and ischemic insults and aggravating the traumatic tissue damage.

The cerebral endothelium is particularly vulnerable to the shearing–stretching forces applied to the brain at the time of trauma. This impact causes the opening of tight junctions that normally isolate the parenchyma from the systemic circulation,
consequently increasing the permeability of the blood–brain barrier (BBB). The resulting diffusion of serum proteins and the transmigration of blood cells into the injured brain tissue are known to exacerbate cerebral damage. Experimental studies from our laboratory using mice subjected to focal TBI have shown that although the passage of large molecules like horseradish peroxidase (44 kDa) injected intravenously occurs only in the acute phase (4–5 hours after TBI), smaller molecules (286 Da or 10,000 Da) are still able to penetrate the brain at 4 days [7]. Understanding the time course of BBB dysfunction is of particular relevance because such knowledge allows the identification of a therapeutic window for the administration of drugs that cannot cross the intact BBB under physiological conditions.

As an interface between the periphery and the brain, the endothelium constituting the BBB is strongly affected by both cerebral and systemic changes, which can significantly affect brain function. Endothelial cells are immunologically active and express a variety of cytokines and cell adhesion molecules that regulate adherence and infiltration of leukocytes into the injured site. The breakdown of the BBB plays a profound role in the development of brain edema following TBI and the consequent increase in intracranial pressure. Additional physiological events can affect the integrity of the BBB by participating in the development of brain edema. One such insult is posttraumatic hypoxia, which can significantly affect patients’ outcomes after TBI and is often associated with increased length of hospital stay, increased severity of brain injury, and worse functional outcome. Hypoxia itself is defined as arterial oxygen saturation (SaO$_2$) of $<90\%$ and has been documented in up to 45% of all TBI patients [8]. The imbalance in blood gases contributes to augmentation of cerebral blood volume due to loss of autoregulation (ability of the vasculature to respond to CO$_2$–O$_2$ ratios by dilating or contracting), which exacerbates BBB permeability further, allows the diffusion of serum proteins and blood cells in the parenchyma, and causes cell swelling via excessive intracellular ion influx. Collectively, these processes constitute the complex phenomenon of secondary brain damage [9].

10.3 Molecular Cascades Mediating Cell Death after TBI

The pathological alterations resulting from brain injury trigger multiple biochemical cascades that lead to the release of neurotoxic substances that ultimately exacerbate neuronal cell death [10]. Occurring from the early hours to days following TBI, these events establish a useful time frame for therapeutic intervention intended to block their irreversible effects. The reduction of secondary brain injury through a modification of these molecular pathways is the clinician’s major task in the management of TBI.

Much research effort has been dedicated to establishing the roles of excitotoxicity, the oxidative pathway, and cerebral inflammation after brain injury [11]. Recent human studies have described, by volumetric brain MRI analysis, an ongoing progressive brain atrophy up to 1 year following TBI, which correlated with altered brain metabolism (lactate/pyruvate ratio) measured in the acute phase by microdialysis [12–14]. These findings corroborate the significant tissue loss and delayed neuronal death observed in TBI rodents that paradoxically coincides with neurological improvement [15,16].
Apoptotic cell death predominantly occurs in neurons surrounding the lesion in focal TBI models such as controlled cortical impact injury, fluid percussion injury, or closed head injury, with peak levels occurring at 1–2 days postinjury and slowly decreasing in subsequent days [15,17–20]. Several factors have been identified as potential triggers of apoptosis, one of which seems to be the excessive production of glutamate, the main excitotoxic neurotransmitter in the brain. Glutamate is abundantly released within minutes after TBI as a consequence of increased neuronal production, release by cell rupture, and the inefficiency of astrocytes to take up glutamate, which is normally metabolized intracellularly into harmless glutamine. Binding of glutamate to N-methyl-D-aspartic acid (NMDA) receptors increases the influx of intracellular Ca\(^{2+}\) that causes mitochondrial overload and activation of caspase-dependent and independent cell apoptosis. A great deal of experimental research, including a vast number of randomized clinical trials, has focused on the neutralization of glutamate, specifically by targeting its main receptor NMDA, with the aim of avoiding and improving tissue and neurological damage. Unfortunately, most of these clinical trials were unable to demonstrate therapeutic efficacy in humans, despite the abundance of successful pre-clinical experiments in animal models [3]. Of note, based on findings from a mouse model of TBI, Shohami’s group recently speculated that the failure of such NMDA-inhibiting compounds may be due to the fact that NMDA receptor hyperactivation is only a transient phenomenon (within 1 hour), which is rapidly followed by a profound and long-lasting loss of function [21]. Consistent with their hypothesis, the administration of exogenous NMDA in TBI mice improved motor and cognitive recovery.

Apoptotic cell death occurs mostly via the extrinsic and intrinsic pathways. The extrinsic pathway of apoptosis differs from the intrinsic pathway in that it is activated by the interaction of a ligand with a membrane receptor. The death receptor Fas is the most scrutinized mediator of extrinsic apoptosis, and implications from these studies is relevant to TBI because Fas is expressed on neurons, rendering these cells highly susceptible to death. Delayed neuronal loss is particularly likely in the region surrounding the lesion and more distally within the hippocampus. Following Fas-ligand binding, the receptor undergoes trimerization, followed by the recruitment of FAS-Associated protein with Death Domain (FADD) and activation by cleavage of caspases-8, which in turn activates the executioner caspase-3 to cleave the enzyme DNase, allowing it to enter the nucleus and break DNA into fragments (detected histologically by TUNEL staining). Fas expression has been identified in dying TUNEL-positive neurons surrounding the brain lesion and also on other cells, including astrocytes and microglia. The pivotal role of Fas in mediating cell death and neurological deficit following TBI is revealed by experiments on specific mouse strains with mutated, nonfunctional expression of either Fas (lpr) or Fas ligand (gld). A neuroprotective effect was demonstrated in ischemic and spinal cord injury models using lpr mutants, which displayed reduced tissue damage and neuronal resilience to apoptosis [22,23]. However, in a TBI model of controlled cortical impact injury, such benefits were noted only when both Fas and the related pro-inflammatory cytokine tumor necrosis factor (TNF) were simultaneously knocked out [24].

In our laboratory, a sustained improvement of neurological deficit and reduction of lesion volume were observed in lpr mice using a closed focal TBI model. These
improved outcomes corresponded to a decreased number of pericontusional apoptotic neurons and altered cortical cytokine production. A marked enhancement of TNF was measured in both the injured and uninjured lpr mice, implicating TNF as a neurotrophic mediator in recovery after TBI (Ziebell et al., unpublished observations).

The relevance of Fas in human TBI is indicated by several research groups’ detection of sustained elevation of soluble Fas and Fas ligand in the cerebrospinal fluid (CSF) of severely injured patients [25,26]. Interestingly, in pediatric TBI, higher concentrations of the anti-apoptotic factor Bcl-2 measured in CSF correlated with better outcomes [27]. This group also showed that expression of apoptotic factors was associated with neuronal cell death in adult TBI brain tissue. Specifically, an increase in Bcl-2, cleavage of caspase-1, up-regulation and cleavage of caspase-3, and DNA fragmentation were found in cells having both apoptotic and necrotic morphologies [28].

Although apoptosis and inflammation were previously considered to be independent molecular cascades, evidence to date suggests a close link between Fas-mediated cell death and cytokines, particularly related to the molecular structure and role of the Fas/Fas ligand and TNF/receptor protein systems. For example, both Fas and one of the two TNF receptors (p55) are type I transmembrane members of the TNF/NGF receptor superfamily, and share homologous sequences in the extracellular regions [29,30]. In addition to its role in apoptosis, Fas displays some neuroimmunomodulatory functions. In fact, Fas ligation on monocytes and macrophages induces a pro-inflammatory cytokine response, resulting in potent neutrophil chemoattractant bioactivity [31]. Likewise, the cytokines interleukin-(IL)-1, IL-6, TNF, and interferon gamma had the ability to enhance constitutive Fas and Fas ligand expression in cultured human astrocytes [32]. Taken together, these studies substantiate the complex relationship between inflammation and the pathways of cell apoptosis, indicating that more work is required to fully elucidate the molecular mechanisms involved in neuronal cell death secondary to TBI.

10.4 Cellular Inflammatory Response and Axonal Damage

The complex inflammatory responses elicited in the injured brain constitute distinct and well-ordered stages of cell activation and immune mediator production. The breakdown of the BBB that occurs rapidly after TBI allows the transmigration first of neutrophils, then of macrophages and, in limited amounts, lymphocytes. All rodent models of focal cortical damage have consistently reported a peak of neutrophils accumulated in and around the lesion by 1 day [17,33], whereas a massive infiltration of resident microglia and blood macrophages does not reach a peak until 4–7 days after TBI [17].

The role of infiltrated neutrophils in contributing to secondary brain damage is controversial. Some studies attribute a detrimental role to neutrophils in relation to BBB function, because after activation these cells sustain inflammation and oxidative stress via the release of reactive oxygen species that damage cell membranes, proteins, and DNA. However, other work has demonstrated that depletion of neutrophils in a TBI model does not alter the extent of edema in the injured hemisphere or the
infarct size [34,35]. Similarly, increased numbers of peripheral neutrophils induced by the injection of granulocyte colony-stimulating factor caused an increase in BBB damage; however, this did not alter the accumulation of neutrophils in the brain or have any impact on edema, suggesting the involvement of other mechanisms in the development of brain swelling after TBI [36].

Essential for the adhesion and infiltration of neutrophils is the up-regulation of cell adhesion molecules such as intracellular adhesion molecule (ICAM) at the BBB. We had previously shown that the concentrations of soluble ICAM-1 in the CSF of patients with severe TBI were associated with BBB dysfunction and the contusion size measured on computer tomogram scans [37]. Animal experiments failed to support this relationship: the use of ICAM-1 knockout mice in a model of controlled cortical impact injury reported no differences in the extent of neutrophil infiltration, lesion size, or neurological outcome [38]. Furthermore, this lack of relationship is supported by our animal studies wherein up-regulation of ICAM-1 in brain tissue following a cortical contusion in mice was maximal at 7 days—a late time point compared to the peak in neutrophil infiltration observed at 24 hours [39]. Late expression of ICAM-1 on cerebral vessels of rats subjected to diffuse axonal injury, along with the absence of brain neutrophil accumulation, is a further argument against the involvement of this molecule in leukocyte transmigration [40]. An alternative function for ICAM-1 was proposed by \textit{in vitro} experiments performed in our laboratory demonstrating that both astrocytes and cerebral endothelial cells stimulated with recombinant soluble (s)ICAM-1 induce the release of the neutrophil chemokine MIP-2, the murine homologue of human IL-8 [39]. In addition, a synergistic effect was shown when both cultures were exposed simultaneously to sICAM-1 and TNF, demonstrating alternative signaling pathways. These data indicate that by promoting chemokine production, ICAM-1 may sustain leukocyte activation and passage into the injured brain.

\textit{Microglia} are the resident macrophages of central nervous system (CNS) parenchyma; they originate from the bone marrow during development of the nervous system. The shape of these cells differs considerably in both health and disease states. Microglia activation follows a specific pattern of morphological changes characterized by distinct combinations of surface molecules [41]. In a resting phase, they are barely detectable by immunohistochemistry, and display a ramified morphology. However, following stimulation microglia become fully activated and identical to ameboid macrophages. Upon challenge, microglia secrete a large number of inflammatory cytokines, reactive oxygen species, and glutamate, and are rapidly stimulated by serum proteins to display phagocytic function.

Using the closed head injury mouse model, our laboratory group demonstrated that the presence of pericontusional microglia/macrophages persisted up to 4 weeks; however, the area of cell spread around the lesion at this late time point was significantly reduced [42] (Figure 10.1). These cells appear heavily vacuolated, having phagocytosed the cell debris of the dead tissue. When neuronal staining is compared with astroglial immunohistochemistry at 4 weeks postinjury, it appears that reactive astrocytes surround the lesion core, which is completely devoid of neurons. Among all brain cells, microglia are believed to contribute most significantly to neurotoxic secondary
processes, whereas astrocytes seem to play a more protective role. However, beneficial removal of dead cells by activated microglia/macrophages is crucial for tissue repair, as it sequesters proteins that would otherwise perpetuate the inflammatory response and myelin components that are known to detrimentally affect regeneration. Activated astrocytes support repair mechanisms by releasing neurotrophic factors that sustain neuronal survival as well as proliferation and differentiation of neural progenitor cells, thus promoting neurogenesis. In contrast, the glial scar has been implicated in hampering neuronal and axonal regeneration, likely by the expression of inhibitory proteins. Like the activation of microglia/macrophages, astrocyte reactivity is a long-lasting process observed after both human and rodent TBI.

Although moderately present in the area surrounding a focal contusion, axonal damage is mostly predominant in human or animal models of diffuse brain trauma. The acceleration–deceleration impact injury model established by Marmarou et al.

Figure 10.1 Representative microscope images of the injured hemisphere following experimental focal closed head injury in the mouse. Immunohistochemistry against neurons (NeuN) (A, D), astrocytes (GFAP) (B, E), and macrophage/microglia (F4/80) (C, F) on brain sections detected neuronal loss, astrocyte activation, and macrophage accumulation, respectively. Note that by 7 days postinjury (A, B, C), F4/80-labeled macrophages are closely compacted into the lesion core, which is devoid of viable neurons or astrocytes. By 28 days postinjury (D, E, F), tissue damage is evident distally from the cortical lesion; increased astrocyte (E) and microglial reactivity (F) in the dorsolateral thalamus corresponds to reduced neuronal density in this region. Scale bar = 1,000 µm.
[18,43] in the rat still remains the best and most widely characterized model for reproducing widespread injury to the axonal structures in the white matter. Early markers of axonal damage, the amyloid precursor protein (APP) and heavy chain neurofilament (200kDa), almost identically overlap in immunohistochemical staining, where the axons present with swelling and retraction bulbs. Preliminary experiments in our laboratory have demonstrated abundant axonal damage following diffuse traumatic axonal injury, particularly within the corpus callosum and the pyramidal tract of the brainstem, which are subject to high forces during the traumatic impact. This axonal damage is exacerbated when a 30-minute hypoxic insult is superimposed on the TBI (Figure 10.2). Povlishock’s group has investigated this model over several years and found that axonal dysfunction is a process that begins early after trauma and slowly progresses, leading to impaired retrograde transport, ionic dysregulation, axonal swelling, and ultimately axonal disconnection [44]. The underlying mechanisms include axonal Ca\(^{2+}\) influx and increased permeability of the axolemma, followed by mitochondrial dysfunction and activation of apoptotic caspases. Although axonal perturbation can be reversed, when the point of no return is reached, it proceeds into total axotomy. Compared to focal TBI, diffuse axonal injury does not involve a massive accumulation of blood leukocytes, but only infiltration of macrophages/microglia in discrete regions such as subarachnoid spaces and the

![Figure 10.2](image)

**Figure 10.2** Representative microscope images demonstrating axonal damage in the rat brain at 1 day following diffuse traumatic axonal injury (TAI). Axonal damage was detected using immunohistochemistry for neurofilament (200kDa heavy chain), which is vulnerable to compaction and accumulation in axons following TAI. Distinct patterns of damage, in the form of retraction bulbs and axonal swelling, are present in the pyramidal tracts of the brainstem (A, B, C) and corpus callosum (D, E, F) in rats that have undergone TAI in combination with hypoxia (A, D) or TAI alone (B, E). Note that the concomitant insult of hypoxia results in a far greater level of axonal injury than that observed in TAI alone. This severity of injury is evident when taken in the context of sham-treated animals (C, F), which are completely devoid of neurofilament accumulation. Scale bar = 100\(\mu\)m.
corpus callosum [45]. Surprisingly, even in the most severe diffuse injury (450 g from 2 m) cell death is hardly detectable in the brain, although the animals show protracted motor impairment in sensorimotor tests as well as the rotarod [40,46]. This may be explained by the dysfunction of the synaptic transmission at the damaged axons, which impairs neurological function, rather than a significant loss of neuronal cell bodies. Astroglial reactivity also occurs relatively early even in the milder form of injury (250 g from 2 m), and persists for at least 2 weeks after injury [45].

10.5 Cerebral Cytokine Network

The aseptic humoral inflammatory cascade evolving in the injured brain is milder than the reaction typical of an infectious disease elicited in either the CNS or peripheral organs. This is likely due to the refractory nature of the brain, once considered an “immune privileged site,” which dampens any immune reaction in order to minimize inflammatory-mediated tissue damage. This conclusion is supported by experiments showing that higher doses of the endotoxin lipopolysaccharide (LPS) injected in the brain are required to induce brain cytokine production, as compared to subcutaneous inoculation [47]. Similarly, in vitro, higher concentrations of LPS are classically used to stimulate cultured astrocytes or microglia, as opposed to stimulation of peripheral macrophages. The ultimate role of cerebral inflammation after TBI is to repair the damage, but there is sufficient evidence to suggest that when protracted over time or when released at high concentrations, cytokine release in the injured brain can generate further harm.

Cytokines are small peptides with a molecular weight of approximately 20 kDa. They are produced by all cells of the CNS in a complex network to regulate a variety of cellular functions, including activation, migration, differentiation, regeneration, and death. The expression of selected and specific cytokine receptors at the cerebral endothelium suggests that cytokines can cross the BBB from the bloodstream to the brain and vice versa, via a saturable carrier-mediated system [48]. Cytokines themselves can disrupt the BBB and be produced by endothelial cells and astrocytes located at the barrier. This transport system renders the brain heavily vulnerable to systemic infections (for instance, encephalopathy, which is often encountered in septic patients). In contrast, cytokines produced in the brain, such as IL-6, may influence the function of peripheral organs (e.g., the liver) by inducing the posttraumatic acute phase reaction [49]. This continuum demonstrates that isolation of the brain by the BBB is limited and subject to specific conditions in health and disease.

Over the past years, it has been established that cerebral inflammation elicited following TBI plays a dual function: it can exacerbate tissue damage, but it can also promote the processes of repair [11,50–52]. Summarizing the large body of work produced in the past two decades is not an easy task. Therefore, this chapter focuses on the most relevant studies, with an update on the current literature concerning this topic. Table 10.1 summarizes some of the pivotal studies, beginning with the early evidence that cytokines are synthesized in the brain of animals subjected to various forms of TBI, to the first substantiation of cerebral cytokine release into human ventricular
### Table 10.1 Detection of Inflammatory Mediator Production and Function in Rodents and Humans Following Traumatic Brain Injury

<table>
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<tr>
<th>Experimental Approach</th>
<th>IL-1</th>
<th>TNF</th>
<th>IL-6</th>
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<tbody>
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<td>Human CSF/serum/microdialysate</td>
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<td>Rodent brain/microdialysate</td>
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<tr>
<td>Application of binding protein/soluble receptors or receptor-antagonists, antibodies</td>
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<td>[85]</td>
<td>[87]</td>
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<tr>
<td>Anti-inflammatory agents: pentoxifylline, dexabinol (NMDA antagonist), IL-1ra, ICE-inhibitor, IL-10, minocycline, EPO, hypothermia</td>
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(Continued)
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We also list studies on brain tissue protein and mRNA synthesis and brain microdialysis, as well as experimental, therapeutic, or genetic strategies to inhibit cytokines’ action either to treat secondary brain damage or to further explore their role in such damage.

Several years ago, we first began to characterize longitudinal patterns of cytokine production (pro-inflammatory: TNF, IL-6, and IL-8; anti-inflammatory: IL-10, TGF-β) in patients with severe TBI up to 3 weeks postinjury [11,17,40,45,49,53–58]. It became evident that contrary to what was observed in the rodent brain, the secretion of cytokines in ventricular CSF was elevated over a long period of time, although peaks coincided in the early days after injury. What came as a surprise to us in the early 1990s was the finding that CSF cytokine measurements exceeded the levels detected in blood serum, supporting the relatively novel concept that the injured brain has the ability to mount a profound inflammatory response in which intrathecal cytokines do not originate via diffusion from the periphery through the BBB but are in fact produced by neural cells themselves.

For instance, IL-6, the main inducer of the acute phase reaction, was found to be abundantly released in the CSF of TBI patients. In addition, serum IL-6 correlated with both CSF IL-6 and the levels of acute phase proteins (C-reactive protein, α1-antitrypsin, and fibrinogen), in concomitance with a severe dysfunction of the BBB [49]. This data from our laboratory suggested that the increased permeability of the BBB likely allows the diffusion of this cytokine into the bloodstream. Our study found no association between IL-6 and either better or worse neurological outcome; however, other groups have shown that IL-6 levels in human brain microdialysate or CSF were associated with improved recovery, whereas measurements of IL-6 in serum correlated

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<th>Experimental Approach</th>
<th>IL-1</th>
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<td>KO mice/transgenic mice</td>
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<td>Cytokine-receptor KO mice</td>
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Table 10.1 provides an overview of the studies providing evidence for the production and function of cytokines in patients with severe brain trauma or animal models of TBI. References are also cited describing strategies used for the neutralization of cytokines in TBI models using specific blockers or general immunosuppressive drugs, as well as the use of cytokine gene knockouts or transgenic mice. IL=interleukin; TNF=tumor necrosis factor; NMDA=N-methyl-D-aspartic acid; ICE=IL-1β-converting enzyme; EPO=erythropoietin; CSF=cerebrospinal fluid; KO=knockout.
with poor prognosis [59–62]. Such apparently contradictory correlations should be interpreted with caution, as the TBI population is notoriously heterogeneous because of individual differences in patterns of brain damage, number of patients recruited in the studies, and the selection of values used for statistical analyses.

In animal models of TBI, IL-6 has been detected mostly in brain tissue as early as 1 hour, peaking between 2 and 8 hours postinjury, though in some studies the cytokine was elevated between 1 and 6 days postrauma [57,63–65]. In numerous laboratories, IL-6 has been detected after focal TBI in the CSF [65], in brain tissue at the mRNA/protein level [63,64,66,67], or in microdialysates [68,69]. After diffuse TBI, IL-6 was found to be elevated in CSF as well as detectable on brain sections by immunohistochemistry and in situ hybridization [57]. In vitro, IL-6 seems to possess multiple beneficial properties, as it inhibits glutamate excitotoxicity and TNF production, and induces IL-1 receptor antagonist (IL-1ra), which counteracts the detrimental effects of IL-1. IL-6 also promotes angiogenesis and astrocyte growth, thereby aiding tissue repair. In addition, we have previously shown that IL-6 has the ability to stimulate synthesis of the neurotrophic factor nerve growth factor (NGF) by cultured astrocytes, in response to incubation with IL-6-containing human CSF collected from TBI patients [53]. Experiments on IL-6 knockout mice have shown that astrogliosis, together with the recruitment of macrophages, is reduced after cryolesion coinciding with enhanced oxidative stress and neuronal cell death [70,71]. To the contrary, our laboratory found that IL-6 deficiency had no effects on outcome following focal TBI [72]. Alternatively, astrocyte-driven overexpression of IL-6 conferred neuroprotection after TBI, whereas other studies demonstrate that uninjured IL-6 transgenic mice exhibit spontaneous neurodegeneration [73,74]. Collectively, these findings only add to the controversy created by studies using different models of brain damage (cryolesion versus focal cortical contusion) and genetic manipulation of cytokine expression with either deletion or overexpression in injured and naïve mice.

Since the early 1990s, the function of TNF after TBI has been the focus of a multitude of studies with various and opposing outcomes [75]. TNF is a key initiator of inflammation capable of inducing a large number of pro- and anti-inflammatory cytokines and chemokines. Though considered to be the main neurotoxic cytokine mediating cell death in cultured neurons and oligodendrocytes, the actual role of this important immune mediator remains obscure. In models of TBI, large number of studies have shown amelioration of CNS damage following therapeutic blockage of TNF. The deleterious functions of TNF are mostly mediated by one of the two receptors (p55), thus implicating TNF in the pathogenesis of neurological diseases and cell apoptosis. However, the opposing beneficial effects are likely attributable to the alternative receptor (p75), which seems to be involved in remyelination and repair. After brain trauma, an early production of TNF was shown at protein and mRNA levels in brain tissue [63,64,76–78], or protein in rodent and human CSF and microdialysate [45,65,79–81]. However, no changes in TNF expression were found in a mild diffuse TBI rat model [82], or in our protein and mRNA analysis following focal brain injury in the mouse [17,83]. Lack of TNF expression in our experiments contradicts previous findings (Table 10.1) and may relate to the difference in TNF expression between mice and rats, as most of the initial studies were
performed in rat models. Generally, TNF production seems to be more elevated in rats than mice, suggesting that genetic differences may influence immune responses and that the choice of strain may be crucial to the outcome of the experiments [84].

In clinical studies, TNF has been detected in both CSF and serum of human patients with severe TBI [55,79–81]. In our patient group, TNF was detected in CSF only at low concentrations (picogram/ml range), and its pattern did not show an evident peak, but rather numerous rises above control over 3 weeks [55]. However, in this patient cohort IL-6 and TGF-β were markedly more elevated in CSF compared to serum, whereas TNF levels were very low and of similar concentration in both fluids. The contribution of TNF to secondary brain damage is supported by the improvement in outcome following its neutralization with the administration of soluble binding proteins, soluble receptors, or immunosuppressive drugs [77,85,86], whereas injection of anti-TNF (or anti-IL-6) antibodies had no effect on motor and cognitive outcome [87] (Ziebell et al., unpublished results).

The role of TNF after TBI became confused as a result of studies employing TNF or TNF-receptor-gene knockout mice in focal injury paradigms. Deletion of TNF expression led to a milder deficit in the posttraumatic acute phase but failed to provide long-term neurological recovery. At 4 weeks, TNF knockout mice also presented larger lesion volumes compared to wild-type mice [88]. In our study using TNF/lymphotoxin-α double-knockout mice, other than an increased early mortality, there were no differences in BBB dysfunction, neurological outcome, cell death, or neutrophil infiltration compared to wild-type TBI controls [72]. Equivalently exacerbated tissue and BBB damage in TNF-receptor (p55 and p75) knockouts corroborated the essential neurotrophic properties of TNF in the pathophysiology of TBI, possibly via activation of the transcription factor NFκB, which regulates the expression of antiapoptotic genes [89]. In addition, earlier we demonstrated that TNF injected into normal mouse brain reduces constitutive synthesis of the pro-inflammatory cytokine IL-18 (a member of the IL-1 family), thus reducing brain inflammation [90]. Altogether, these recent studies suggest that TNF’s function may differ in the acute and the delayed phase after TBI; it seems to act as a potent immune mediator initially, but as a protective, neurotrophic factor later on, which is required for CNS repair.

First identified as a potent endogenous pyrogen, subsequent research revealed that IL-1 is a multifunctional cytokine playing a wide range of functions in neuropathological diseases, including Alzheimer’s, multiple sclerosis, ischemia, depression, and TBI. Its relevance as a promoter of neurodegeneration predominantly derives from early studies on brain ischemia [51,91]. IL-1 is rapidly secreted by microglia and its receptors are constitutively expressed by neuronal cells. Among its functions, IL-1 regulates the secretion of several pro-inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species, and modulates the activation of matrix metalloproteinases, thus acting as an initiator of the inflammatory cascade and tissue damage. The duality in the role of brain inflammation seen with TNF also applies to IL-1. Although microglial IL-1 is required for astrocyte production of neurotrophins (EGF, CNTF) that promote regeneration, the deletion of IL-1 in knockout mice failed to achieve proper remyelination, and these mice showed impaired astrocyte activation that was linked to increased BBB permeability [92].
In TBI, a large number of studies have demonstrated up-regulated expression of IL-1 mRNA and protein from the early hours up to days after focal and diffuse TBI in animal models. Increase of IL-1 was detected using a variety of molecular techniques, including immunohistochemistry, microarray, multiplex, and microdialysis; and was associated with BBB dysfunction, hippocampal cell death, and motor deficit [64,66–69,82,93–95]. Although IL-1 promotes the synthesis of neurotrophic factors, this cytokine is generally believed to be neurotoxic in neuropathology, as shown by inhibitory experiments utilizing IL-1ra which improved outcome when injected peripherally or centrally after ischemia or TBI [96]. In contrast, one study has shown that transplantation of fibroblasts transfected to release IL-1ra into the contusion, although improving outcome, actually reduced NGF production [97]. In another study, mice lacking IL-1 receptor expression exhibited reduced glial activation and decreases in Cox-2, IL-1, and IL-6, though TNF and NGF remained unchanged [98]. In addition, IL-1ra transgenic mice showed attenuated acute IL-1, TNF, and IL-6 levels after TBI, coinciding with improved neurological recovery [99]. Preclinical animal experiments that tested immune suppressive drugs such as minocycline or erythropoietin (EPO) attributed the neuroprotective mechanisms of these compounds to the reduction of brain IL-1 synthesis after TBI [17,83,100].

Although IL-1 can be difficult to measure in human fluids, there are several studies reporting elevation of IL-1 concentrations in CSF or microdialysates of TBI patients [101–108]. In pericontusional brain tissue acquired from patients undergoing surgery after TBI, IL-1 expression has been shown using both immunohistochemistry and in situ hybridization, and up-regulation of IL-1 was found in both the acute and delayed stages [105]. In some clinical studies, a rise in IL-1 levels seems to correlate with poor neurological outcome [103,109]. However, dissociation between improvement of outcome and IL-1 increase was suggested following mild hypothermia in pediatric TBI. Although hypothermia successfully decreased intracranial pressure in this patient population, it did not attenuate enhanced production of IL-1, nor of IL-6 or IL-10, in ventricular CSF [108].

10.6 Role of Chemokines in TBI

Chemokines, or chemotactic cytokines, contribute to neuroinflammation following TBI by initiating the recruitment of peripheral leukocytes into the injured brain parenchyma. The function of these mediators in the migration and activation of leukocyte subsets is well established; however, their relative contributions to both physiological homeostasis and pathological conditions are still being identified. Chemokine expression is not restricted to hematopoietic cells; rather, both glia and neurons are capable of secreting a range of chemokines, and constitutively express a wide variety of chemokine receptors [110,111]. In the CNS, chemokine actions are crucial, not only in disease states, but also in neuronal migration during development, cell proliferation, protection against neuronal cytotoxicity, and intercellular communication. Gene deficiency studies have clearly demonstrated that several chemokines act as vital, nonredundant mediators in inflammatory responses, such as that resulting from TBI.
Following brain injury, chemokines are rapidly released by activated resident microglia and astroglial cells. Chemokine expression is regulated by cytokines such as TNF and TGF-β [112,113]. Studies over the past two decades, with both patients and experimental models, have reported the release of several chemokines following TBI (Table 10.2). The elevation of IL-8 (known by systemic nomenclature as

**Table 10.2** Detection of Chemokine Production and Function in Rodents and Humans Following Traumatic Brain Injury

<table>
<thead>
<tr>
<th>Experimental Approach</th>
<th>Human CSF, Serum, or Brain Tissue</th>
<th>Rodent Serum or Brain Tissue</th>
<th>Gene Knockout or Inhibition with Anti-Sense Oligonucleotides</th>
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<td>Fractalkine (CX3CL1)</td>
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Table 10.2 is an overview of studies providing evidence for the production and function of chemokines in patients with severe TBI or experimental animal models of head injury. Note that studies investigating IL-8 in rodents predominantly focus on MIP-2 (CCL2) and KC (CXCL1), which are considered the functional homologues of human IL-8 (CXCL8). Listed in parentheses beside each traditional chemokine name is the systematic nomenclature.
CXCL8), a potent neutrophil-attracting chemokine, has been detected in the CSF of both adults and children following severe TBI [54,114], and is associated with BBB dysfunction and increased mortality. Likewise, the murine IL-8 analogues MIP-2 (CXCL2) and KC (CXCL1) are induced in CNS tissue following experimental brain injury [115–117].

Another key chemoattractant in neuroinflammatory responses is monocyte chemoattractant protein-1 (MCP-1, also known as CCL2). The exuberant monocyte-rich response characteristic of trauma to the CNS may be attributed to this chemokine, which plays a nonredundant role specifically in the recruitment of blood-borne monocytes from the periphery, as well as in the activation and migration of resident microglia to the lesion site. An elevation of MCP-1 in the injured brain has been demonstrated by our group, with protein concentrations peaking as early as 4 hours in models of closed head injury and diffuse axonal injury [40,42]. Astrocytes and endothelial cells are the primary sources of this chemokine soon after injury [118,119], indicating that chemokine synthesis constitutes an important aspect of the early reactive astrogial response. Once recruited, macrophages and activated microglia are also able to secrete MCP-1, suggesting an autocrine loop of activation that may perpetuate the ongoing cell migration. We have recently demonstrated that MCP-1 gene-deficient mice exhibit reduced neuronal loss, macrophage infiltration, and astrocyte activation, and improved neurological function following closed head injury [42], suggesting that macrophages recruited in response to MCP-1 play a primarily detrimental role following TBI.

10.7 Anti-Inflammatory Intervention after TBI

10.7.1 The Role of Erythropoietin as a Neuroprotectant in Humans and Animal Models

The targeting of single end products of neurotoxic pathways failed to confer neuroprotection or improve outcomes after TBI. Alternative options in clinical trials may rely on the administration of drugs with multiple modes of action. One such promising therapeutic is erythropoietin (EPO), a 30kDa molecule to which has been attributed immunomodulatory and neuroprotective properties in CNS pathology [120,121]. EPO is synthetized by neurons and astrocytes; its receptor is expressed constitutively by neurons and endothelial cells and is up-regulated following brain hypoxia and injury [122]. Studies in injury models of the CNS have demonstrated neuroprotective and neuroregenerative functions of EPO via reduction of apoptosis, inflammation, oxidative stress, and excitotoxicity, and have also revealed its ability to decrease lesion volume, reduce brain accumulation of leukocytes, and improve motor and cognitive function [123,124]. EPO rapidly crosses the BBB following intravenous administration in humans and mice [125–127], and its beneficial neurological effects have been proven in patients with stroke, schizophrenia, and multiple sclerosis [120,127,128]. Recently, there has been a remarkable increase in the number studies demonstrating the neuroprotective role of EPO in animal models of neurological diseases [121]. In TBI, most groups have investigated the role of EPO after focal injury
[129,130]; only one study employed the rat model of diffuse TBI [131]. After cortical contusion, EPO improved motor deficit and cognitive function, while reducing inflammation, axonal degeneration, and the number of apoptotic neurons [124]. A potential mechanism underlying the regenerative potential of EPO is enhancement of neurogenesis, as EPO has been shown to increase the proliferation of progenitor cells and neuronal differentiation in uninjured mice [132].

10.7.2 Minocycline, a Controversial Immunosuppressive Neuroprotectant

Another drug abundantly tested in models of neurological diseases is minocycline, a tetracycline derivative that has been shown to be therapeutically effective at reducing inflammatory and apoptotic processes. Minocycline treatment conferred neuroprotection following spinal cord injury [133], excitotoxicity [134], and ischemic injury [135–137]. In TBI, Sanchez Mejia et al. found that minocycline injected after a controlled cortical impact injury resulted in improved neurological function, decreased lesion volume, and attenuated production of cerebral IL-1β [100]. In a recent study, we demonstrated that high doses of minocycline treatment in a model of focal TBI resulted in only brief, transient neuroprotection by reducing secondary brain damage, concomitant to an early reduction in microglial activation and cerebral synthesis of IL-1β and IL-6 [17]. However, in a subsequent study, the use of lower doses of minocycline administered over a longer period of time was more effective in producing a sustained improvement of neurological deficit over 6 weeks postinjury (Bye et al., unpublished observations). Genomic analysis of TBI mice, by DNA microarray, indicated that treatment with high minocycline dosage reduced the expression of 27 inflammatory genes and transcription factor genes that were up-regulated in saline control mice following TBI [83]. Surprisingly, minocycline was also found to enhance the expression of apoptotic genes in mouse brain, implying a negative impact of chronic immune suppression after TBI. In another preclinical study, protracted administration of ibuprofen [138] showed that total inhibition of cerebral inflammation after TBI impairs the beneficial effects of cytokines in the modulation of repair processes. The recent reports investigating the role of minocycline have shown inconsistent and detrimental effects of this drug in models of neurodegeneration; these results underscore the importance of further testing of minocycline to establish its viability as a potential therapy following TBI.

10.8 Conclusions

The overview in this chapter is intended to highlight the controversies concerning the role of brain inflammation elicited after brain trauma; in doing so, we gave a series of examples offered over the years by animal and clinical studies. The complexity of this field is due first and foremost to the lack of understanding of a pathology that is only beginning to be discovered. Only when the intrinsic differences between diffuse and focal TBI are fully clarified will we be able to fully comprehend the actual role
of all the cellular and molecular components involved in these complex networks. The variability in outcomes of animal data reflects our dependence on specific experimental parameters for proper evaluation of results. Cellular and molecular responses to TBI, including inflammation, may vary according to genetic patterns, use of animal strains, and the methods used to produce brain injuries. It is clear that cryolesion can hardly be compared to fluid percussion injury or diffuse axonal damage. Also, individual laboratories use distinctly different behavioral tests to assess outcomes, making the comparison of studies almost impossible. Despite this experimental variability, the common consensus today remains: Inflammation possesses both beneficial and detrimental properties, as complete ablation of cytokines and cytokine receptors generates exacerbated tissue and neurological damage after TBI. New therapies aimed at reducing the burden of TBI should focus on minimizing injury by modifying but not eliminating the inflammatory response and creating the conditions that are optimal for regeneration.

Acknowledgments

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11 Defence and Defeat Reaction: Central Control and Peripheral Effects

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11.1 Introduction

When the sophisticated psychosocial challenges of today’s modern life evoke human emotion, they engage the same age-old behavioural-neuroendocrine response patterns, which in primitive life serve to protect self and species (Figure 11.1). These response patterns are since eons-of-time imprinted in the limbic-amygda-hippocampus section of the mammalian brain (paleocortex), as well as in the subordinate hypothalamic and brainstem centres. Among these patterns are the so-called defence and defeat reactions which are our age-old emotional response patterns, intended to reinforce behavioural expression such as running away or fight.

Largely all parts of the brain are engaged in the control of emotion and their expressions, and the somatomotor-behavioral, autonomic and neuroendocrine efferent links are in various ways combined into “triads-of-expression”; designed to cope with whatever challenge and threats life can offer. The difference between humans and other species depends particularly on our sophisticated neocortex, allowing us to intellectually cope with different situations. Further, once emotions are induced, we can learn, at least to certain extent, to voluntarily suppress or modify the somatomotor-behavioural responses, while the autonomic and hormonal expressions cannot be suppressed voluntarily in the same manner. Therefore, if often repeated, the activation of these emotional response patterns may in the long run have sinister consequences, as largely all tissues and organ systems, including the immune system, become exposed to neuroendocrine mobilisations that no longer serve to support fight or flight and physical exertion, and hence could disturb the balance in physiological systems or “homostasis”, to use Walter Bradford Cannon’s term [1].

There is now increasingly strong evidence that chronic activation of physiological stress responses can contribute to several lifestyle related disorders, like primary (essential) hypertension and the so-called metabolic syndrome (abdominal obesity,
insulin resistance, hypertension and dyslipidaemia) [2,3]. Chronic psychosocial stress, with sustained activation of predominantly the defeat reaction, has also been shown to be linked to diabetes type-2 [4] and recent data from the British Whitehall study confirm the link between chronic stress and the metabolic syndrome, showing a dose-response relationship over 14 years period between work stress and the risk of developing the metabolic syndrome [5]. Furthermore, the large worldwide Interheart study shows that presence of psychosocial stressors is associated with increased risk of acute myocardial infarction [6]. In a recent paper describing the cardiovascular toll of stress, the authors conclude that, despite relatively few well-controlled studies, evidence exist for a strong and consistent association of acute and chronic psychosocial stress and cardiovascular risk factors such as hypertension and insulin resistance [7].

The decisive importance of the psychosocial environment is maybe best demonstrated in two separate studies on genetically and culturally homogenous populations clearly showing the impact of the social environment on rise in blood pressure with age [8,9]. Timio and co-workers showed in a 30 year follow-up study how the differences in the social life situation almost entirely explained the blood pressure rise in a group of women exposed to vivid Italian life-style compared to 150 nuns belonging to a secluded order in the same area [8]. Hollenberg and co-workers came to a similar conclusion when studying the Kuna Indian tribe in Panama, comparing a group still living the traditional life-style in their home islands compared to Kuna Indians who lived in a suburb to or in the big-city environment of Panama City. Age-related increase in blood pressure was not seen in Indians living traditional life-style, while the other groups
showed an increase in blood pressure with age [9]. The lifestyle situations between these groups naturally differed, though their entirely different psychosocial situations, with respect to environmental stimuli, were the most obvious one.

One factor thought to be of a great importance to restore the balance of unhealthy lifestyle factors, such as diet and psychosocial stress, is regular physical exercise. Extensive evidence now exists that physical activity is an important preventive tool concerning development of cardiovascular and metabolic diseases, and sedentary lifestyle is now generally considered to be a major risk factor for cardiovascular diseases [10,11]. After tens-of-thousands of hunter-gatherer generations, physical activity is no longer a requirement for daily living in the 21st century [12]. Therefore, the lack of the preventive effects of physical activity could be one important contributor, explaining some of the consequences of chronic psychosocial stress, not only on metabolism and cardiovascular system but also on the immune system, and recent data even suggest a protective effect of exercise on cancer [13–15]. One can say that human genes and modern live-style are to some extent incongruent, as our genome was originally selected for daily physical exertion [12].

### 11.2 Historical Landmarks

Already Charles Darwin was struck by how closely similar emotional expressions are in higher species, realizing that they must have been imprinted in the basal brain-section quite early in evolution, as outlined in his book from 1872 “The expression of emotion in man and animal” [16]. The pioneering experimental analyses of the cerebral responses to environmental stimuli were made by three eminent physiologists, Ivan Pavlov, Walter Bradford Cannon and Walter Rudolf Hess. Their classical studies were followed by the work of many others, including the particularly important work of Hans Selye. From a quite different angle of approach he contributed to the knowledge of how physical and mental noxious stimuli can via the brain induce profound neuroendocrine changes. Selye also coined the widely used (and misused) term “stress” [3,17].

For his work, concerning the cerebral control of gastric secretion, Pavlov was already in 1904 awarded the Nobel Prize in Physiology or Medicine to which comes his subsequent discovery how conditioned reflexes are severely disturbed if animals are exposed to intense mental stress. Hess received the same prize in 1949 for his experimental mapping of the brain as coordinator of a wide range of emotional response patterns. Had Cannon not died a few years earlier, he would probably have shared the prize with Hess for his pioneering work, covering largely all aspects of the sympatho-adrenomedullary axis, from the highest brain centres to peripheral transmitter mechanisms [18]. Cannon was the first to introduce the concept of homeostasis and the theory that the sympathetic nervous system serves as a first line of defence against disturbing stimuli [1].

The overwhelming complexity of the brain certainly fascinated these great physiologists – perhaps best expressed by Pavlov in his 1927 book “Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex” (Figure 11.2).
Hans Selye’s approach was quite different, starting with his clinical observations during his medical studies in Prague in the 1920-30s. He noticed how severely sick or badly traumatized patients often exhibited closely similar symptoms, independent of insult, including signs of adrenocortical enlargement and atrophy of lymphatic tissues. Emigrating to Montreal, he later got the opportunity to test his observations on patients in laboratory animals and gradually it became clear that an intense engagement of the hypophyseal-glucocorticoid system was a key link in explaining his findings. In 1936, Selye introduced “the syndrome that was produced by diverse nocuous stimuli” [17], though unfortunately he labelled the observed syndrome “stress”. The term “strain” would, however, have been more-to-the-point as stress causes strain, like these terms are used in the field of physics. Actually, Selye later said jokingly that had his English been better in younger years, he would have been world known as “father of strain” instead of “father of stress” [19].

Thus, while Cannon and Hess emphasized the hypothalamic-sympatho-adrenomedullary axis as a major cerebral command link, Selye’s work was of key importance for revealing the equally important hypothalamic-pituitary-adrenocortical axis (HPA-axis). These pioneer findings of Selye started a new research field and over the past half century the glucocorticoid (GC) effects have been intensely studied, revealing that their roles are much more complex than Selye may have thought. Thus, the GC action in stress is a picture of extraordinary diversity, depending on the physiological endpoints, and later research has shown that GC effects can act both suppressive and stimulatory as well as permissive, depending on situation, timeframe and which organ is affected [20]. An important part of this diversity was published by Hench and co-workers already in 1949 when they showed the remarkable anti-inflammatory action of GC [21], which...
did not fit into the Selye’s paradigm concerning stress. However, these findings can on the other hand be considered as an important step in suggesting that the brain may also influence the immune system.

11.3 Extending the Pioneer’s Findings and the Discovery of Neural-Immune Connection

In recent decades, the mentioned pioneer findings have been greatly extended by a range of refined techniques and explorations concerning how psychosocial stimuli and challenges can decisively contribute to cardiovascular, metabolic and immunological disturbances and disorders. Of particular importance are James P Henry’s fascinating studies of mice colonies along 1960-90s [22]. By imposing various kinds of prolonged psychosocial challenges, he could induce either defence or defeat reactions in the animals, showing how sustained psychosocial stress could elicit neurohormonal response patterns which, in the long run, could induce disorders closely mimicking man’s primary hypertension, and the metabolic syndrome. He further showed how genetics, sex and earlier experience could decisively influence the outcome [23,24]. In addition, induced defeat reaction led in some cases to an increase of an endemic type of mammillary tumours. However, his brief report of these observations was refused by leading cancer journal, because at this time, it was thought to be impossible that a mental state could have anything to do with local tumour development.

Later, other studies opened the door for such mechanisms, and in 1975 Ader and Cohen published a paper introducing the term psychoneuroimmunology [25]. In 1981, Felten and co-workers discovered a network of nerves which could influence cells of the immune system. Thus, nerves were found in the thymus and spleen terminating near clusters of lymphocytes, macrophages and mast cells, all of which help control immune function. This discovery provided one of the first indications of how neuro-immune interactions occur [26].

Henry’s approach to explore the effects of psychosocial stimuli in group-living animals has also been most successfully applied to primates where, particularly, the studies of Kaplan and co-workers in cynomolgus monkeys have shown how prolonged induction of defeat reactions could induce coronary atherosclerotic changes, even when diet was kept unchanged [27]. As previously mentioned, the findings that psychosocial stress can affect the development of the metabolic syndrome and atherosclerosis and even cause myocardial infarction have later been confirmed in humans [2,5,6,28].

In general, the fields of psychoneuroendocrinology and psychoneuroimmunology have expanded tremendously and a vast number of studies add to present knowledge of how the central nervous system can influence the function of peripheral organ systems, including the immune system [29]. The development of brain imaging methods has also increased the possibility of studying brain function in awake human subjects, including how the brain is activated during acute mental stress and how psychosocial stress is perceived and processed [30].
11.4 Principal Organisation of the Defence and Defeat Reactions

Regulation of body function is organized at different hierarchical levels, where those of the central nervous system (CNS) are of decisive relevance when it comes to environmental stimuli. Thus, CNS constitutes the highest level in the hierarchy of control systems that direct the responses to both internal and environmental stimuli. Figure 11.3 illustrates in schematic form the hierarchic organisation in higher organisms, from neocortex down to intracellular organelles and the genetic code. At each level of control, functions are balanced-off by negative feedback mechanisms, according to the principles of “homeostasis”.

Figure 11.3 Outline of the six main levels of hierarchical organisation and control in higher organisms (adapted from Folkow B. Ann Behav Med 1993;15(4):236–244).
The enormous complexity of thought processes and control actions makes the CNS unique, where e.g. modern psychosomatic research shows how powerful thought processes and emotions are in controlling bodily functions, resulting in both positive and negative health effects [29,31]. This includes the fascinating data on the placebo effects, for instance how cognitive activation with placebo analgesia engages similar neuronal network as opioid analgesia [32].

Recent development within the fields of genomics and molecular biology has vastly increased the knowledge of the regulatory function at cellular and molecular levels. However, among the many levels of organisation, the brain is unique; besides obtaining all intrinsic information from the entire organism, where the amount of afferent fibres is ten times as many as efferent ones, to which comes a range of blood-borne hormone messages, the brain also receives continuous information about the extrinsic environment via e.g. vision, hearing and olfaction.

The pathways from the brain to bodily expression can characteristically be divided into previously mentioned efferent links: The somatomotor system, the autonomic nervous system and the endocrine system. These triads of response patterns, organised at the paleo-cortical-hypothalamic levels and principally equal in man and animals, are phylogenetically very old. The terms “defence” and “defeat” reaction are derived from their intense engagements in animal experiments. This may give the erroneous impression that they are reserved for catastrophe situations only, but actually, they are in mild forms, and in various constellations, parts of daily life in modern society. Maybe one of the best models to describe these two reactions is the one of “hawks” and “doves,” describing how animals cope with stress with different behavioural strategies. Hawks are the aggressive individuals with a fight and flight reaction strategy while doves show freeze and hide behavioural strategies or outright withdrawal and defeat [33,34].

11.4.1 Defence Reaction

The age-old defence reaction is, as mentioned, commonly induced in mild form in modern society life, e.g. whenever man is exposed to interesting, irritating, or otherwise challenging mental stimuli. When acutely induced, a differentiated sympathetic-adrenomedullary discharge increases cardiac output and blood supply to the skeletal muscles, myocardium and brain, while blood flow and functions are reduced in organ systems not needed for vigorous motor activity, e.g. kidneys and gastrointestinal tract. As a net result, blood pressure is raised, usually to a considerable extent. The increased secretion of adrenaline causes release of glucose from the liver that increases blood glucose levels, while glycolysis is facilitated in skeletal muscles. Activation of the sympathetic-adrenomedullary system also increases the release of lipids to the circulation, boosts the coagulation system by e.g. facilitating thrombocyte aggregation [35] and stimulates the immune system in a different manner [36]. All these effects serve as anticipatory adjustments to favour flight or fight and physical exertion. Enhanced mental activity and alertness are also a common features of the defence reaction which is partly due to increased blood-borne catecholamines.

At maximal engagement, this implies an all-out mobilisation of the organism’s resources to cope e.g. with life-threatening situations, were the gain of a split second
can determine the outcome. But, as already mentioned, mild engagements of defence reactions occur frequently in modern life, inducing the same, though, milder bodily changes, indicating an appropriate balance between stimulus strength and extent of engagement. The acute defence reaction is thus an important survival reaction and by nature advantageous for the organism. One important exception is some pathological situations, particularly in the presence of coronary insufficiency or myocardial disorder, and recent studies show that patients with coronary artery disease may show exaggerated platelet and hemodynamic reactivity to mental stress, compared with healthy individuals, partly explaining the pathophysiological processes underlying emotional triggering of acute cardiac events and, in worse cases, sudden death [37]. Further, as previously mentioned, chronic or frequent episodic activations of defence reactions can in the long run seriously contribute to cardiovascular diseases such as primary hypertension [3,38]. One should, however, be aware that this relationship is complicated and many factors such as genetics, individual differences in vulnerability, interpretation, experience and physiological reactivity all contribute to the acute and the chronic outcome of environmental stimuli.

11.4.2 Defeat Reaction

This characteristic reaction pattern – which in view of serious long-term effects may even be more important than the defence reaction – is induced, for example, when animals are exposed to situations that overwhelm their coping mechanisms. The behavioural side is characterized by withdrawal and suppressed physical activity. In man, this reaction is strongly involved in e.g. deep sorrow or when situations are experienced as being beyond hope and rescue or, in milder forms, as frustrating or bleak. In animal research, the defeat reaction is linked to an activation of the HPA-axis, as explored by e.g. Henry and others [39]. In situations experienced as overwhelming, withdrawal and passivity, typical for defeat reactions, is appropriate and generally beneficial. However, intense and prolonged activation due to long-lasting effects of stressors or inability to turn off stress responses can in the long run have serious consequences for health and end up in disease.

The disturbed release of glucocorticoids can contribute to severe suppression of the immune system, which in a number of ways can affect health, as discussed in other chapters of this book. The metabolic disturbances typical of the metabolic syndrome have also been found to be related to disturbances in the HPA axis [40]. The metabolic syndrome is to a great extent lifestyle-related, as both diet and inactivity play significant roles for the development and the clinical consequences of the syndrome. Moreover, recent data from the Whitehall study further support that psychosocial factors are of great relevance for the development of the metabolic syndrome [5] and together with predisposing genetic elements, the situation becomes most complex, to which comes that glucocorticoids affect food intake.

It should be remembered that “civilisation disorders” like primary hypertension and the metabolic syndrome are rare, indeed, in primitive human society, while they in modern hectic life are becoming more and more prevalent even in younger individuals, and hypertension is often seen as the most prevalent component of the metabolic
syndrome [41,42]. However, the prevalence of the metabolic syndrome varies considerably between studies, mainly due to different definitions used, but also due to other factors such as differences between gender and ethnic groups [42,43].

Thus, having a “thrifty gene” favouring survival in primitive life may in our modern society “back-fire” as e.g. the effects of glucocorticoids are often combined with high caloric intake and physical inactivity [34]. Individuals frequently responding with defeat reactions and the associated activation of the HPA axis may thus be more vulnerable to metabolic diseases and also to immunological disturbances.

Post-traumatic stress disorder (PTSD), which may be considered to be a decisive consequence of prolonged defeat reaction in man, is frequently comorbid with the metabolic syndrome, and particularly if the patients also suffer from depression [44]. Furthermore, several studies indicate that people with severe PTSD show a dysregulation in HPA-axis function. Briefly, glucocorticoid levels are usually considerably lower in PTSD patients than in healthy controls, believed to be a result from an enhanced negative feedback by glucocorticoids, which is secondary to an increased sensitivity of glucocorticoid receptors in target tissues [45].

One distinct difference between the defeat and defence reactions, seen in e.g. animal experiments is that gonadotrophins and testosterone as well as growth hormone are suppressed in defeat reactions, while they are increased in defence reactions [46]. In humans, the pictures are often far more complex and data concerning the effects on sex steroids are not consistent, which may reflect that in complex modern life the defence and defeat reactions can frequently shift from the one-to-the-other as daily life situations change from day to day. However, animal experiments are commonly so designed as to promote “the-one” or “the-other” in an “either-or” fashion to simplify analyses.

11.5 The Complexity of Defence and Defeat Reactions in Modern Man

Translating the defence and defeat reactions to humans and life challenges in today’s society is far from easy, as in reality present-day man can be faced with many situations even during the same hour, causing shifts between defeat or defence reactions. While the so-called freezing reaction or playing dead reaction, as explored in animals [3] (the equivalents of vigilance response and emotional fainting in man) usually are very brief (seconds or minutes), defence reactions, and particularly defeat reactions, can in modern life be quite sustained and frequently induced, though usually in mild forms. Even though their physiological expressions differ considerably, defence and defeat reactions are in man’s situation often “blended” in various constellations, depending on situation, earlier experience, genetics, etc. For such reasons the net outcome in man can be complex, indeed, and quite intricate to analyse, and this was in fact also the case in Henry’s mice colonies.

In today’s modern society, millions-of-bits of information reach the brain each minute, though, thanks to blissful inhibitory mechanisms at subcortical and cortical levels, consciousness is not overwhelmed by such bombardments. In fact, recent developments in neuroscience suggest that stimulating the brain through training of
e.g. memory is generally positive as there is evidence for cognitive and neural plasticity across the adult life span [47]. Modern society, including the growing development of technology and the amount of information available, can thus in some sense be stimulating for the brain, given that the individual will be able to emotionally cope with this situation and organize the amount of available information and stimuli. One example of this is the use of computer game task in children with attention-deficit/hyperactivity disorder (ADHD) to train working memory which also reduces the symptoms of hyperactivity and inattention [48].

11.6 Concluding Remarks

The physiological response patterns to emotional and environmental stimuli are phylogenetically very old. These response patterns, including the defence and defeat reactions are activated repeatedly, though usually in mild form, on every day basis in modern society. This calls for an almost continuous engagement of emotional and behavioural responses, but fortunately the highly developed neocortex is usually able to cope with such situations.

In reality, distinguishing between the activation of the defence and defeat reactions in modern life is difficult, as man can be faced with many situations even during the same hour, causing shifts between defeat or defence reactions. Since the physiological expressions differ considerably between these reactions, the net outcome can sometimes be difficult to interpret, including measurements of the biological responses to psychosocial stress. This could partly explain the discrepancy in the literature regarding measurements of biological markers of psychosocial stress and health outcome such as immune function.

Our brain is bombarded with millions-of-bits of information each minute, though, thanks to blissful inhibitory mechanisms, consciousness is usually not overwhelmed by this. Modern research on brain plasticity has also revealed the brain’s remarkable ability to adapt and modify its structure, and this is probably one of the key explanations why the brain has adapted quite well to modern society. However, due to sustained environmental stimuli in combination with vulnerability and maybe most importantly lack of recovery, quite-a-few individuals can develop emotional problems, trying to cope with the stream of challenges in modern, hectic life. Such situations invite to more or less sustained engagements of the defence and/or defeat reactions, with consequences of both mental and somatic disturbances. To this comes, quite often, unhealthy life style including inactivity and too high caloric intake, which further increases the risk of cardiovascular, metabolic and/or immunological disorders in various constellations, as outlined above.

After all, from a strictly biological point-of-view, modern hectic life is often on collision course with how the genetic code of our species, via the tough principle of “survival of the fittest” is organized. To paraphrase the colourful metaphor of the outstanding exercise physiologist, P.O. Åstrand, the long history of our species can be compared with a 42 195 meter long marathon run, where our own run can be considered to start when the first primitive tools were produced. In such a perspective, agriculture is
like the final 150 meters of the run, the “machine age” like the last few meters and present day “computer life” like the last decimeters (unless it should be written in UK-English?). Considering this, it is perhaps not so strange that neocortical abilities and paleocortical instincts and emotions sometimes come in conflict, resulting in major consequences such as mental disturbances and associated somatic disorders.

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References


Section F

Behavior

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12 Cytokines, Behavior, and Affective Disorders

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12.1 Introduction

For a long time the brain was considered an immunologically privileged site, having a reduced responsiveness to immune challenges. Evidence for this viewpoint originated from observations that the brain lacks an adequate lymphatic system for the capture of antigens, is protected from the general circulation by the blood–brain barrier, and was thought not to exhibit the classic inflammatory response, which is characterized by early invasion of macrophages and leukocytes. However, more recent developments in the field of neuroimmunology have challenged this idea [1], and it is now established that the brain can exhibit many of the classic signs of inflammation following infection or injury. These include edema, activation of microglia, local invasion of circulating immune cells, and cytokine production.

Before considering a possible role for cytokines in influencing behavior, one must consider how cytokines might reach their central sites of action, particularly as, under normal conditions, cytokines produced in the periphery cannot readily cross the blood–brain barrier. Four different mechanisms have been proposed [2]. (1) Passive transport into the brain at circumventricular sites lacking a blood–brain barrier (e.g., the organum vasculosum of the lamina terminalis [OVLT]). (2) Binding of cytokines to receptors in the cerebral vascular endothelium, followed by the induction of prostaglandin and nitric oxide synthesis. These, in turn, can exert a direct influence on the brain. Cytokines may also affect the blood–brain barrier by the induction of cell adhesion molecules, such as intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in the central nervous system (CNS) endothelium, thus increasing the potential for circulating T lymphocytes, especially CD4+ T lymphocytes, to cross the blood–brain barrier. (3) Uptake into the CNS, via a carrier-mediated, saturable transport system. Specific uptake mechanisms, for example for interleukin (IL)-1α and IL-1β, have been demonstrated at the luminal surface of the blood–brain barrier. (4) By direct activation of peripheral (vagal) afferent terminals following local release. For example, hepatic macrophages can release cytokines and other pro-inflammatory mediators following challenge with bacterial lipopolysaccharide (LPS), and these mediators can then activate adjacent sensory paraganglia of the
hepatic vagus nerve. Afferent stimuli can thus reach the nucleus tractus solitarius, via the dorsal nucleus of the vagus in the brainstem. The former projects to the hypothalamus and hippocampus, via ascending noradrenergic pathways.

Pro-inflammatory cytokines can also be synthesized by microglia, astrocytes, and blood vessels within the brain, either constitutively or following an immune challenge [3,4]. Furthermore, various parts of the brain, including the hypothalamus and hippocampus, express mRNAs for pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, and TNF-α [5,6]. Specific neuronal binding sites for cytokines are present in brain regions such as the area postrema, hypothalamus, amygdala, hippocampus, and prefrontal cortex. Thus, cytokines can have a direct effect on neuronal function and are ideally placed to play a role in modulating behavioral as well as autonomic and neuroendocrine responses [7,8].

Specific neurochemical changes occur within the brain in response to an immunological challenge or following the administration of pro-inflammatory cytokines. Systemic or intracerebral administration of IL-1 can enhance the turnover of noradrenaline in the hypothalamus and hippocampus, 5HT in the hippocampus and prefrontal cortex, and dopamine (DA) in the prefrontal cortex [9–12]. Peripheral administration of IL-2 produces an increase in noradrenaline metabolism in the hypothalamus and hippocampus and of DA metabolism in the prefrontal cortex and striatum. However, IL-2 does not affect 5HT function [12], although it inhibits acetylcholine release from the hippocampus and frontal cortex [13]. Furthermore, IL-2, unlike IL-1, does not significantly increase plasma corticosterone concentrations. Chronic IL-2 treatment also produces neuronal loss and degenerative changes in the hippocampus and a reduction in memory [14]. IL-6 increases DA and 5HT turnover in the hippocampus and prefrontal cortex, without affecting noradrenaline in the hypothalamus [12]. However, noradrenaline itself can stimulate astrocytic IL-6 release, raising the possibility that noradrenaline released during activation of the autonomic nervous system will activate the cytokine cascade [15]. There are many similarities between the neurochemical changes evoked by IL-1 and IL-6 in the brain. For example, both cytokines enhance 5HT turnover in the hippocampus and prefrontal cortex, and increase the 3,4-dihydroxy-phenyl acetic acid (DOPAC:DA) ratio in the latter region. However, IL-6, unlike IL-1, does not affect hypothalamic noradrenaline (NA) or plasma corticosterone concentrations [9,12]. The acute release of tumor necrosis factor (TNF)-α stimulates noradrenaline secretion, whereas chronic release inhibits it, for example, in the median eminence [16]. Thus, pro-inflammatory cytokines can differentially affect neurochemical function in brain regions that mediate neuroimmune interactions and that are important in the regulation and integration of the behavioral and neuroendocrine responses to physical and psychological stressors.

12.2 Cytokines and Sickness Behavior

Following exposure to an immune challenge, pro-inflammatory cytokines induce a series of symptoms that are known collectively as sickness behavior. These are adaptive behaviors which, together with fever and the associated neuroendocrine changes,
Cytokines, Behavior, and Affective Disorders represent a highly organized strategy designed to fight infection [17]. Sickness behavior also includes a range of neuropsychiatric effects, so it is not surprising that some researchers have proposed that cytokines may play a role in the etiology of certain psychiatric disorders, in particular the affective disorders (see Section 12.4). Studies on the neuroimmunological and behavioral profiles of animals suffering from infective and inflammatory disorders have demonstrated that these conditions give rise to a specific pattern of physiological responses (increased body temperature, increased slow-wave sleep, weight loss, decreased plasma iron and zinc) and behavioral responses (anorexia, decreased psychomotor activity, huddled posture, decreased sexual and exploratory behavior, social withdrawal) [18,19]. These responses are consistent and similar within and across species, indicating not only that, biologically, they have a fundamentally important adaptive function, but also that they are mediated by similar mechanisms. In elucidating the role of the pro-inflammatory cytokines in sickness behavior, attention has focused primarily on IL-1, IL-6, and TNF-α as well as the interferons IFN-α and IFN-γ.

Peripheral and central administration of IL-1β evokes all the components of the acute phase reaction, including the behavioral effects [20]. IL-1 injected into the rabbit brain has three distinct effects. Firstly, it has a somnogenic action, augmenting non-REM sleep and EEG slow-wave activity and inhibiting REM sleep in a dose-dependent manner [21,22]. Secondly, it is pyrogenic, inducing short monophasic febrile responses unrelated to its sleep-enhancing properties [23]. Thirdly, it can activate the hypothalamus–pituitary–adrenal (HPA) axis via a stimulant effect on corticotropin-releasing hormone (CRH), which is known not to be secondary to changes in body temperature because it still occurs with subpyrogenic doses of IL-1 [24].

Sickness behavior gives rise to behavioral responses that are traditionally interpreted as an inability to carry out normal activities due to debility. However, these responses have remained unchanged throughout evolution, and it seems reasonable to hypothesize that these behavioral and physiological mechanisms have developed to help fight infection and illness and to promote recovery. Until relatively recently, studies of behavior in sick animals have been comparatively few. Most have concentrated on which cytokines are behaviorally active and how their effects are exerted at the neuronal level. More recently, sickness behavior per se has been studied as an independent variable in order to determine the way in which it is organized and regulated. It is suggested that the behavioral changes accompanying an infective or inflammatory episode are expressions of motivational reorganization, geared specifically toward countering the invading pathogen(s). In this instance, motivation has been defined as “a central state that orientates the perception and actions of a sick animal” [25]. Indeed, cytokines decrease responses for rewarding intracranial self-stimulation, thus suggesting the possibility of an action on motivation and arousal. However, this was by no means conclusive evidence for a specific effect on motivation, as reduced responding could be due to a variety of other factors. An important feature of a motivational state is that it competes with other motivational states for behavioral output. This results in the normal expression of behavior having a hierarchical structure of motivational states, which is continuously updated according to the stimulus input. When a potentially life-threatening infection occurs, an animal
must adapt its behavior so that it is optimized for overcoming the disease. If a sick animal is exposed to danger, for example, it is still able to make the appropriate behavioral response to enable it to avoid the danger. Thus, fear-motivated behaviors can take precedence over sickness-motivated behaviors when necessary. Sick animals are not completely incapacitated or debilitated, and are still able to express complex behaviors and to evaluate accurately any situation to which they are exposed and to deal with it in an appropriate manner. Once this has been done, they will re-engage in recuperative behavior.

12.2.1 Cytokines and Body Temperature

Moderate increases in body temperature augment the immune response and are the result of a rise in the hypothalamic thermal set point evoked by a change in neuronal activity of the preoptic area. This effect is mediated by IL-1 and IL-6, which function as endogenous pyrogens. Mice deficient in either IL-1β or IL-6 are resistant to turpentine-induced fever and do not demonstrate the increases in circulating prostaglandin E (PGE)_2 that are normally noted in wild-type mice. These pyrogenic cytokines induce the expression of PGE_2, which acts as the final mediator in the initiation of fever. IL-6 is particularly important, as LPS (except at very high doses), IL-1β, and TNF-α do not evoke fever in IL-6-deficient mice, although this effect can be reversed by intracerebral administration of IL-6. Nevertheless, IL-6 knockout mice still have normal circadian body temperature variations. Conversely, IL-10 (an anti-inflammatory cytokine) inhibits the synthesis of pro-inflammatory cytokines and IL-10-deficient mice have a markedly enhanced pyrogenic response to LPS.

The fever that occurs following infection is an important means whereby the host combats infection. Fever increases the mobility and activity of neutrophils and the production and activity of interferon, which is itself pyrogenic. IL-1, an important initiator of the acute phase response, exerts a stimulatory effect on T lymphocytes, which is enhanced at febrile temperatures. Finally, fever, coupled with a decrease in plasma iron concentration, slows the rate of bacterial growth [26]. Reduced activity and somnolence (see Section 12.2.2) enable energy conservation that allows the full development of a fever that plays an essential role in recovery from infection. Thus, a body temperature ranging from 38°C to 40°C potentiates immune-cell function while diminishing the growth rate of numerous viral or bacterial pathogens. For example, rabbits exposed to infection have an optimal survival rate when their body temperature increases by 2°C. A metabolic increase of 13% is required to raise body temperature by 1°C, so it is not surprising that energetically expensive behaviors (such as locomotor activity) are reduced, thereby minimizing heat loss.

12.2.2 Cytokines and Sleep

Increased fatigue and somnolence are common features of the acute phase response. In animal models of infection, biphasic changes in non-REM sleep have been reported, followed by prolonged decreases lasting for more than 24 hours. Sleep is significant in host defense, and animals that exhibit enhanced sleep have fewer signs
of severe infection and a greater probability of survival. Muramyl dipeptides (the monomeric subunits of peptidoglycan that make up bacterial cell walls), endotoxin, and bacterial LPS can stimulate IL-1 release, which in turn produces somnolence. Plasma IL-1 concentrations reach a peak at the onset of slow-wave sleep in healthy individuals [27], and cerebrospinal fluid (CSF) concentrations of IL-1 increase during sleep [28]. TNF-α and IFN may also have somnogenic actions, although IL-2, IL-6, and TNF-β do not [22]. Failure to sleep during infection is associated with an increased severity of clinical signs and a poorer prognosis. Accordingly, animals that are chronically sleep-deprived tend to have a greater incidence of bacterial infection.

12.2.3 Cytokines and Ingestive Behavior

Loss of appetite during infection may seem to be maladaptive; it appears illogical that appetite should decrease at a time when the body is expending energy to increase body temperature. However, loss of appetite in infection is, in the short term, an adaptive behavioral response, although in the long term it may lead to malnutrition and excessive weight loss. In the wild, an animal must expend energy to obtain food, and in these circumstances loss of appetite allows the animal to conserve its energy, which is required to mount a fever and fight off infection. By remaining relatively immobile, an animal is able to curl up and conserve heat, which would otherwise be lost by convection. Finally, by not foraging an animal is less likely to encounter predators at a time when it is least able to defend itself.

The decrease in food intake may also facilitate decreases in the plasma concentrations of substances such as iron, which are important for bacterial growth. It is commonly believed that malnutrition compromises the immune system, but a controlled study, in which subjects underwent a loss of 20% of their original body weight over a 24-week period, found that these individuals did not exhibit an increased incidence or severity of infection (e.g., respiratory infection) compared with controls. Also, various reports from places where individuals may have been suffering from malnutrition, such as prisons, showed that the most malnourished individuals succumbed to fewer infections than their well-fed counterparts [29]. Studies in endotoxin-treated mice showed that those animals demonstrating the greatest degree of anorexia and weight loss in response to the infection were the least likely to die. Conversely, normalizing the caloric intake of infected animals by intragastric feeding actually increased mortality, with 93% of force-fed animals dying, compared with 43% given the same dose of LPS but allowed to regulate their own food intake. Central or peripheral administration of pro-inflammatory cytokines also reduces feeding, suggesting that these cytokines may play a role in anorexia. Bacterial LPS and IL-1β reduce operant food intake, a measure of food-seeking behavior. TNF-α knockout mice have normal circadian variations in food intake and body weight gain and develop obesity following the administration of a high-fat or high-calorie diet. In contrast, mice that overexpress TNF-α exhibited wasting and chronic inflammation, suggesting that this cytokine affects feeding during pathological conditions. Interferon therapy for chronic conditions such as cancer and multiple sclerosis is associated with a reduction in appetite and progressive weight loss. The effect of
IFN-γ is more marked after peripheral administration and is believed to be due to the secondary release of cytokines such as IL-1. Additionally, interferons are potent inducers of somnolence and fever, and it has been suggested that these neurobehavioral responses also contribute to the observed reduction in feeding. IL-6 is a major inducer of the acute phase response and has a direct central inhibitory effect on feeding. However, this effect is less marked than that of IL-1, interferons, or TNF-α, suggesting that IL-6 plays a contributory role in cytokine-induced anorexia rather than being a major mediator. Studies have shown that low doses of IL-1 administered centrally mainly affect meal size and duration but not frequency, whereas higher doses affect all feeding parameters. The effects appear to be specific, as they are blocked by co-administration of IL-1 receptor antagonist (IL-1ra) [30].

Cytokines cause anorexia by peripheral humoral mechanisms as well as direct action on the hypothalamus [31,32]. Although subdiaphragmatic vagotomy can attenuate the effects of peripheral LPS or IL-1β on fever, reduced social exploration, taste aversion, and other aspects of sickness behavior, there is little evidence for a major role of the vagus in anorexia [33]. There is some evidence that the anorexic effects of cytokines may be secondary to the release of cholecystokinin (CCK), glucagon, and insulin in the periphery [32]. IL-1 increases circulating CCK and decreases food intake and gastric emptying. However, recent evidence [34] indicates that the CCK<sub>A</sub> receptor antagonist, devazepide, did not block the anorectic action of IL-1β, thus failing to support a role for CCK in IL-1β-induced anorexia. Nevertheless, the effect of IL-1β in delaying gastric emptying is antagonized by the central administration of a CRH receptor antagonist [35].

Although cytokines may not be the sole factors responsible for the anorexia and weight loss noted in pathological conditions, it is interesting that effects on feeding and body weight are present in disorders involving chronic dysfunction of the immune system, such as cancer, AIDS, and inflammatory bowel disease. In humans, nausea and anorexia often accompany cytokine treatment, and patients with anorexia nervosa have been found to have one or more factors in their sera that can stimulate cytokine production.

12.2.4 Cytokines and Anhedonia

Experiments have also been undertaken to study the relationship between sickness and hedonic processes. Hedonism in animals is commonly assessed through preference and/or consumption of sweet solutions. The effects of LPS have been assessed on taste reactivity patterns in rats (specific tongue and mouth movements) to threshold and standard concentrations of saccharine, sucrose, and quinine. Reactivity patterns to quinine and sucrose were unaffected by LPS. A standard concentration of quinine (0.1 mM) evoked the same degree of aversion, and sucrose (90 mM) the same intensity of hedonic reaction, as they did in the absence of LPS. However, LPS altered the reactivity pattern to saccharine, in that treated rats showed fewer ingestive and more aversive responses to a standard (5 mM) concentration of saccharine compared with controls. (At a threshold concentration of saccharine, the responses of LPS- and saline-treated rats did not differ.) Although the results of this study support
the idea that there is a relationship between cytokines and sensory pleasure, they may also be interpreted as being due to an increase in “finickiness” rather than anhedonia. For example, LPS-treated rats had the same hedonic patterns in response to sucrose as controls, whereas animal models predictive of a reduction in hedonism (e.g., models of depression) are characterized by a decrease in sucrose preference and ingestion.

Feeding is regulated on the basis of the taste and composition of nutrients, and there is a significant correlation between the sweet taste of food and its calorific value, and between the bitter taste of food and its toxicity. The attraction to sweet nutrients and aversion to bitter food are particularly important for an omnivore such as the rat in terms of survival value, more so since the rat lacks the ability to elicit an emetic reflex. The fact that LPS-treated rats responded to quinine in the same way as controls could be interpreted as suggesting that sick rats are still able to reject bitter-tasting foods. Conversely, LPS-treated rats still respond with positive appetite to sucrose, suggesting that sickness does not interfere with the perception of hedonic (ingestive) value. However, saccharine has a mixture of quinine- and saccharine-like properties and LPS-treated rats will reject a concentration of saccharine that would normally be ingested by controls. This may be interpreted as an increased sensitivity of LPS-treated rats to the aversive component of saccharine and/or a decreased responsiveness to its hedonic component. This phenomenon would prevent sick animals from ingesting amounts of a potentially toxic compound that would normally be tolerated by healthy animals. This interpretation fits with Cabanac’s hedonic theory of motivation, whereby hedonism supports behavioral responses to useful stimuli and displeasure facilitates the avoidance of potentially dangerous stimuli [36].

12.2.5 Cytokines and Cognitive Function

Infection or treatment with pro-inflammatory cytokines, such as IL-1β or IFN-α, can adversely affect cognitive function in humans and animals. Central or peripheral administration of IL-1β can produce learning deficits by impairing retrieval of information; for example, impairing performance on spatial memory tasks and interfering with escape learning and enhancing fear conditioning. The latter can be blocked by intracerebroventricular (ICV) treatment with IL-1ra prior to exposure to inescapable foot shock [37]. Conversely, brain-derived neurotrophic factor (BDNF) in the hippocampus plays a positive role in cognition, and intrahippocampal IL-1β can attenuate the increase in BDNF mRNA in the hippocampal CA1, CA2, and dentate gyrus (DG) regions that is normally induced following contextual fear conditioning [38]. This effect of IL-1β may be direct, or may involve glucocorticoid effects, as the concentration of glucocorticoids is elevated following IL-1β administration, and the detrimental effects of IL-1β on memory may be prevented by co-administering the glucocorticoid receptor antagonist RU486 [39]. IL-6 may adversely affect memory processing, and IL-6 knockout mice exhibited a facilitation of radial maze learning compared with wild-type mice, in terms of smaller working memory errors, faster acquisition, and a higher percentage of animals attaining the criterion [40]. IL-2 also plays a role in cognition, and mice deficient in this cytokine exhibit defects in spatial learning and memory when tested in the Morris water maze.
12.2.6 Cytokines and Other Behaviors

Individual differences in the effects of cytokines or endotoxin on behavior have been reported. LPS and IL-1β inhibit sexual behavior in female but not male rats, an effect independent of cytokine influence on ovarian hormones [41–43]. Highly aggressive ICR mice increase their startle response to mild social investigation after LPS treatment, whereas mice from the low-aggression line show a decrease in startle amplitude [44]. IL-6-deficient mice are more aggressive in an inter-male aggression paradigm and also show absence of stress-induced analgesia, implying changes in the opioid system [45]. IL-6 knockout mice also have an increased susceptibility to the convulsant effects of N-methyl-D-aspartic acid (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate, suggesting that the excitatory amino acid system is more active in these animals compared with normal controls. This also raises the possibility that IL-6 knockout mice may be useful as a model for epilepsy.

The effects of peripherally administered IL-1 on sickness behavior are believed to be NF-κB dependent, as central administration of NF-κB inhibitor peptide blocks the sleep-inducing and pyrogenic effects of IL-1. However, following central administration, IL-1β activation of mitogen-activated protein kinase (MAP kinase) pathways seems to be involved in the neural effects of this cytokine.

12.3 Specific Disorders in Which Cytokines May Produce Behavioral Effects

The following subsections describe data relating to the behavioral effects of cytokines evoked either following an external immunological challenge, or in the presence of an inherent disorder of the immune system, as in autoimmune disease.

12.3.1 Acute Viral Infections

Upper respiratory tract infection, influenza, gastroenteritis, Epstein–Barr virus, and cytomegalovirus are associated with behavioral symptoms that include anorexia, lethargy, somnolence, irritability, poor concentration, reduced motor activity, and depressed mood. The impairment of cognitive function associated with influenza has been studied [46], and a similar effect has been noted following infusion of IFN-α in healthy individuals [47]. Cognitive impairment was also noted in subjects with laboratory evidence of influenza infection but an absence of other clinical symptoms. The time course of the neuropsychiatric symptoms noted subsequently in acute viral illness parallels that of physiological changes such as fever and somnolence.

12.3.2 Recurrent and Chronic Viral Infections

Herpes zoster and chronic hepatitis B are examples of recurrent and chronic viral infections that are also associated with neuropsychiatric symptoms. Patients with recurrent herpes zoster infections commonly report the onset of symptoms such as
lethargy and depressed and irritable mood 24–48 hours prior to recurrence of skin lesions. Thus, patients’ neuropsychiatric symptoms parallel their response to immunological challenge, of which cytokine production is an integral part. Patients treated with IFN-α for chronic viral hepatitis or malignant diseases also exhibit depressive symptoms.

### 12.3.3 Postinfective Fatigue and Chronic Fatigue Syndrome

Chronic fatigue syndrome (CFS) typically follows relatively minor influenza-like or mononucleosis-like illnesses. It is characterized by the presence of chronic and disabling fatigue, in the absence of neurophysiological evidence of actual muscle fatigue or neuromuscular abnormalities [48]; and by neuropsychiatric symptoms such as sleep disturbance, impaired concentration and memory, mood changes, headaches, and musculoskeletal pain [49,50]. It manifests many of the symptoms of atypical depression, and 21–47% of CFS patients subsequently experience major depressive episodes [51]. Although extensive studies have been carried out on immunological, neuroendocrine, neurological, and psychological function in this disorder [49,52], it remains to be resolved whether this is a nonspecific disorder which is present during different disease states, or whether particular subsets of CFS with specific pathophysiological characteristics can be identified [50,53]. Of particular relevance to the possible involvement of cytokines in this disorder are findings that the symptoms of CFS are similar to those of sickness behavior or the side effects noted during the therapeutic administration of cytokines [19,54]. Many patients exhibit CFS following an infective illness, and there is evidence that in these individuals there is a persistent low-level activation of the immune system. This includes a reduction in delayed hypersensitivity skin responses, impaired lymphocyte responses to mitogens, depressed natural killer (NK) cell activity, increased activity of activation markers, and the presence of autoantibodies to insoluble cellular antigens [49,55–57]. It has therefore been suggested that the symptoms of CFS are due to enhanced CNS cytokine production and release in genetically predisposed individuals in response to antigenic challenge [49,58]. However, studies involving the determination of absolute concentrations of cytokines in the sera have failed to detect any differences between patients with CFS and controls [58–61]. Nevertheless, studies on the release of cytokines from mononucleocytes in vitro have demonstrated increased IFN-α production in response to viral antigen [62] and increased endotoxin-stimulated release of IL-1β, IL-6, and TNF-α in patients with CFS [58]. It has also been suggested that the neuropsychiatric symptoms noted in CFS could be due to altered cytokine production by glial cells in the CNS. However, this evidence was obtained from patients who already had well-documented neuropathology, including those with multiple sclerosis, HIV infection, and Alzheimer’s disease.

### 12.3.4 Systemic Autoimmune Diseases

Systemic autoimmune diseases are often associated with neuropsychiatric phenomena. However, evidence that these behavioral effects may be due to direct CNS pathology
must not be ignored. For example, patients with systemic lupus erythematosus (SLE) may have cerebral vasculitis, production of autoantibodies with cross-affinity for neural tissue resulting in cerebral inflammation and/or tissue damage, and destruction of the integrity of the blood–brain barrier. Nevertheless, neuropsychiatric symptoms have also been reported in disorders not typically associated with direct cerebral involvement (e.g., Sjogren’s syndrome), and where other evidence of cerebral involvement is lacking (e.g., absence of abnormalities seen on EEG or with enhanced neuroimaging techniques). It is, however, pertinent to note that autoimmune disorders are overrepresented among groups of patients with treatment-resistant depression [63].

12.4 Cytokines and Affective Disorders

The potential link between immune alterations and affective disorders has been recognized for many years. Early work in the field of psychoneuroimmunology tended to concentrate on whether psychological stress or psychiatric disorders such as depression could result in changes in humoral or cell-mediated immunity, and the possible mechanisms underlying such occurrences. Indeed, there is significant evidence to suggest that psychological stress or depression can directly stimulate the production of pro-inflammatory cytokines that influence a range of conditions, particularly those that are more prevalent during aging, such as cardiovascular disease, osteoporosis, arthritis, type 2 diabetes, certain types of cancer, and functional decline. Depression can also down-regulate the cellular immune response; as a consequence, processes, such as prolonged infection and delayed wound healing, that fuel sustained production of pro-inflammatory cytokines may be promoted. It is not surprising, therefore, that antidepressant drugs have been shown to possess anti-inflammatory effects [64,65]. It has now become accepted that CNS–immune system interactions are in fact mutual and that the converse also applies: namely, that disturbances of immune function may play a role in the etiology of psychiatric disease, including depression [66]. For example, a mild, non-sickness-inducing inflammatory stimulus (salmonella typhi vaccine) can evoke negative mood changes in healthy individuals, which are correlated with increases in IL-6 production [67]. Furthermore, neuropsychiatric symptoms such as psychomotor slowing, subjective fatigue, and subjective cognitive impairment are common not only in major depression but also in specific infective and autoimmune disorders, such as lupus, scleroderma, and Sjogren’s syndrome, thus suggesting that these symptoms may be due to a common central action of cytokines. Endogenous psychoses also show several other parallels with autoimmune diseases, including early onset, genetic predisposition, waxing and waning of symptoms, and (in the case of depression) sex ratio.

12.4.1 Stress and Anxiety

Experimental and epidemiological data have revealed that psychological and physical stresses are capable of altering the function of immunocompetent cells [68,69], although the precise nature of the alteration depends on the type and severity of the
stress, whether it is acute or chronic, and whether the individual is perceived to have any degree of control over it. The effects are also dependent on the tissue and particular cytokine under investigation. Acute psychological or physical stress produces an increase in CD8 T lymphocytes and NK cells and a decrease in B lymphocytes, and increases the stimulated production of TNF-α and IL-6 [70,71]. Individuals exposed to chronic stress have fewer circulating B cells, T cells, and granulocytes, as well as a reduced capacity for mitogen-induced lymphocyte proliferation and decreased NK cell activity compared with controls [72–74]. However, there are some inconsistencies. For example, patients with panic disorder or social phobia have increased NK cell numbers, and the former group also has increased numbers of B lymphocytes and human leukocyte antigen-D-related (HLA antigen-DR)-presenting cells. Subjects who show clear signs of anxiety during an academic exam have significantly higher IFN-γ, IL-1ra, and soluble interleukin-6 receptor (SIL-6r) levels in the serum compared with those with low anxiety scores [75]. However, results from another study demonstrated decreased IFN-γ production accompanied by increased IL-10 expression during exam stress [76]. Interestingly, treatment of neonatal male rats with endotoxin, on postnatal days 3 and 5, can alter anxiety behavior in adulthood compared with vehicle-treated controls. These observations underlie the importance of early life exposure to immunological challenges in the development of emotional behavior, and further suggest that neonatal infection may be an important predictor of susceptibility to anxiety-related disorders in adulthood [77].

12.4.1.1 Depression

One of the most consistent abnormalities in patients with major depression is dysregulation of the HPA axis [78,79]. Basal plasma cortisol or adrenocorticotropic hormone (ACTH) concentrations tend to be normal or slightly increased. However, there is a significant increase in 24-hour urinary cortisol excretion [80] and in CSF CRH concentrations [81]. Elevated CSF CRH and plasma cortisol concentrations have been reported in drug-free patients with major depression, compared with healthy subjects or those with other psychiatric disorders, whereas these values were within the normal range for depressed subjects treated with the antidepressant fluoxetine. Postmortem studies have shown elevated CRH concentrations and CRH mRNA in the hypothalamic paraventricular nucleus (PVN) of depressed patients. Impaired glucocorticoid negative feedback control of the HPA axis exists in a significant proportion of depressed subjects, as evidenced by nonsuppression of cortisol secretion after dexamethasone administration [82]. A reduction in the number of glucocorticoid receptors (GRs) in the CNS has also been noted in depressed patients.

Pro-inflammatory cytokines such as IL-1 and IL-6 are potent stimulators of CRH synthesis and thus are activators of the HPA system. As CRH hypersecretion is often present in major depression, it may be hypothesized that HPA dysfunction in these patients may be a consequence of an increase in the release of these cytokines. Conversely, in accordance with the bidirectional nature of CNS–immune system interactions, the chronically increased CRH drive can itself influence the production and action of central cytokines [83].
The therapeutic use of cytokines has been associated with a range of dose-dependent neuropsychiatric symptoms, including symptoms similar to those noted in depressive disorders: fatigue, altered sleep patterns, irritability, loss of appetite, weight loss, low mood, anhedonia, apathy, and mental retardation [84,85]. These symptoms are most common in patients treated with IFN-α, occurring in up to 36% of cases, and are often of sufficient severity to necessitate discontinuation of therapy or the co-administration of antidepressant drugs. In rarer cases, IFN-α may cause psychosis, delirium, and persistent manic-depressive illness, the recurrence of post-traumatic stress disorder (PTSD) or suicidal behavior. The severity of the observed effects tends to be related to the dose and duration of treatment, as well as to individual differences between patients, including previous psychiatric history. Although it is possible that those patients who became depressed during the course of cytokine therapy were predisposed to this condition, the neuropsychiatric symptoms disappeared upon cessation of treatment. In animals, cytokines also evoke anhedonia, anorexia, a decrease in social behavior, psychomotor retardation, altered sleep patterns, and impaired cognitive function. Some of these alterations in behavior are associated with HPA overactivity and glucocorticoid resistance.

These changes are similar to those noted during chronic immune activation, and it has been difficult to distinguish what is due to “sickness” from what may be due to specific psychiatric effects. However, this distinction has been achieved for two of the symptoms, namely anhedonia and helplessness. Anhedonia (the inability to experience pleasure) is one of the essential features noted in major depressive episodes with melancholia. It can be demonstrated experimentally in animals by attenuated responses to rewarding intracranial self-stimulation (ICSS), a reduced preference for sweet solutions, and an impairment of sexual behavior. Interleukin-1β, TNF-α, and IL-2 can all cause anhedonia in rats and mice [86,87]. In experimental animals, LPS administration, which induces production of pro-inflammatory cytokines and IL-6, also induces anhedonia, as evidenced by disrupted sexual behavior in female rats, decreased response to rewarding lateral hypothalamic self-stimulation, and a decreased consumption of saccharine [88]. The latter effect could be attenuated by chronic imipramine treatment [89]. It is also pertinent that the nonsteroidal anti-inflammatory drug diclofenac attenuates LPS-induced reductions in reward behavior, as we know that cytokines can induce prostaglandin synthesis [90]. Helplessness, another key symptom of depression, can be studied in animals by using a “learned helplessness” behavioral model. This relates to learning the lack of contingency between behavior and its consequences and occurs, for example, when animals are exposed to a situation from which they cannot escape. Both LPS and IL-1 have been found to increase immobility time in two such models, the forced swim test and during exposure to inescapable electric shocks. The effects of IL-1 in the latter test are abolished by administration of IL-1ra, thus emphasizing the importance of this cytokine in mediating these behavioral effects [91].

The activation of inflammatory, autoimmune, and cell-mediated immune responses may accompany depression. Increased plasma concentrations of positive acute phase proteins, and a reduction in the levels of negative acute phase proteins, have been noted. As well as the presence of an acute phase response, enhanced
expression of antinuclear and antiphospholipid autoantibodies may indicate an auto-
immune response. The main findings that indicate activation of cell-mediated immu-
nity are those that show an increased number of T helper cells (CD4+), T memory
cells (CD4+CD45RO−), activated T cells (i.e., CD25 + T and HLA-DR + T cells),
and B cell subsets, as well as an increased ability of monocytes and lymphocytes to
produce cytokines such as IL-1β, IL-2, IL-6, IFN-γ, and TNF-α, all of which have
been noted in patients with depression [92–98].

Although it has been suggested that the increase in pro-inflammatory cytokines
in patients with major depression correlates with illness severity and indices of HPA
hyperactivity [99,100], these observations have not always been replicated by oth-
ers [101,102]. Furthermore, some authors consider the possible association between
cytokines and depression to be highly speculative [103]. Most authors have, how-
ever, confirmed that there are increases in the plasma concentrations of acute phase
proteins [104]. This observation, along with reports of mild leukocytosis, neutro-
philia, and elevated C-reactive protein and components of the complement system
[105], suggests the presence of a mild inflammatory response in depression, possi-
bly initiated by cytokines. Functional markers of cellular immunity have been stud-
ied in patients with major depression (MD), with or without melancholic features.
Patients who had MD with melancholia produced significantly less IL-2 than did
MD patients without melancholia; the latter did not differ from normal subjects.
Melancholia patients also showed reduced IFN-α and IL-10 production. IL-12 levels
of both groups with MD were higher than the control group, and decreased signifi-
cantly after 8 weeks of antidepressant treatment [106]. IFN-γ is produced in larger
amounts by lymphocytes of patients with MD than by those of healthy subjects.
The higher plasma IFN-γ concentrations in such patients are accompanied by lower
plasma tryptophan availability [97]. This occurs because IFN-γ is capable of induc-
ing the enzyme indoleamine 2,3-dioxygenase, which converts tryptophan to kynure-
nine. The reduced plasma tryptophan concentrations will ultimately lead to reduced
synthesis of 5HT in the CNS. Pro-inflammatory cytokines also up-regulate the 5HT
transporter, causing a reduction in extracellular 5HT concentrations. These effects on
central 5HT, coupled with effects on noradrenergic and HPA function, could underlie
the depressogenic actions of the pro-inflammatory cytokines. Conversely, the anti-
inflammatory cytokine IL-4 reduces 5HT uptake.

12.4.1.2 Effects of Antidepressants on Immune Function

If cytokines are involved in the etiology of depression, then cytokine antagonists
should have antidepressant actions. Similarly, the antidepressant drugs that are used
clinically should be able to modify the production and/or actions of cytokines. The
former possibility has not been tested extensively, but it is known that centrally
administered IL-1 antagonists can block learned helplessness [91] and can reduce
cytokine-induced decreases in social exploration [107].

The effects of antidepressant drugs on immune function have been studied in
more detail. These have been shown to have an inhibitory effect on the release of pro-
inflammatory cytokines from activated monocytes and macrophages, and to enhance
the expression of anti-inflammatory cytokines [108,109]. Repeated administration of imipramine in the rat reduced the ability of splenocytes to produce IL-1 and IL-2 following an 8-week exposure to unpredictable mild stress [110]. Imipramine, clomipramine, and citalopram can also suppress IL-2 production by stimulated lymphocytes, and IL-1β and TNF-α by stimulated monocytes. Single or repeated administration of desipramine (10 mg/kg) to naïve mice increased the ability of splenocytes to produce the anti-inflammatory cytokine IL-10 [111,112], and repeated administration of imipramine induced IL-1ra mRNA in the rat brain [113]. Human subjects with PTSD and depression had significantly greater IL-1r and lower IL-2r levels compared with controls. These values were normalized after treatment with the selective serotonin reuptake inhibitors (SSRIs) citalopram and sertraline [114]. Inhibition of IL-6 release from monocytes but increased release from lymphocytes has been reported following incubation with antidepressants such as imipramine, clomipramine, and citalopram. It is thus possible that these suppress IL-6 production by monocytes but increase its production by lymphocytes. The cells involved in IL-6 production possess a range of functional receptors, including those for 5HT, and changes in receptor function/density induced by alterations in neurotransmitter concentrations may subsequently affect their activity [115]. For example, an association between activation of 5HT2B/2C receptors and an increase in IL-6 production has been shown [116]. Thus, it may be speculated that increases in available concentrations of 5HT and in the density of 5HT receptors, induced by antidepressants such as fluoxetine, may play a role in the increased production of this cytokine. These findings must nevertheless be interpreted cautiously, as only a small number of patients were studied and data from the depressed patients before and after successful fluoxetine treatment were not available.

In vitro incubation of whole blood from healthy subjects with SSRIs such as fluoxetine and sertraline, the selective noradrenaline reuptake inhibitor venlafaxine, tricyclic antidepressants (imipramine, clomipramine), a reversible monoamine oxidase inhibitor (moclobemide), and L-5HTP significantly increased the production of IL-10 and/or decreased that of IFN-γ, thus decreasing the ratio of IFN-γ to IL-10 [117,118]. In addition, the 5HT system can inhibit IFN-γ-induced major histocompatibility (MHC) expression [119] and mitogen-induced T-cell proliferation [120]. SSRIs also have inhibitory effects on acute phase protein production [121] and on immune system activation in general [122,123]. More recently, in vitro studies have been carried out using whole blood from fluoxetine-treated patients with treatment-resistant depression, age-matched healthy controls, and younger healthy volunteers, following stimulation with phytohemagglutinin (PHA) and LPS for 48 hours, with or without antidepressants. The main findings were that imipramine, venlafaxine, 5-HTP, and a combination of 5-HTP and fluoxetine increased IL-6 production, whereas none of the drugs affected TNF-α. The production of IL-6 was higher in depressed patients than in the age-matched controls, whereas TNF-α production was significantly higher in the older volunteers than in the younger subjects [124]. The observed stimulant effect of antidepressants on IL-6 production does not negate the hypothesized relationship between the therapeutic activity of antidepressants and their immunomodulatory effect, as IL-6 can inhibit the secretion of the two major pro-inflammatory cytokines TNF-α and IL-1, both of which have established depressogenic effects, and can enhance 5HT neurotransmission in several brain regions, which is also of benefit
in depression. Fluoxetine has also been shown to suppress IFN-\(\gamma\) production in human whole blood. Clinical studies suggest that all classes of antidepressants can exert immunosuppressant effects, as they reduce the production of pro-inflammatory cytokines such as IL-1, TNF-\(\alpha\), and IFN-\(\gamma\), and stimulate the production of negative immunoregulators such as IL-10 and IL-1ra.

However, in some animal models only the tricyclic antidepressants, and not the SSRIs or venlafaxine, normalize changes in immune function by an action on TNF. These differences may be due to the limitations of the animal models of psychiatric disorders. Nevertheless, chronic antidepressant treatment abolished LPS-induced anhedonia in rats [87], an effect believed to be due to a reduction in the capacity of monocytes and macrophages to produce pro-inflammatory cytokines.

More recently, it has been shown that IL-6 is necessary for the antidepressant action of Hypericum perforatum (HP; St. John’s wort), and that activation of CNS 5HT metabolism following HP treatment may be attributed to increased IL-6. HP extract reduced immobility in a model of depression (forced swim test) in wild-type, but not IL-6 knockout, mice. It also induced significantly higher levels of 5HT and 5HIAA in the diencephalon of wild-type mice compared with IL-6 knockout mice [125].

Thus, evidence obtained with a wide range of antidepressant drugs and from depressed subjects, as well as from healthy volunteers, supports the contention that antidepressant drugs are able to affect cytokine production and that altered cytokine concentrations may be related to depressive illness. However, there is a paucity of data relating to predepression cytokine concentrations in patients, and it is difficult to determine which came first, the depression or the elevated concentrations of pro-inflammatory cytokines. Studies involving the use of cytokines in immunotherapy appear to lend support to the idea of a role for cytokines in the etiology of depression. However, the doses used for immunotherapy are high and in excess of concentrations that may be achieved endogenously. Furthermore, such patients are already seriously ill, and the effects of severe stress caused by the presence of the illness cannot be ruled out. It is known that stressors can influence pro-inflammatory cytokine function, and it is therefore possible that the altered cytokine activity observed in depression may be secondary to an increase in stress or stress perception.

Indeed, all subtypes of depression are associated with an increase in stress and/or stress perception, as well as excessive emotionality, reduced efficiency of coping strategies, diminished quality of life, anhedonia, reduced interpersonal skills, and reduced social buffering. Thus, cytokine changes may be secondary to the real or perceived stress associated with the disease. Additionally, comorbid features (e.g., anxiety, personality disturbances, and self-neglect [126]) are often present in depression, which could influence cytokine concentrations. At present, the correlative relationship between cytokines and depression requires further investigation, but it is possible that the elevated cytokine concentrations contribute to symptoms such as somnolence, muscle fatigue, and reduced food intake that are noted in this condition. There is a consensus of opinion that depression is characterized by changes in neuroendocrine and immune system function. Novel antidepressants that affect the central corticotropin system and HPA activity, the CRH-R1 antagonists, are already undergoing clinical trials. It seems worthwhile, too, to develop antagonists of pro-inflammatory cytokines (e.g., IL-1 antagonists) that would reduce HPA hyperactivity
and alleviate depressive symptoms. It may also be beneficial to attempt the opposite approach: namely, to develop compounds that increase the production of the anti-inflammatory cytokines.

12.4.1.3 Schizophrenia

Schizophrenia comprises several disease entities, some of which are as yet undefined. However, there is a substantial degree of heterogeneity among them, which indicates the existence of overlapping etiological factors. The disorder manifests itself in the form of abnormal mental function and disturbed behavior. These features usually appear in the second or third decades of life, as a heterologous group of clinical symptoms. Positive symptoms include psychotic symptoms such as delusions, hallucinations, and thought disorganization. Negative symptoms include loss of motivation and flattening of affect. Disturbances in basic cognitive functions such as attention, executive function, and specific forms of memory are also consistently noted, and are now thought to be central to the behavioral disturbance and functional disability that occur in this illness. Additionally, a number of patients have concomitant mood symptoms, including depression and anxiety, which may contribute to the relatively high (10%) lifetime incidence of suicide in schizophrenics.

Early work on the possible association between schizophrenia and autoimmune disorders [127–130] lost favor to the more widely accepted dopamine hypothesis of schizophrenia [131]. Subsequently, an attempt was made to integrate the two, working with the suggestion that the disorder might result from dopamine receptor-stimulating autoantibodies [132]. Several inadequacies of the dopamine hypothesis have now become evident; for example, the facts that the negative symptoms are not ameliorated by classical (“typical”) neuroleptics and that some groups of patients remain treatment resistant.

Alterations in immune system function have long been known to exist in this disorder, and signs of inflammatory disease processes have been observed in treatment-resistant schizophrenic patients [133]. In this group, paranoid or negative symptoms [134,135], disease acuity [136,137], and drug treatment [138–141] were found to influence immunological parameters. Furthermore, infection during pregnancy in mothers whose offspring subsequently develop schizophrenia has been postulated to explain the observation that a statistically significant proportion of schizophrenic patients have birth dates between December and May (seasonality of birth). Exposure to viral or bacterial infection of the CNS in childhood leads to a fivefold increase in the risk of developing schizophrenia in later life.

The suggestion that IL-2 plays a role is supported by the observation that 65% of nonschizophrenic subjects treated with IL-2 exhibited symptoms such as delusions, severe cognitive impairment, and some degree of affective change [142]. However, in vitro lymphocytes from schizophrenic subjects show a reduction in mitogen-stimulated IL-2 production [143,144], which appears to correlate inversely with the negative symptoms and to be associated with a lower age of disease onset [145]. The decreased production of IL-2 in vitro could reflect the increased production of IL-2 [146]. However, it might also be the consequence of a reduced capacity of lymphocytes to produce IL-2. The soluble IL-2 receptor (SIL-2r) has also been studied
in schizophrenia. Concentrations were found to be increased and associated with early age of onset and negative symptoms of the disease. Similarly, levels of IFN-γ in blood from schizophrenics are dependent on the type of psychopathology present. Patients having mainly positive symptoms (delusions, hallucinations, bizarre behavior, and thought disorder) show evidence of increased levels of IFN-γ, whereas those with negative symptoms (asocial behavior, withdrawal, flattened affect, impaired attention, and apathy) exhibit a decrease in IFN-γ production [147–149].

Elevated plasma concentrations of IL-6, which parallel the duration of the disease, have also been noted [150–152]. More recently, Garver et al. [153] assessed differences in IL-6 concentrations in the CSF of two distinct subgroups of schizophrenic patients (delayed responders and poor responders) during neuroleptic-free periods in which their psychotic symptoms were present. These were subsequently compared with the data from a group of normal subjects. Interleukin-6 concentrations in the CSF were found to be significantly higher in the delayed responders compared with the poor responders and the controls, thus indicating that immune activation is present in patients suffering from a distinct subtype of schizophrenia. Furthermore, high SIL-6r levels in the serum and CSF are correlated with paranoid-hallucinatory symptoms [141]. These studies indicate that abnormalities of the IL-6 system tend to be associated with an unfavorable course of the disease, with respect to duration, treatment resistance, or more marked paranoid-hallucinatory symptomatology. IL-6 is a product of macrophage-monocyte activation and also of activation of the Th2 system [154]. Supporting the suggestion that this system is activated in schizophrenia are observations that levels of other cytokines produced by these cells, such as IL-10 and IL-4, are also altered. For example, an increase in IL-10 concentrations related to negative symptoms has been reported [155,156]. Additionally, an increase in CSF IL-4 levels has been reported in juvenile schizophrenics [157]. Increased levels of immunoglobulin E (IgE), a sign of an increased Th2 response, were found in schizophrenic patients compared with controls [158]. Thus, it has been postulated that in schizophrenia there is a reduction in activity of the Th1 (cellular immune) system and an activation of the reciprocally linked Th2 (humoral immune) system [159]. This shift is particularly evident in patients having predominantly negative symptoms and/or a poor therapeutic outcome [160]. However, other investigators [149] have concluded that the deficient production of Th1 cytokines in schizophrenia is not due to either a change in the number of immunocompetent cells or to counter-regulation of the Th2 cytokine IL-10. Studies concerning levels and/or production of IL-1 and TNF-α in patients with this disorder have so far been inconclusive.

In vivo, the pro-inflammatory cytokines can significantly inhibit cortical neuron dendritic development in a manner consistent with the neuropathology noted in schizophrenia [161]. Furthermore, a study [162] examining the effect of peripheral cytokine challenge on neurobehavioral development demonstrated that IL-1α, IL-2, IL-6, and IFN-γ all affect physical development. This is accompanied by behavioral changes related to fear/anxiety levels and in sensorimotor gating at different stages of development, suggesting that challenge with pro-inflammatory cytokines during early postnatal development could give rise to future psychobehavioral and/or cognitive impairments with various latencies.
12.4.1.4 Effect of Neuroleptics on Immune Function

Some *in vitro* studies have reported that neuroleptics can cause immune activation [163,164], whereas others have demonstrated the opposite effect [152,159]. Patients treated with haloperidol have a positive correlation between CSF IL-10 concentrations and symptom severity, particularly with respect to negative symptoms [156]. Chlorpromazine (CPZ) inhibits TNF-α production and protects mice from IL-1 toxicity and endotoxin-induced TNF-α toxicity [165,166]. Studies *in vitro* with mitogen-stimulated lymphocytes have demonstrated that haloperidol, CPZ, and flupenthixol inhibit production of IL-2 but not IL-1 [167]. CPZ also reduces mRNAs for IL-2, IFN-α, and TNF-α in human T cells and thymocytes, as well as inhibiting the release of activating cytokines *in vitro* [168,169]. Conversely, the concentrations of SIL-2r are increased by neuroleptic treatment, although this effect was originally thought to be a consequence of the disorder [143,148,170–172]. A significant decrease in SIL-6r levels has also been noted during antipsychotic therapy [140,141,151,152,173]. The typical antipsychotics (e.g., CPZ and haloperidol) appear to have negative immunoregulatory effects, whereas the atypical antipsychotics (e.g., clozapine, risperidone) have more complex effects on the immune system. Clozapine treatment can reduce to normal the elevated blood concentrations of TNF-α noted in schizophrenic patients [174], and can produce agranulocytosis in some patients [175]. A controlled clinical trial, which administered the COX-2 inhibitor celecoxib or placebo to schizophrenic patients who were also receiving risperidone, indicated a benefit from celecoxib. The data showed a significant decrease in total positive and total negative syndrome scale scores, a significant decrease in negative symptoms [176]. Although the magnitude of improvement was modest, these data add further support for the involvement of inflammatory processes in the etiology of this disorder, and will encourage further research into the use of therapies employing immunomodulatory compounds.

12.5 Concluding Remarks

The behavioural effects of inflammatory mediators, such as the cytokines, in response to an immunological challenge or in the presence of an inherent disorder of the immune system (such as autoimmune disease) have been examined in detail. These include sickness behavior, effects on thermoregulation, sleep, ingestion and cognition. The link between altered immune function and the possible role of inflammatory mediators in the aetiology of affective disorders such as depression and schizophrenia has also been examined. This has led to the suggestion that manipulations of the immune system (such as the use of interleukin receptor antagonists or COX-2 inhibitors) may lead to novel therapies which would be of benefit in these conditions.

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13 Increased Type 1 Helper T Cell Functions and Reward Stimulation

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13.1 Introduction: Psychological Variables Do Correlate with Immune Alterations

A recent meta-analytic study showed that psychological stress does correlate with immunosuppression [1]. This report covers more than 300 empirical articles describing a relationship between psychological stress and parameters of the immune system in human participants. Acute stressors were associated with potentially adaptive up-regulation of some parameters of natural immunity and down-regulation of some functions of specific immunity. Brief naturalistic stressors (such as school examinations) tended to suppress cellular immunity while preserving humoral immunity. Chronic stressors were associated with suppression of both cellular and humoral responses. Effects of event sequences varied according to the kind of event (e.g., trauma versus loss). Subjective reports of stress generally did not associate with immune system changes. Cell populations that were very sensitive to stress included natural killer (NK) cells and T cells. In addition, NK cytotoxicity, type 1 T helper (Th1) cell function, and T-cell proliferation to mitogen were all sensitive to various type of stresses. In contrast, some effective psychosocial interventions succeeded in augmenting immune function, especially of those immune cells that were sensitive to stress [2–5].

13.2 Neural Regulation of Immunity

The meta-analytical findings suggest that immune function is regulated by the central nervous system (CNS). In terms of immune suppression, brain destruction studies have shown that hypothalamic areas are relevant to immune modulation [6,7]. Thus, we performed several electrical brain stimulation studies [8–10]. Because we were interested in the augmentation of immunity, we focused on electrical stimulation of
the lateral hypothalamus (LH), which is one of the centers for reward behavior and sensations [11].

13.3 Reward Center/Pleasure and NK Cells

13.3.1 Lateral Hypothalamus

Wenner et al. found that NK cell activity in Wistar–King–Aptekman (WKA) and SD rats was significantly higher following electrical stimulation of the LH as compared to animals that underwent sham operations [8]. There was no such difference between sham-operated rats and those receiving electrical stimulation in the frontal cortex as a control. Operations were performed under sodium pentobarbital anesthetic, and NK activity against YAC-1 target cells was measured 20 hours later using the $^{51}$Cr release assay. Because the LH area stimulated in these experiments is a potent reward center, and stimulation of this point increased NK activity, it has been suggested that pleasure plays a role in cellular immunity. Later, Iimori et al. [9] showed that electrical stimulation of the LH in rats increased splenic NK cell activity, whereas electrical ablation of the LH decreased it. However, the percentage of NK cells in the spleen, as detected by the anti-NKR-P1 monoclonal antibody (mAb), did not change significantly. These results suggest that the LH does not modulate splenic NK cell activity by increasing NK cell numbers, but rather by increasing NK cell activity. In addition to the acute electrical stimulation of the LH of anesthetized rats, Wenner et al. [10] showed that splenic NK cell activity was significantly higher following chronic, uncontrollable electrical stimulation of the LH in fully conscious rats, compared to sham-operated rats [10]. All rats demonstrated that the electrode site had self-stimulating properties, which supports the possibility that the experience of reward may be implicated in NK cell activity augmentation.

Recently, Wrona and Trojniar and Wrona et al. [12,13] tested the effects on both spleen and blood NK cells of acute (1 day) and chronic (21 days) electrical stimulation of the LH, and examined large granular lymphocyte (LGL) numbers in conscious, freely behaving animals. Chronic stimulation of the LH caused significant augmentation of NK cell activity. Rats responding to LH stimulation with feeding showed a slightly greater effect than those responding with a locomotor reaction. The observed effects were anatomically specific. The involvement of the LH in reward phenomena was shown to be associated with NK cell augmentation as well.

13.3.2 Ventral Tegmental Area

It was also discovered that chronic electrical stimulation of the midbrain ventral tegmental area (VTA) increases spleen NK cell cytotoxicity in rats [14]. The LH belongs to the so-called brain reward system, a collection of the central structures the activation of which produces positive emotions. The midbrain VTA is another prominent reward-relevant structure. In the present work, chronic electrical stimulation of the VTA (constant current 0.1-ms duration cathodal pulses delivered at frequency 50Hz
Increased Type 1 Helper T Cell Functions and Reward Stimulation during 60 minutes daily session for 14 consecutive days) in rats caused an increase in NK cell cytotoxicity (measured by chromium release assay) in the spleen but not in the peripheral blood; there was no simultaneous effect on the number of LGLs (measured by the morphological method) or plasma level of prolactin (PRL), growth hormone (GH), corticosterone (COR), and testosterone (TST).

Thus, we now have enough data to assume that pleasure or reward behavior evoked by electrical stimulation in the LH and VTA is linked with augmentation of NK cell activity. These findings might give some insight into the underlying mechanisms by which psychological intervention that enables recovery from depressive symptoms or improvement of coping ability can ameliorate the repression of NK cell activity.

13.3.3 Reward Center and T Cell

As described earlier, T-cell and Th1 cell functions are sensitive to stress. However, we have not obtained any data that illustrate the correlation of reward center with T cells and with their function. Recently we tried to investigate whether T-cell cytokine production via CD3 stimulation is modulated by LH electrical stimulation.

13.3.3.1 Methods

We used inbred and pathogen-free WKA male rats (Clea Japan, Tokyo, Japan), 7–9 weeks old, weighing approximately 380 g. On the date of the operation, they were anesthetized with sodium pentobarbital (50 mg/kg) and fixed on a stereotaxic apparatus (Narishige Instruments, Tokyo, Japan).

The rats were divided into four groups (n = 11 each); the LH-stimulated rats had an electrode inserted into the unilateral LH area, 3 mm posterior to bregma, 1.7 mm lateral to the right side, and 8.5 mm below dura. These animals received acute electrical stimulation at 100 mA × 0.5 seconds × 50 Hz every 3 seconds for 30 minutes. The LH sham-stimulated rats had an electrode inserted into the same LH area but were given no stimulation. The cortex-stimulated rats had an electrode inserted into the unilateral cortex area, 1.5 mm posterior to bregma, 1.5 mm lateral to the right, and 2 mm below dura and were given acute electrical stimulation as for the LH-stimulated rats. The cortex sham-stimulated rats had an electrode inserted into the same cortex area but were given no current.

Additional rats were divided into four groups (n = 6 each): LH-ablated rats had electrodes inserted into the same positions bilaterally as the LH-stimulated rats, and were subjected to local ablation by electrical current of 2 mA, 50 Hz for 15 seconds. The LH sham-ablated rats had electrodes inserted into the same LH area bilaterally, but underwent no actual ablation. The cortex-stimulated rats were subjected to the same local ablation as the LH-ablated rats. The cortex sham-operated rats had an electrode inserted into the same cortex areas but were given no current. All treatments were carried out under sterile conditions. The operations were performed from 10:00 AM to 2:00 PM.

The rats were sacrificed by CO2 asphyxiation 24 hours after the operation and their spleens were collected for immunological analyses. Single-cell suspensions
were prepared in DMEM (GIBCO BRL Life-technologies, Gaithersburg, MD, USA), supplemented with 10% FCS (Boehringer Mannheim, Australia), $5 \times 10^{-5}$ 2-mercaptoethanol and 100 mg/l kanamycin, which was used as the culture medium throughout this study. Red blood cells were removed by hypotonic shock. Viable splenocytes were counted by trypan blue exclusion assay.

Splenocyte suspensions were passed through a G10 column and adherent cells were eliminated. Nonadherent splenocytes ($1 \times 10^8$ cells per 2 ml of DMEM 10% FCS) were suspended onto the dishes coated with anti-CD3ε mAb (1 μg/ml) (Endogen, Cambridge, MA, USA) and incubated for 24 hours. Interferon-γ (IFN-γ) and interleukin-4 (IL-4) productions in the culture supernatants were measured by ELISA (Endogen, Cambridge, MA, USA). FITC-conjugated antirat CD4 mAb, CD8 mAb, and PE-conjugated CD45Rc mAb (Endogen, Cambridge, MA, USA) were used for flow cytometry.

After sacrifice, the rat brains were excised and immersed in PBS containing 10% formalin and 20% sucrose for 10–14 days. Cryostat sections were prepared and examined for the position of the electrode tip and ablated areas [15].

Statistics were done using SPSS software.

13.3.3.2 Results

13.3.3.2.1 T-Cell Subpopulations in the Spleen After LH Stimulation
As shown in Table 13.1, the percentages of CD4+ T cells and CD4+CD45Rc+ T cells in the LH-stimulated rats were significantly higher than those in the LH sham-operated rats. No significant changes were detected in any other groups.

13.3.3.2.2 Cytokine Production After LH Stimulation and Destruction
As shown in Table 13.2, IFN-γ production by T cells in LH-stimulated rats was significantly higher than in the LH sham-operated rats. IL-4 production from the T cells in the LH-stimulated rats was significantly lower than in the LH sham-operated rats. No significant changes were detected in any other groups. In addition to stimulation studies, Table 13.3 shows that the IFN-γ production by T cells of the LH-ablated rats was significantly lower than in the LH sham-operated rats and that IL-4 production from the T cells in the LH-ablated rats was significantly lower than in the LH sham-operated rats. No significant changes were detected in any other comparisons or groups.

13.3.4 Reward and Increased T Helper 1 Function
These results strongly suggest that excitation of the LH is linked with reward behavior and that pleasure increases type 1 T-cell functions. Ablation of the LH decreased Th1 activity and augmented Th2 activity. The direction of the effects of LH treatment on T-cell function coincides nicely with the effects on NK cells. It might now be safely said that reward links with increased Th1 activity and that, together with NK cell activity, reward or pleasure plays an important role in anticancer, antiviral, and cellular immunity via T-cell cytokine production.
Table 13.1 The Percentage of T-Cell Subpopulations from Brain-Stimulated Rats Compared with Those from Sham-Operated Rats

<table>
<thead>
<tr>
<th></th>
<th>CD4+ (SD)</th>
<th>CD4+ CD45Rc+ (SD)</th>
<th>CD8+ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH-stimulated</td>
<td>34.1(1.4)</td>
<td>23.7(1.6)</td>
<td>28.7(0.5)</td>
</tr>
<tr>
<td>LH sham-operated</td>
<td>30.8(1.6)</td>
<td>19.9(1.2)</td>
<td>30.0(0.9)</td>
</tr>
<tr>
<td>Paired t test: p value</td>
<td>0.035</td>
<td>0.002</td>
<td>0.18</td>
</tr>
<tr>
<td>Cortex-stimulated</td>
<td>28.3(1.8)</td>
<td>20.8(1.7)</td>
<td>22.5(1.8)</td>
</tr>
<tr>
<td>Cortex sham-operated</td>
<td>28.1(2.3)</td>
<td>21.5(1.3)</td>
<td>21.4(1.7)</td>
</tr>
<tr>
<td>Paired t test: p value</td>
<td>0.83</td>
<td>0.61</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Table 13.2 IFN-γ and IL-4 Productions from the Splenocytes of Brain-Stimulated Rats Compared with Sham-Operated Rats

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ (pg/μl, SD)</th>
<th>IL-4 (pg/μl, SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH-stimulated</td>
<td>5913(1106)</td>
<td>52.3(14.5)</td>
</tr>
<tr>
<td>LH sham-operated</td>
<td>3070(645)</td>
<td>122.3(26.0)</td>
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<td>Paired t test: p value</td>
<td>0.01</td>
<td>0.017</td>
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<tr>
<td>Cortex-stimulated</td>
<td>2825(64)</td>
<td>111.4(18.5)</td>
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<tr>
<td>Cortex sham-operated</td>
<td>3381(787)</td>
<td>125.2(17.7)</td>
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<tr>
<td>Paired t test: p value</td>
<td>0.66</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 13.3 IFN-γ and IL-4 Productions from the Splenocytes of Brain-Destructed Rats Compared with Sham-Operated Rats

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ (pg/μl, SD)</th>
<th>IL-4 (pg/μl, SD)</th>
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</thead>
<tbody>
<tr>
<td>LH-destructed</td>
<td>2083(806)</td>
<td>72.3(18.5)</td>
</tr>
<tr>
<td>LH sham-operated</td>
<td>3120(712)</td>
<td>118.3(24.7)</td>
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<tr>
<td>Paired t test: p value</td>
<td>0.02</td>
<td>0.03</td>
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<tr>
<td>Cortex-destructed</td>
<td>3028(702)</td>
<td>128.4(20.3)</td>
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<td>Cortex sham-operated</td>
<td>3291(907)</td>
<td>120.2(19.7)</td>
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<td>Paired t test: p value</td>
<td>0.78</td>
<td>0.84</td>
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</table>

13.4 Conclusion

This report summarizes the effect of the LH and reward behavior on the augmentation of cellular immunity. In addition to NK cell population and function, T-cell populations and Th1 functions are modulated centrally by the LH and by reward
stimulation. Further studies are required to elucidate the exact pathways from the LH to immune cells and to identify the mediators of this phenomenon.

References