# Without nerves, immunology remains incomplete – *in vivo veritas*

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**Summary**

**Interest in the interactions between nervous and immune systems involved in both pathological and homeostatic mechanisms of host defence has prompted studies of neuroendocrine immune modulation and cytokine involvement in neuropathologies. In this review we concentrate on a distinct area of homeostatic control of both normal and abnormal host defence activity involving the network of peripheral c-fibre nerve fibres. These nerve fibres have long been recognized by dermatologists and gastroenterologists as key players in abnormal inflammatory processes, such as dermatitis and eczema. However, the involvement of nerves can all too easily be regarded as that of isolated elements in a local phenomenon. On the contrary, it is becoming increasingly clear that neural monitoring of host defence activities takes place, and that involvement of central/spinal mechanisms are crucial in the co-ordination of the adaptive response to host challenge. We describe studies demonstrating neural control of host defence and use the specific examples of bone marrow haemopoiesis and contact sensitivity to highlight the role of direct nerve fibre connections in these activities. We propose a host monitoring system that requires interaction between specialized immune cells and nerve fibres distributed throughout the body and that gives rise to both neural and immune memories of prior challenge. While immunological mechanisms alone may be sufficient for local responsiveness to subsequent challenge, data are discussed that implicate the neural memory in co-ordination of host defence across the body, at distinct sites not served by the same nerve fibres, consistent with central nervous mediation.**

**Keywords:** contact sensitivity; haemopoiesis; memory; nerve fibres; neuroimmunology

## Neuroimmunology

It is first necessary to highlight increasingly troublesome issues of definition in this area of research. As a greater number of peptide mediators common to the immune, nervous and neuroendocrine systems are discovered, terms such as 'cytokine' and 'neuropeptide' become progressively more misleading. Cytokines and neuropeptides are potential mediators between the nervous and immune systems, as well as existing within each system. As evidence of this 'neuro-immune dialogue' accumulates, a point may be reached where a major reclassification of molecules becomes necessary. There are also problems regarding functions ascribed to specific mediators. Glucocorticoids are not just immunosuppressive (a phenomenon associated with chronic stress and high levels of these molecules), but are also involved in optimizing immune responses at low levels in acute stress (see below). Ablation of the sympathetic nervous system, or treatment with adrenergic agonists, can exacerbate or ameliorate symptoms in animal models of arthritis. In an adjuvant-induced model, β-agonists were found to be pro-inflammatory in the earlier, asymptomatic stage, but anti-inflammatory in the later, symptomatic phase.[1] Chemical sympathectomy had a similar time-dependent effect in antigen-induced arthritis.[2]

Such issues can be confounding and add to the confusion concerning sources and targets of these mediators *in vivo*. These issues aside, there is a significant body of evidence supporting the integration of these systems in a

co-ordinated host defence network. In this review we will concentrate on neural co-ordination of host defence and present evidence to show that this co-ordination is important in the orchestration of immunological activities.

## Neuroimmunology encompasses both homeostasis and pathology

How do nervous, endocrine and immune systems interact? There have emerged several discrete categories of research into fields that each uses the term 'neuroimmunology.' Here we briefly review these groupings and identify some of the difficulties associated with their classification.

One area concerns 'inappropriate' immune responses – pathological processes involving injurious effects on the nervous system – including autoimmune reactions, such as multiple sclerosis,[3] as well as cytokine mediation of inflammatory injury in stroke.[4] Although the origin of cytokines involved in inflammatory brain injury may not necessarily be immunological, this highlights a semantic problem throughout all fields of neuroimmunology regarding peptide mediators. The pejorative terms 'cytokines' and 'neuropeptides' need careful use, as neither is limited to immune or nervous origin. Similarly, catecholamines, although predominantly sourced from sympathetic nerves and the adrenal medulla, can also be sourced from immunocytes (see below and Table 1). Such issues can be confounding and add to the confusion concerning the source and target of these mediators *in vivo*.

A second, broad category includes the study of 'appropriate' or homeostatic neuroendocrine mechanisms, the core of which is an adaptive effect of short-term (acute) stress-activation of the hypothalamus–pituitary–adrenal (HPA) axis[5,6] that is sufficient to terminate inflammatory responses and steer T-helper cytokine balance. Immunological status appears to be coupled to homeostatic endocrine control via cytokine levels, including interleukin (IL)-1, which mediate activation of the HPA axis.[7–9] Mediators in this axis involve hypothalamic corticotropin-releasing hormone (CRH) evoking adrenocorticotrophic hormone (ACTH) release from the anterior pituitary, which, in turn, stimulates corticosteroid release from the adrenal cortex (Fig. 1).

Another difficulty in understanding 'neuroimmune' mediation is that generalizations of function cannot easily be ascribed to specific mediators. For example, corticosteroids are not simply immunosuppressive; at extremes, low-level corticosteroid release may sustain immune system function,[10] while chronic stress can elevate susceptibility to disease via secondary immune deficiency.[11–14]

A third category involves homeostatic activity, mediated not by neuroendocrine outflow, but by direct, 'hard-wired' interactions between the nervous and immune systems that are the focus of the present article.

Since we last reviewed this subject,[15] every major category of peripheral nerve has been shown to be involved in the modification of an immunological activity (Fig. 1). We therefore provide a brief review of the ability of peripheral nerve fibres and specific cells of the host defence system to engage in cross-talk at the cellular level, followed by two examples, from our own work, on the involvement of peptidergic nerves on:

- haemopoietic cell differentiation and release from bone marrow; and
- the availability of hapten-specific memory in contact sensitivity responses in the skin.

Furthermore, we develop a theory concerning the wider role of c-fibres in the co-ordination of immune function.

## Defining and establishing functional connectivity (synaptic or otherwise) between cells

To 'prove the principle' that productive interactions at a cellular level between peripheral nerves and the immune system take place, evidence needs to be accumulated that the criteria established for synaptic connectivity are met. Not only must a mediator be synthesized by a cell, it must be released, be detected by a receptor expressed by another cell, and induce a functional alteration within that cell as a result. Past research has clearly established that the immune and nervous systems share common mediators (Table 1). Not only can cells in both systems synthesize and release these mediators, they both show physiological responses based on the presence of specific receptors. This presents both the evidence for physiological cross talk between these systems and a major problem for neuroimmunologists, as to establish *in vivo* connectivity it must demonstrate that the source of a mediator is cells of one system and the target is the other system. Experiments utilizing pharmacological agents *in vivo* therefore present problems for the isolation of neuroimmune interactions, as shared mediators could be affecting either one or both systems. Similarly, tools previously thought to be highly specific for one system may eventually be shown to affect both systems; capsaicin being a case in point. This molecule (an extract of chilli peppers) had been thought to exclusively target specific vanilloid receptors on small-diameter, unmyelinated sensory nerve fibres, but these same receptors have recently been shown to exist on a range of cells in the skin and immune system,[16–19] requiring a re-evaluation of data from experiments using capsaicin. It can be appreciated, then, that the design of experiments is critical for establishing connectivity between these two systems that share so many chemical mediators. Moreover, while *in vitro* experiments can establish the presence of functional receptors and physiological responses, they cannot define the *in vivo* source and target of the specific chemical mediator, or

**Table 1.** Mediators of neuroimmunomodulation

| Name | Source(s) | Targets, functions, effects | Refs |
|---|---|---|---|
| Substance P | Central nervous system, peptidergic nerve fibres (c-fibres), macrophages, granulocytes, lymphocytes | Released antidromically as part of nociception. Increases lymphocyte output from lymph nodes, stimulates lymphocyte proliferation, causes mast cell degranulation. Blocks T-cell adhesion to fibronectin and can augment cytokine secretion from antigen-exposed cells *in vitro*. Neurokinin receptors present on endothelia, smooth muscle, lymphocytes, Langerhans' cells, mast cells. Neurokinin receptor activation causes rises in intracellular $Ca^{2+}$. Related peptides neurokinin A and B are also released by c-fibres. | 24,63,64,68, 69,164–168 |
| Calcitonin gene-related peptide (CGRP) | c-fibres, lymphocytes | Induces vasodilation, plasma extravasation, mast cell degranulation. Inhibits antigen presentation by Langerhans' cells. Alters T-cell cytokine secretion and adhesion to fibronectin. CGRP receptor present on T-cell surface. Stimulation of CGRP receptors on Langerhans' cells brings about signalling through adenylate cyclase and cAMP. | 16,100–102,169 |
| Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) | Produced at nerve terminals in the CNS and PNS, as well as in lymphoid organs | Suppresses the induction of contact hypersensitivity. Inhibits Langerhans' cells antigen presentation *in vitro*. Down-regulates expression of macrophage costimulatory molecules. Released by sensory nerves upon damage/inflammation. Receptors present on cutaneous nerves, macrophages, Langerhans' cells and T cells. Receptor activation induces cAMP formation in Langerhans' cells. | 77,170,171 |
| Neuropeptide Y | Nerve terminals in the CNS and PNS, lymphoid organs, lymphocytes and monocytes | Suppresses natural killer cell activity. Co-released with norepinephrine from sympathetic terminals following depolarization, participating in neurogenic inflammation. NPY receptors expressed by dorsal root ganglion neurones. | 172–175 |
| Somatostatin | Nerve terminals in the CNS and PNS, lymphoid organs | Specific somatostatin receptors present on lymphocyte surface, Langerhans' cells. Along with CGRP and NPY, directly induces T-cell adhesion to fibronectin. Also directly stimulates T-cell cytokine secretion. Can enhance or reduce lymphocyte proliferation. | 112,124,176–178 |
| GnRH-I, -II (gonadotrophin-releasing hormone) | Neuronal cells, T lymphocytes | Increases surface expression and transcription of a laminin receptor in T cells. Laminin receptor thought to be important in facilitating T-cell extravasation and holding of memory T lymphocytes in areas of challenge. Also induces chemotaxis towards SDF-1/CXCL12. | 179 |

**Table 1.** (*Continued*)

| Name | Source(s) | Targets, functions, effects | Refs |
|---|---|---|---|
| α-melanocyte stimulating hormone (α-MSH) | Keratinocytes, Langerhans' cells, melanocytes | Synthesis by keratinocytes up-regulated by immunological challenge. May induce hapten-specific tolerance by modulating levels of interleukin-10. Decreases endothelial cell adherence, dendritic costimulatory molecule expression, antagonizes interleukin-1 activity. Melanocortin receptors expressed throughout the skin. POMC peptides stimulate melanogenesis. | 126,180–187 |
| Corticotropin-releasing hormone (CRH) and urocortin | Throughout the skin, may be transported to the skin via sensory nerves in rodents. Lymphocytes | Keratinocytes, mast cells, endothelia all carry CRH receptors. Can prompt mast cell degranulation, despite antinflammatory and antiproliferative properties. Stimulates POMC activity in cutaneous fibroblasts. | 125–128,130,131 |
| Adrenocorticotrophic hormone (ACTH) | Anterior pituitary, skin cells, leucocytes | Derived from POMC. Cells throughout the skin express melanocortin receptors responsive to ACTH and MSH. Induces expression of preprotachykinin and neurokinin receptor. POMC peptides stimulate melanogenesis. | 126,187–189 |
| Nerve growth factor (NGF) | Mast cells, fibroblasts and other cell types at sites of injury/inflammation | Binds TrkA receptors on sensory afferents. Essential for nerve fibre growth/development/ maintenance. Can influence mast cell activity. | 126 |
| Serotonin (5-HT) | Afferent nociceptors, mast cells (rodents only). Stored by lymphocytes and platelets | Enhances plasma extravasation induced by capsaicin. Proinflammatory, vasodilator. Noradrenergic termini in lymphoid organs may take up 5-HT for later release. Immune cells, keratinocytes, unmyelinated axons all thought to express HT receptors. | 86,190 |
| Endorphins and enkephalins | Langerhans' cells, mononuclear leucocytes, keratinocytes | Antinociceptive properties. Production stimulated by proinflammatory stimuli (e.g. TNF-α). Enhances mononuclear cell chemotaxis and interleukin-2 production. Opioid receptors present on keratinocytes. | 191–195 |
| Epinephrine and norepinephrine | Lymphocytes, macrophages, sympathetic neurones, keratinocytes, adrenal medulla (under acute stress) | Norepinephrine co-localizes with neuropeptide Y in lymphoid organs. Intradermal injection can suppress the sensitization and elicitation phases of contact sensitivity. Decreases proinflammatory cytokine expression, promote anti-inflammatory cytokine production. Can be an activator of the immune response; depends on the type of recipient cell and its activational and maturational state. Stimulation with physiological concentrations of catecholamines stimulates adenylate cyclase activity and cyclic AMP production. Keratinocytes, almost all leucocytes, including Langerhans' cells and mast cells, express adrenoreceptors. $A_2$-agonists inhibit interleukin-12 secretion by dendritic cells and therefore T helper 1 cell development. This inhibition is mediated through increases in intracellular cyclic AMP concentration. | 196–198 |



**Table 1.** (*Continued*)

| Name | Source(s) | Targets, functions, effects | Refs |
|---|---|---|---|
| Histamine | Mast cell granules, basophils. Platelets, dendritic cells and T cells produce histamine *de novo* | Potent vasodilator. Activates submucosal neurones. Increasesinterleukin-10 secretion, inhibits interleukin-12 secretion by dendritic cells and T helper 1 cell development as a result. This inhibition is mediated through increases in intracellular cAMP. Increased levels of CD80, CD86 and MHC class II on dendritic cells. Histamine receptors: expressors include neurones, endothelia, dendritic cells, T and B cells. T helper 1 cells predominantly express H1R. H2R is primarily coupled to adenylate cyclase – also capable of signalling through PLC. T helper 2 cells predominantly express H2R. | 199–202 |
| Acetylcholine | Cholinergic innervation, keratinocytes | Cholinergic receptors present on keratinocytes and lymphocytes. Induces norepinephrine secretion from lymphocytes. Depresses TNF-$\alpha$ secretion without affecting interleukin-10 levels. | 80,81,203,204 |
| Melatonin | Nervous system, immune system | Mammalian skin produces melatonin from serotonin. Melatonin receptors expressed in mammalian skin. Modulates lymphocyte proliferation and the T helper 1/T helper 2 cytokine balance. | 190,205,206 |
| ATP and ADP | Keratinocytes, nociceptors (in response to noxious stimuli) | Nucleotide (P2X/Y) receptors present on sensory nerve fibres – ATP responsive? ATP may exert an inhibitory effect on dendritic cell migration, prolonging exposure to antigen. | 207 |
| Potassium | Nervous system and other tissues (in response to noxious stimuli) | Activates T-cell adhesion to fibronectin, facilitating migration. Brings about $\beta_1$-inegrin adhesion in the same way as 'neuropeptides'; T-cell membrane depolarization and opening of the T-cell Kv1.3 voltage-gated $K^+$ channel. | 208,209 |

CGRP, calcitonin gene-related peptide; CNS, central nervous system; NPY, neuropeptide y; POMC, pro-opiomelanocortin; PNS, peripheral nervous system; PLC, phospholipase C; SDF-1, stromal cell derived factor-1; TNF-$\alpha$, tumour necrosis factor-$\alpha$.

whether the elements for signalling pathways demonstrated *in vitro* may in fact be utilized *in vivo*. Although imperfect, the neurotoxic actions of capsaicin have improved our understanding of functional connectivity between nervous and immune systems *in vivo*.

## Co-ordination of host defence mediated by c-fibres

Although primarily recognized as afferent nerve fibres for the sensation of damaging or painful stimuli, c-fibres have been identified to have important roles in conveying sensibility to immunological stimuli as well as providing efferent drive for inflammatory processes and homing cues for dendritic cells (DCs) in most peripheral sites and organs of the body. Previously thought to have 'free' nerve endings, c-fibres are associated with a variety of specialized cells in the organ systems, notably with modified macrophages [e.g. Langerhans' cells (LCs), DCs and stromal cells][16] and mast cells.[17]

Current dogma states that the immune system is widely regarded as autonomous and self-regulating. While at the

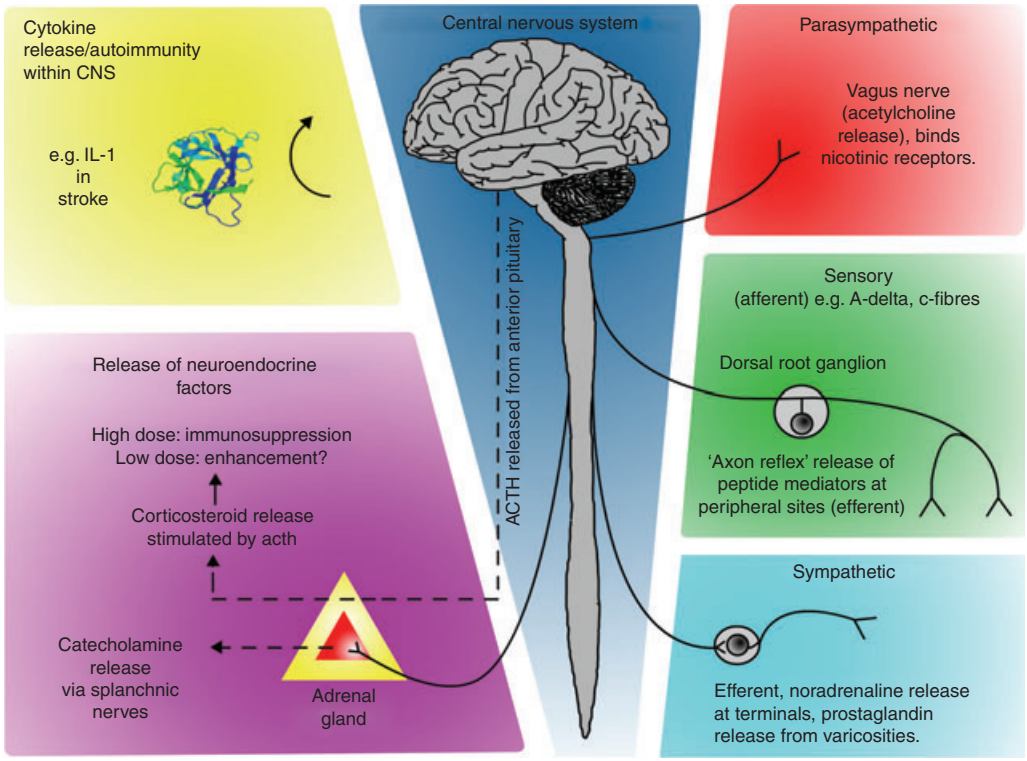



**Figure 1.** Immunomodulation by the nervous system. Adrenocorticotrophic hormone (ACTH) released from the pituitary (centre) acts upon the adrenal cortex to stimulate the release of glucocorticoids (bottom left), which typically suppress immune function, especially biasing towards T helper 2 (Th2) cells. The adrenal medulla receives sympathetic innervation from the splanchnic nerves, which can provoke release of catecholamines into the circulation. Cytokine release and autoimmune processes within the central nervous system (top left) are also considered to be a form of neuroimmunomodulation. Sympathetic innervation (bottom right) is present in all lymphoid organs, as well as tertiary sites. Sensory innervation (centre right) is similarly pervasive and is characterized by peptide neurotransmitter release and capsaicin sensitivity. Parasympathetic innervation, (top right) principally from the vagus nerve, releases acetylcholine. Leucocytes have been shown to harbour receptors for and respond to all of these chemical mediators. CNS, central nervous system; IL-1, interleukin-1.

cellular level there is little doubt that immune cells are capable of regulating each others' activities, recent experimental evidence suggests that neural pathways are essential for the co-ordination of immune activity *in vivo*. In an attempt to resolve the function of c-fibres in neuroimmune co-ordination, we must first propose a regulatory loop and gather evidence to test whether the elements of this circuit are connected and regulate immune function. If c-fibres are to exert any direct influence over an immune response, the first requirement is that they are present and functional throughout the host defence system, including in the bone marrow and secondary lymphoid organs, as well as at the sites of potential antigen exposure (the skin, the surfaces of the airways and digestive tracts, for instance), such that the static nervous system has as many contacts as possible with a highly mobile immune system through its more static, regulatory components. Second, immune cells must be capable of responding to neurotransmitters released by these nerve fibres, to products of neurotransmission or to factors generated by the stimulation of other non-neural cells. Third, there must be sensory monitoring of host challenge and immune activity such that the nervous system is 'informed' of host status and/or host response to challenge so that it can effectively co-ordinate host defence activity. Below we give examples of data that support the presence of such an integrated and co-ordinated host defence system.

## Neurogenic inflammation

It was first documented by Bayliss, in 1901, that stimulation of neurones in dorsal root ganglia results in vasodilation[18] (reviewed in refs 19 and 20), suggesting that these afferent (sensory) nerves also had an efferent (motor) function. The role of neural afferents and efferents in the inflammatory response, and in specific inflammatory conditions, has received a great deal of attention, with the identification of substance P (SP), calcitonin gene-related peptide (CGRP) and nitric oxide as key neurotransmitters. SP is synthesized in dorsal root ganglion neurones[21] and is also found in the hypothalamus, substantia nigra,

the dorsal horn of the spinal cord and in small Aδ and unmyelinated c-fibres in the periphery[22,23] (reviewed in ref. 24). It has long been accepted in clinical dermatology and gastroenterology that peptide mediators released from cutaneous c-fibres are potent proinflammatory agents, giving rise to neurogenic inflammation and underlying a range of conditions, including dermatitis, psoriasis, eczema, Crohn's disease and colitis. Collateral c-fibre axons provide an efferent route for inflammatory cues to spread laterally from afferent terminals in the form of a local reflexive arc, termed the 'axon reflex' (Fig. 1). A major component of allergic reactions both in the airways[25,26] and in the skin,[27–30] neurogenic inflammation may be initiated by the release of SP and CGRP. Additionally, and probably more important in promoting vasodilation and vascular permeability,[31–34] leading to plasma extravasation, sympathetic nerve fibres release potent inflammatory mediators, including norepinephrine, from varicosities along the nerve fibre as well as from the nerve terminals.[28] Nowhere is this more evident than in synovial joints.[35] c-Fibres are also intimately associated with mast cells throughout the body (see below) and stimulate the release of histamine, further aggravating inflammatory conditions. These observations indicate that both direct and indirect interactions between nerve fibres and immune cells, as well as independent neural effects on peripheral targets, are activated in inflammatory conditions.

## Anatomical and functional evidence of lymphoid innervation

Studies of lymph node innervation were conducted as long ago as 1899, by Tonkoff,[36] that hinted at the presence of sensory nerves in lymph nodes. Similar conclusions were arrived at in the late 1980s, when electron microscopy enabled the definition of partially myelinated axons entering the node, as well as potential sensory nerve terminals associated principally with the vasculature[37] but also branching into cortical and paracortical regions, terminating among lymphocytes.[38,39] Dense plexuses of adrenergic fibres in association with blood vessels and the surrounding periarterial lymphatic sheath in lymph nodes have been demonstrated, as have occasional fibres with no apparent vascular association in the cortical, paracortical and medullary regions of lymph nodes.[40] Staining for a number of peptide mediators has proven positive, showing potential co-localization between SP and CGRP, among others, reflecting the association seen in the skin.[41,42] In contrast to noradrenergic fibres that are mainly associated with the vasculature and perivascular areas, SP/CGRP-positive nerves are often seen in the cortical regions of lymph nodes, as well as in the vasculature and lymphatics. Some evidence already exists to suggest that the activity of these and other sympathetic nerve fibres is immunomodulatory, both in lymph nodes and skin. For example, rats that had undergone sympathetic ganglionectomy prior to immune challenge showed decreased lymphocyte proliferation in response to concanavalin A (Con A).[43] Co-cultures of neonatal superior cervical ganglia and adult murine tissue revealed significant outgrowth of neurites from the ganglia towards lymphoid tissue, which was diminished by administering anti-nerve growth factor (NGF).[44] Interestingly, neurite outgrowth to mesenteric lymph nodes was enhanced in this study when the lymphoid tissue was taken from mice infected with nematode worms. This observation is consistent with evidence that showed an increase in the density of lymph node innervation following antigenic stimulation;[45] increases in the numbers of axonal profiles within the medulla were observed 4 months after the subcutaneous injection of bovine serum. In a contact sensitivity (CS) model, antigenic challenge induced an increase in the length of cutaneous protein gene product 9·5-reactive nerve fibres and their NGF content.[46] Similarly, nerve fibre density has been found to be enhanced in psoriatic lesions.[47] The spleen is invested with a similarly dense innervation, where release of IL-6 and tumour necrosis factor-α (TNF-α) is 'fine-tuned' by sympathetic neurotransmission.[48] Consistent with a close functional relationship between peripheral nerves and key immunocytes within lymphoid tissue, including the gut, which are proposed to mediate homeostatic benefit, the closeness of sympathetic innervation to follicular DCs has been correlated with their rate of neuroinvasion by prions.[49]

## Sympathetic and neuroendocrine influences on leucocytosis

A number of studies have shown that acute psychological stress induces the mobilization of lymphocytes into the blood[50] and that catecholamines, namely epinephrine, play a critical role in the induction of this stress-induced redistribution of lymphocytes, known as lymphocytosis. First described 100 years ago,[51,52] catecholamine-induced lymphocytosis is characterized by a rapid (10-min) increase in lymphocyte numbers in the circulation; the numbers of 'large granular lymphocytes', which would come to be known as natural killer (NK) cells, were found to increase to a greater extent, by ≈400%.[53] This reproducible lymphocytosis is typically followed by a more variable increase in granulocyte numbers.[50] There exist several possibilities to explain the source of this rapid increase in immune cell numbers. As the effect is too rapid and too great in magnitude to be explained by proliferation, cells loosely adherent to venule walls (the marginal pool), the lungs, spleen, bone marrow and lymph nodes, are all possible reservoirs[50] and are all served by adrenergic and, indeed, peptidergic nerve fibres.[42] Neuropeptide Y and catecholamines can be secre-

ted simultaneously in these regions, consistent with neuropeptide Y (NPY)-mediated leucocyte mobilization.[54,55] Cortisol secretion in response to stressful stimuli also prompts a redistribution of lymphocytes.[56] In a wider context, sympathetic leucocytosis can be thought of as a less-recognized facet of the 'fight-or-flight' response.[57] Mobilization of immune cells into the tissues readies the organism to deal with physical trauma and any potential infection that may result.[50] Catecholamine and cortisol-induced leucocytosis is a well-documented phenomenon, but it seems that this is not the sole level of neural control over immune-cell trafficking; an indication of more widespread neural control comes from studies of the effects of anaesthetics.

## Effects of anaesthetics and vasoactive mediators in leucocyte trafficking

It has long been suspected that general anaesthesia has a deleterious effect on the immune system, both in the suppression of resistance to infection and enhanced tumour growth,[58] an idea supported by more recent evidence suggesting that NK cell number and activity can be depressed by general anaesthesia.[59] Studies with cannulated ovine lymph nodes show that the lymphocyte output from this node into the efferent vessel is reduced by over 99% by barbiturate or halothane (i.e. general) anaesthesia. Similar findings were reported with local (lidocaine) anaesthetic.[58] Confirming a neural involvement in this effect, stimulation of the sympathetic chain prompted an increase in lymphocyte and lymph output from the node.[60] These experimental findings suggest that there is control of leucocyte retention and/or trafficking through this sympathetic neural pathway. Although peripheral lymphatic vessels are little more than an endothelial cell monolayer, larger collecting lymphatic vessels often contain a layer of lymphatic smooth muscle along with connective tissue and nerve terminals, which enable contractility to the point where the lumen can transiently disappear,[61] propelling lymph along the vessels. However, the control over lymphocyte retention in nodes is likely to be based more on the modulation of adhesive mechanisms than on mechanical gates.

The list of vasoactive substances that have been found to alter lymphocyte flow has steadily grown. Serotonin (5-HT), SP (Met)enkephalin and phenylephrine prompt an approximate doubling in lymphocyte flow over 1–4 hr, whereas neurotensin, vasoactive intestinal peptide (VIP) and carbachol all reduce lymphocyte numbers by $\geq 50\%$ over a similar timescale (see Table 1).[62,63]

The exposure of peripheral blood lymphocytes to physiological concentrations of somatostatin, CGRP, NPY or dopamine specifically increases integrin-mediated adhesion of these lymphocytes to fibronectin by 10–30 times above background levels.[64–66] By contrast, SP inhibits leucocyte adhesion, regardless of whether it has been induced by a 'pro-adhesive' peptide mediator, a number of chemokines, or stimulation with phorbol ester.[64,65]

Leucocyte trafficking is determined by the expression of adhesion molecules, not only by leucocytes but also by endothelia. A series of studies using human dermal microvascular endothelial cells have shown that SP, acting primarily through a neurokinin receptor (NK-1R), induces an increase in intercellular adhesion molecule type 1 (ICAM-1) mRNA and ICAM-1 surface expression, as well as vascular cell adhesion molecule-1 (VCAM-1).[67–69] Similar SP-induced ICAM-1 expression has been reported in keratinocytes.[70] It would appear that such an increase is of immunological significance, as levels of neutrophil adhesion to human umbilical vein endothelial cells rise to $\approx 250\%$ above background with exposure of both cell types to $10^{-15}$ or $10^{-10}$ M SP.[71] SP also induces neutrophil chemotaxis in inflammatory responses, presenting a potential means of control over one of the major events in cell-mediated inflammation.[22,72,73] This idea was elaborated upon with the finding that SP (or a NK-1 receptor agonist) cannot induce neutrophil accumulation in skin in the normal state, but it can potentiate neutrophil accumulation when inflammation is induced by IL-1β.[74]

## Peptide mediators and cytokine shift

VIP inhibits the proliferative T-cell response normally seen with exposure to mitogens, such as ConA,[75] and it also decreases the level and delays the onset of IL-2 production by these cells.[76] It now seems that VIP promotes T helper 2 (Th2) cells and suppresses the development of T helper 1 (Th1) cells through at least two different mechanisms. The pretreatment of macrophages *in vitro* with VIP enhances their ability to induce Th2-type cytokines in $CD4^+$ cells and inhibits the induction of Th1 cytokines.[77,78] Accordingly, it appears that Th2 effectors are the sole source of lymphocyte-derived VIP,[79] suggesting that this forms part of a Th2-polarizing loop. VIP is also present in nerve fibres present in lymphoid organs, providing a potential neural pathway for stimulation of this cytokine shift.

## Parasympathetic influences

The majority of neurotransmitters implicated in the modulation of inflammation are neuropeptides, but a role does seem to exist in this regard for the parasympathetic neurotransmitter, acetylcholine (ACh). In human macrophage cultures stimulated with lipopolysaccharide, nanomolar concentrations of ACh dramatically decrease the secretion of the pro-inflammatory cytokines TNF, IL-1β, IL-6 and IL-18, without affecting the levels of the anti-inflammatory cytokine, IL-10.[80] This would fit with the expression of cholinergic receptors by keratinocytes and

the functional downstream signalling that ACh induces in these cells.[81] Furthermore, electrical stimulation of the vagus nerve (within which ACh is the principal efferent neurotransmitter) was found to inhibit levels of TNF both in the liver and serum of rats, preventing the manifestation of endotoxic shock.[80] Some of these immunomodulatory effects may be exerted indirectly by CGRP, as administration of ACh at lower or higher concentrations to isolated rat mucosa could inhibit or potentiate CGRP release, respectively.[82] The expression of α7 nicotinic receptors by DCs has also been reported.[83] Although high-dose immunosuppressive effects of nicotine are known, Aicher *et al.* showed nicotine ($10^{-7}$ M) to be pro-inflammatory by three measures:

(1) Nicotine enhanced antigen-presenting cell (APC) or DC expression of surface molecules involved in inflammation.

(2) Nicotine promoted the homing of DC to atherosclerotic plaques.

(3) Nicotine-stimulated APCs showed a greater capacity to stimulate T-cell proliferation and cytokine secretion.

Their findings included the stimulation of CD40, CD86, major histocompatibility complex class II (MHC II), CD54 and IL-12 expression through this nicotinic pathway. Such findings ought to encourage a closer look at the effects of parasympathetic innervation on adaptive immune response. In contrast to sympathetic nerve fibres, parasympathetic, cholinergic nerve fibres have not been demonstrated in many anatomical associations with immune cells, presenting another case questioning source and target of chemical mediator *in vivo*.

## Peptide mediators in the skin

Nanomolar concentrations of SP (as well as VIP and somatostatin) induce mast cell degranulation and the release of histamine from serosal and connective tissue mast cells,[22,84] as well as inducing the release of TNF-α.[85] The release of peptides such as SP (or other proinflammatory mediators, e.g. serotonin[86] see Table 1) from nerves might initiate or enhance inflammation through contact with mast cells in the skin,[87] and in the gastrointestinal and respiratory tracts.[88] Experiments have shown that peptidergic and/or sympathetic neurones growing in tissue culture are 'attracted' towards a mast cell-like cell line and can form sustained contacts with these cells.[17] This is followed by an inhibition of mitotic activity in these mast cell-like cells and an increase in the number of granules that they harbour. Significantly, this phenomenon was only observed with cells directly contacting a nerve membrane; cells directly adjacent to, but not in contact with, a neurone exhibited neither reduced mitotic activity nor reduced granule numbers.[89] An intriguing interaction was exposed when it emerged that mast cell degranulation up-regulates integrin expression on the surface of LCs,[90] hinting that mast cell activation (by nerves or otherwise) might contribute towards LC migration and subsequent antigen presentation.

It has been reported that SP has no direct effect on the antigen-presenting capacity of epidermal LCs[16,91] and that LC frequency within the epidermis is not altered by SP.[92] However, nanomolar concentrations of neurokinin receptor agonists or antagonists enhance or suppress the CS response, respectively, when injected into murine skin 30 min prior to sensitization,[92] suggesting an indirect route for SP immunomodulation. By contrast, CGRP has been shown to directly inhibit antigen presentation by macrophages through the down-regulation of MHC II and CD86/B7-2 expression,[91,93–96] an observation consistent with the anatomical association between sensory nerves and 70–80% of LCs in the skin[16,97] (reviewed in ref. 98), as well as the induction of tolerance in a CS model by the intracutaneous injection of CGRP.[99] CGRP can also inhibit T-cell proliferation and IL-2 production,[100] as well as peripheral blood mononuclear cell proliferation; the response to tetanus toxin antigen was reduced by as much as 50% by preincubation with CGRP, for example. Interestingly, these immunosuppressive effects can be mitigated by neutralizing IL-10, suggesting this as a channel through which CGRP exerts its effects.[101] This may also explain the apparent production of CGRP by rat and human lymphocytes themselves, in response to mitogenic stimuli,[102,103] as a self-regulatory loop to restrain proliferation. CGRP does induce cAMP production in keratinocytes, although basal levels of cAMP and the increases seen in response to CGRP are both higher in LCs.[91] LCs also contain transcripts of both pituitary adenylate cyclase-activating peptide (PACAP) receptors, VPAC1 and VPAC2.[104] Accordingly, intradermal injection of 5 μM PACAP 15 min prior to hapten sensitization can significantly inhibit ear swelling upon challenge 1 week later.[105] This implies that PACAP, like CGRP, can inhibit antigen presentation by LCs.

Both SP and the neurokinin-1 receptor are expressed by human monocytes and macrophages.[106] SP down-regulates membrane-bound enzymes on monocytes, as well as inducing the release of lipo-oxygenase and cyclo-oxygenase pathway products (i.e. leukotrienes and prostaglandins)[22] and TNF-α.[107] The production of IL-1, IL-6, IL-12 and TNF-α by macrophages is induced by nanomolar concentrations of SP,[108,109] as is the production of keratinocyte IL-1.[110] Significant T-lymphocyte proliferation is provoked by the specific binding of SP at nanomolar levels;[21,111–115] increases in DNA and protein synthesis in such cells are indicative of this proliferative effect[116] (reviewed in ref. 117). Owing to the presence of SP and NK-1 receptors in both immune and nervous systems, the question again arises as to source

and target in *in vivo* functions. This question was directly addressed in a series of elegant experiments by Chavolla-Calderón and colleagues who deleted the *PPT-A* gene (from which the SP transcript is derived) or sensory nerve fibres and found that the immune complex-mediated hypersensitivity response in murine lung tissue was abolished and could not be overcome by reconstituting such mice with wild-type bone marrow.[118] These experiments lend strong support to the contention that the requirement for the *PPT-A* gene in inflammation does not lie within immune cells but requires a neural source.

Perhaps in agreement with the finding that mechanical denervation increases the time that skin takes to heal[119] and reduces macrophage and T-cell infiltrates into the wounded site,[120] platelets have been shown to express neurokinin receptors 1 and 3 and aggregate in response to SP.[121] The numbers of SP-positive fibres associated with blood vessels increase in the skin following burns,[122] implying that this peptide is involved in the recruitment of immunity and the repair of tissue following damage caused by inflammation or injury. Tissue lymphocyte populations have a higher percentage of peptide mediator receptor-positive cells than blood lymphocytes;[123] fluorescent somatostatin conjugates are bound by one-third to one-half of T and B cells from lymphoid sites, this decreases to less than one-quarter in blood lymphocytes.[124] It is unclear whether this reflects induction of this phenotype at a certain site, or selective homing of such a population to the tissues.

## Local neuroendocrine modulation of skin function

Cells within the skin are capable not only of responding to neuroendocrine mediators, but can, in many cases, synthesize and process the same mediators themselves, generating an additional, local level of fine-tuning of immune responses.[125–132] Corticotropin-releasing hormone (CRH) and the pro-opiomelanocortin (POMC)-derived peptides α-melanocyte-stimulating hormone (α-MSH), ACTH and β-endorphins can all be produced by cells in cutaneous compartments (see Table 1).

Along with resident macrophages, keratinocytes harbour β-adrenoreceptors and can also synthesize and produce catecholamines; defects in catecholamine responsiveness have been implicated in vitiligo and atopic eczema.[133,134] Treatment of LCs *in vitro* with epinephrine or norepinephrine inhibits their antigen-presentation capability and diminishes CS responses when administered locally or at a remote site *in vivo*,[135] implying that catecholamines released either from cutaneous nerves or the adrenal gland are capable of reducing LC activity. The same suppressive effect may also occur elsewhere, as $\beta_2$-agonist treatment inhibits mast cell TNF-α release.[136] These observations may explain the inhibition of cutaneous norepinephrine turnover observed in the hours following oxazolone sensitization,[137] effectively relieving cutaneous leucocytes of inhibition in order to initiate a response to the antigen.

## Evidence of neural involvement in the CS response

Given the presence of small, unmyelinated c-fibres in the skin, their involvement in neurogenic inflammation,[138] and their functional association with epidermal LCs and mast cells, it was hypothesized that their removal would impact upon a CS model, a primarily Th1-mediated response to the hapten antigen 2,4-dinitrochlorobenzene (DNCB).[139] In this model, sensitizing hapten is given to the abdomen, or another site, and a challenge with the same hapten given 4 days later to an ear results in a CS response measured as increased ear thickness 24 hr after challenge. This presents a useful model for using to test the role of cutaneous nerve fibres in a specific immune response. Chemical deletion of vanilloid receptor-1 (VR-1)-positive nerve fibres can be achieved with a sufficiently high dose of capsaicin.[140–143] Denervation of the site of sensitization by topical capsaicin application could abolish the response to DNCB at the site of challenge, provided denervation was carried out before sensitization.[139] A similar outcome was observed, regardless of whether all nerve fibres were destroyed by application of a dilute phenol solution, or if sensory nerve fibres alone were destroyed by capsaicin application. The same outcome was observed following denervation of both the sensitization and challenge sites (abdomen and right ear, respectively) (Fig. 2). No evidence of a systemic effect of topical capsaicin application was found, as a sensitizing application of DNCB to the back of a mouse on which the abdomen had been denervated produced a normal CS response measured at the ear. The CS response measured at the ear was also lost if the capsaicin was administered systemically, destroying all sensory nerve fibres. LC migration, and vascular and lymphatic integrity, all appeared to be unaffected in denervated tissue. A separate study carried out some years previously showed that in rats systemically denervated with capsaicin, there was no difference in neutrophil chemotaxis towards SP,[72] suggesting that treatment with capsaicin is not toxic to the immune system and that these cells retain their responsiveness to this mediator, despite its depletion from the skin. However, VR-1 receptors have been found on a range of skin and immune cells, meaning that the possibility of direct effects of capsaicin treatment on immune function remains.[144–147] The time course of denervation experiments suggests that any effects of capsaicin on immune function would need to be non-toxic and very long lasting, something that would be unusual for this chemical agent at the doses used.

In a study of adjuvant-induced arthritis, it was discovered that capsaicin injection into the draining lymph node did not affect manifestation of the disease in the region served by the denervated node, but it did preclude the spread of inflammation to the contralateral limb.[148] The role of lymph node innervation has also been investigated in CS reactions.[149] Denervation of the lymph node draining the site of sensitization similarly compromises the CS response measured at the ear, reducing ear swelling to the same extent as complete removal of the node. Consistent with the anti-adhesive properties of SP,[64,65] subcutaneous injection of the peptide over the node before challenge rescues the response in a dose-dependent manner. It appears, from these experiments, that cutaneous nerve fibres and nerve fibres supplying lymph nodes are required for the normal process of eliciting a CS response. Indeed, cutting off sensitization or challenge sites, or draining lymph nodes from their nerve supply, results in a failure to release specific (memory) cells for the hapten. Immunological theory states that memory cells traffic from the draining lymph node, and memory is eventually present in all lymph nodes, but these experiments suggest that this phenomenon may require neural involvement. Controversially, perhaps, they also suggest that there might be a neural memory for host challenge, as well as an immunological memory for specific antigen, and that this is required to release memory on subsequent challenge by the same antigen. The memory for hapten-antigen from the lymph node draining the site of sensitization is made available to distant (naive) sites of subsequent challenge with hapten via a neural mechanism sensitive to interruption by neurotoxins (capsaicin and phenol), which can be replaced by the administration of SP.

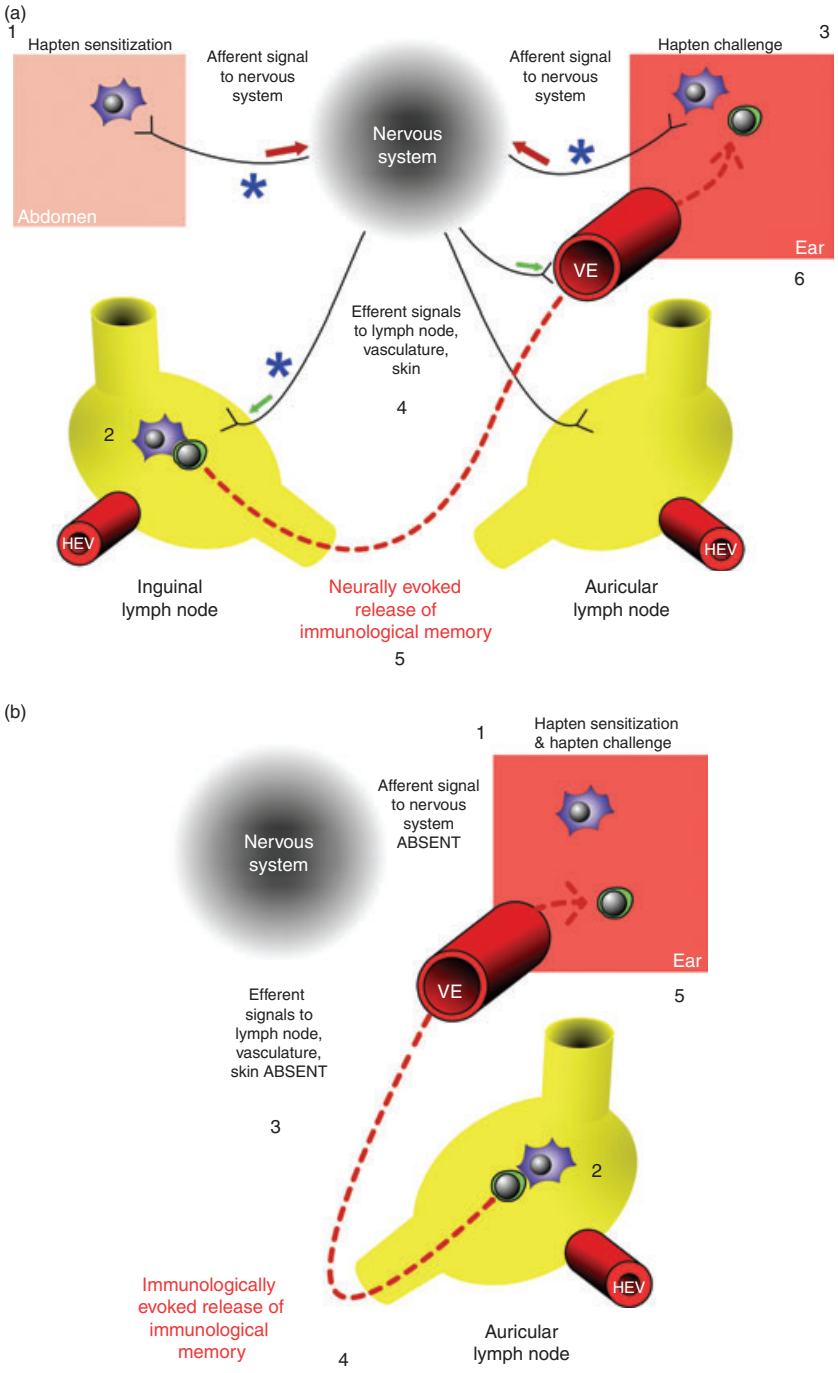
## Evidence of neural involvement in leucocyte release from bone marrow

Bone marrow is innervated with both myelinated and non-myelinated nerve fibres,[42] many of which have been shown anatomically to associate with stromal cells, specifically perivascular cells.[150] As stromal cells are a key source of growth factors and regulatory adhesion molecules for haematopoietic cells, it was hypothesized that this association represents a site of neural control of haemopoiesis[151–153] and consequently of immune responses.

It has been suggested that nerve fibres present in the marrow regulate the mobility of haematopoietic cells and the permeability of the stromal cells that surround the vascular network. Severing the femoral nerve hat supplies the bone marrow in the femur, or chemical sympathectomy with 6-hydroxydopamine causes a marked and prolonged reduction in leucocyte numbers in the bone marrow (by approximately one-third) and a concomitant increase in the circulation.[151] Interestingly, chemical sympathectomy did not provoke mobilization of colony-forming progenitor cells from the marrow, whereas mechanical denervation did, ruling out adrenergic input and implicating other transmitters in the control of adhesion of these cells in the marrow. These findings have been further confirmed by surgical removal of sympathetic ganglia supplying the femur (J. Miyan, unpublished data) indicating that the effects are probably a result of the loss of neural control, rather than direct or indirect effects on immune cells. The authors proposed that nerve fibres can alter the adhesive properties of perivascular cells and perhaps even the haematopoietic cells themselves, controlling the mobilization of such cells into the periphery. Treatment with capsaicin produced a dramatic change in bone marrow haemopoiesis, as measured in *in vitro* colony-forming assays,[152] suggesting a direct role of CGRP, a suggestion confirmed by the finding that this peptide had direct access to haemopoietic progenitors to induce changes in proliferation, while other mediators required indirect pathways, presumably via stromal cells to mediate changes in activity.[152] Neural control of haemopoiesis through c-fibres may underlie the complications seen in burns patients where leucocyte profiles in the peripheral blood are abnormally high and include immature and progenitor cells. Furthermore, spinal injury patients show a loss of bone marrow activity below the level of the spinal lesion, suggesting that this loss of function may result from loss of neural input.[154–156] The indications from all these lines of evidence point to significant neural involvement in bone marrow function and leucocyte mobilization to the circulation. Coupled with the data on neural involvement in peripheral host defence and immune functions, there is convincing evidence supporting a case for neural co-ordination, something that only comes to light in *in vivo* investigations where neural elements are targeted.

## Immune system to nervous system communication

The guinea-pig superior cervical ganglion, located in the neck and the most likely source of the sympathetic innervation of the ears and auricular lymph nodes, contains around 1500 mast cells which degranulate upon antigenic stimulation of the ganglion *in vitro*, releasing histamine, leukotrienes and prostaglandins.[157,158] Following as little as 5 min of exposure to ovalbumin, compound action potentials have been recorded from the nerve trunk leaving the ganglion. This reflects an increase in the number of postganglionic neurones induced to fire, possibly mediated by products of mast cell degranulation.[157] This may represent a feedback loop of neural activity, a notion consistent with pro-inflammatory cytokines as sensitizers of sensory neurone depolarization and of autonomic neuronal involvement in negative feedback control of inflam-




mation. However, it is unclear how and why mast cells should populate the ganglion, or how antigen would be delivered to this site *in vivo*. In different experiments, treatment with TNF-α, IL-1β or IL-6 elevated the release of peptide mediators from isolated sensory neurones in response to low-dose capsaicin,[159] providing evidence that the function of not only sympathetic, but also sensory, nerves can be modified by inflammatory cytokine levels. Several other studies have demonstrated that these same cytokines can inhibit the release of norepinephrine from sympathetic neurones in the myenteric plexus and spleen, whereas ACTH stimulates norepinephrine release.[48]

In patients with minor stroke, a delayed-type hypersensitivity response was markedly weaker when elicited on the side of the body affected by the stroke.[160] This may connect with findings that neocortical stimulation in rats can increase T-lymphocyte output from the thymus, apparently by glucocorticoid-independent means.[161] Using viral *trans*-synaptic tracing methods in rats, a pathway from the bone marrow, spleen and lymph nodes to the

**Figure 2.** Effect of sensory denervation on the manifestation of contact sensitivity (CS). (a) Challenge at a site remote to sensitization: nerves are required to relay activation signals for CS between distant sites. Upon hapten sensitization of the abdomen (1), a signal is generated in the c-fibre associated with the Langerhans' cells (LC). The LC migrate to the draining lymph node (2) and interact with naïve, hapten-specific T lymphocytes. Upon ear challenge 4 days later (3), a second signal reaches the nervous system, prompting efferent signalling (4) to the inguinal lymph node (which harbours memory T cells from sensitization), as well as the vasculature and skin cells at the site of challenge. This stimulus evokes the release of memory T cells into the circulation (5), whereupon they migrate to the site of challenge and secrete a variety of pro-inflammatory mediators, contributing to contact sensitivity (6). When the site of sensitization is denervated with capsaicin 48 hr beforehand (top left asterisk), hapten sensitization of the abdomen does not generate a nervous system signal. However, LC migration to the local (inguinal) node and memory T-cell generation is unaffected. There is now a discrepancy in the system; the animal has immunological memory of exposure to the hapten, but no neural memory was formed. Upon challenge of an innervated site (ear), the nervous system receives the appropriate signal, but fails to mobilize memory T cells from the remote (abdominal–inguinal) lymph node. This, in turn, means that no recordable CS response occurs. Denervation of the site of challenge (top right asterisk) does not affect the propagation of a sensitization signal or formation of immunological memory, but a signal is not received from the site of challenge, meaning that no elicitation of the neural memory occurs, efferent signals are not generated and immunological memory is not mobilized, rendering the CS response absent. Denervation of the lymph node draining the site of sensitization (bottom asterisk) does not prevent the formation of neural or immunological memory, but the signal to the lymph node following challenge cannot be delivered. Consequently, mobilization of immunological memory fails and no CS response takes place. Loss of the CS response is reversed by the artificial infusion of substance P (SP) to the denervated node (data not shown). (b) Sensitization and challenge at the same site: local mechanisms exist for the activation of CS that are independent of nerves. Denervation of the site of sensitization or the draining lymph node does not preclude the formation of immunological memory. Following sensitization (1), a population of memory T cells, specific for hapten, are formed and remain in the draining lymph node (2). If the same site is used for challenge 4 days later, LC harbouring antigen will migrate to this same node, prompting effector T-cell release and migration to the site of challenge (3,4), initiating the response independently of nervous system input (5). The site of challenge and/or the draining lymph node can be denervated in this situation with no visible effect on the CS response. HEV, high endothelial venule; VE, vascular endothelia.

spinal cord, medulla, hypothalamus and insular cortex has been described (ref. 162; Krisztina Kovács, personal communication), reinforcing the hypothesis that bone marrow and immune system function can be brought under the control of the autonomic nervous system. These examples suggest that alternative central and local neural reflexive pathways are associated with immune system function and also illustrate a model of a hierarchy of candidate levels of regulation by nerve fibres. Regulatory loops could be entirely local, depending only on nerve fibres in the periphery, and decisions could be made at the level of the sympathetic ganglia, the spinal cord, or even the brain itself, echoing the theories proposed by Besedovsky & del Rey.[132]

## Conclusions

### Integrated host monitoring and defence

Evidence of peripheral nerve fibres showing close anatomical association and functional effects upon immune cells has not only been documented for lymphoid organs, but also for tertiary sites, such as the skin and mucosal surfaces.[136]

From our own experiments (summarized above) 'memory' of antigen exposure (wherever and in whatever form that memory may reside) is formed in both immune and nervous systems. Second exposure to that antigen at another site elicits the release of peptides from neurones in the draining lymph node, stimulating the release of hapten-specific T lymphocytes (and perhaps any other lymphocytes in the node) into efferent lymphatics and the circulation. In animals with a denervated lymph node, this neural input is missing and the lymphocytes are not mobilized, a situation reversed by the artificial infusion of SP. We speculate that the nervous system retains a memory for prior challenge, although the level of specification of this neural memory is not yet clear. Immune specificity arises from the T-cell receptor for antigen, but such specific receptors are neither present nor required on sensory nerves for antigen to produce neural memory. The mechanism, in this case, is the modality-specific sensing cell (e.g. a photoreceptor, hair cell, chemosensor or, in this case, an APC) that produces a response to the stimulation that is transmitted to the sensory nerve. The pattern of activity in the sensory nerve codes for the pattern produced in the sensing cell (e.g. in a frequency of nerve potentials). In this way the sensory nerve would need no specific mechanism to detect the antigen, relying instead on the membrane changes of the APC as it processes antigen, to produce a characteristic signature for the antigen. Clearly, this requires further experimental investigation, but it is not an unreasonable suggestion given the operation of other sensory mechanisms. We therefore propose that sensory nerves, in conjunction with LCs/DCs, 'sense' exposure to antigen. Speculative although this may seem, we are far from the first to propose that this neuroimmune network be regarded as an additional sensory system.[163]

When theories this controversial are presented, they rightly undergo an extraordinary degree of scrutiny. Although there may always be alternative, non-neural interpretations of some data, the collective body of evidence reviewed here, and other evidence we have previ-

ously reviewed[15] represents a strong case for the existence of a neural co-ordination of host defence activity. As mentioned above, the presence of common mediators and receptors across these two systems, and also shared with the neuroendocrine and endocrine systems, present serious problems in the design of experiments and interpretation of data. The utilization of transgenic models, as well as new techniques to selectively affect one or other systems, may aid in the resolution of these issues.

This review has emphasized neural involvements in the co-ordination of multiple immune activities. It demonstrates the importance of neural signalling in either the promotion or the limitation of innate and adaptive immune responses present *in vivo*. It offers a physiological and pharmacological understanding for therapeutic opportunity in immune modulation.

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