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SIGNALING THE BRAIN IN SYSTEMIC INFLAMMATION: THE ROLE OF PERIVASCULAR CELLS

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1. ABSTRACT

Cytokines released from activated immune cells can act on the brain to elicit a range of centrally mediated acute phase responses. Several lines of evidence point to the barriers between the brain and its fluid environments, mainly cells associated with the cerebral vasculature, as critical sites for the transduction of circulating cytokine signals, and the initiation of brain responses to them by virtue of their capacity to produce local signaling molecules, notably prostaglandins. While it was initially assumed that such functions were the province of the vascular endothelium, recent work has identified a subset of marrow-derived brain macrophages, termed perivascular cells, as exhibiting the greater sensitivity to prostanoid synthesis induced by systemic cytokine or endotoxin challenges. Application of a novel liposome-based targeting method supports a critical involvement of brain macrophages, and their capacity to manifest induced prostanoid synthesis, in the interleukin-1-induced recruitment of control circuitry governing at least one acute phase response (hypothalamo-pituitary-adrenal axis activation), and suggests a two-way interaction between perivascular and endothelial cells in monitoring circulating cytokine signals. The ability to selectively manipulate perivascular cells holds promise for further informing mechanisms of immune-to-brain, and for intervening in pathologies that may result from dysfunction of such interactions.

2. INTRODUCTION

Cytokines released by activated cells of the monocyte/macrophage lineage as a consequence of infection or inflammation act on the brain to elicit a host of acute phase responses. These include such adaptations as somnolence, lethargy, fever, and anorexia, commonly referred to as "sickness behavior" (1,2), and a range of metabolic effects, dominant among which is activation of the hypothalamo-pituitary-adrenal (HPA) axis (3,4). This latter effect has come to be viewed as paradigmatic of the bidirectional nature of interactions between the immune and central nervous systems. Glucocorticoids, the end product of the HPA cascade, are powerful and broad-spectrum inhibitors of immune and inflammatory reactions, and have been exploited clinically for this purpose for decades (5,6). The recognition that interleukin-1 (IL-1) and other proinflammatory cytokines can potently stimulate HPA output (7,8) defined a feedback loop, in which the immune system appears to have co-opted the endocrine mechanism to negatively regulate its own activity (9). That is, to restrain excess production of cytokines and immune cell proliferation in response to infectious/inflammatory events. Dysregulation of the endocrine arm has been implicated in the genesis of a wide range of autoimmune disorders in susceptible animal models (10,11) and in man (12).

Because cytokines are large, hydrophilic molecules, which would normally be excluded from traversing the blood-brain barrier (BBB), the question of how they access the CNS to effect specific responses has remained a critical and daunting one. A variety of alternate routes have been proposed. Separate and saturable transport systems for each of the major proinflammatory cytokines to cross the barrier have been identified [e.g.,(13)], though their capacity to allow entry of biologically significant concentrations in response to acute insults remains open to question. Circumventricular organs that lack a functional BBB are obvious potential points of access that have been implicated strongly in the febrile responses to IL-1 and LPS (14). Transduction by peripheral nerves, particularly the vagus, has been widely explored, and seems to play a major role in challenges given via the intraperitoneal (ip), but not the intravenous (iv) or intracerebroventricular (icv), routes (15,16). Finally, transduction by non-neuronal elements associated with the cerebral vasculature, with subsequent release of local messengers, such as prostanoids (17,18), nitric oxide (19,20) and/or cytokines, themselves (21,22), has been prominently implicated as a means by which circulating cytokines may access CNS mechanisms. Sorting among

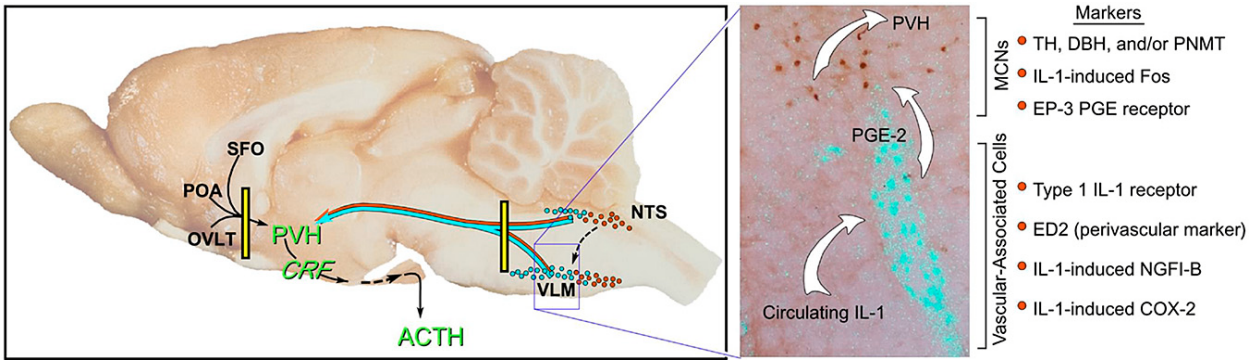

Figure 1. Involvement of medullary catecholamine neurons in IL-1 influences on PVH. Left: Organization of adrenergic (red) and noradrenergic (blue) projections from the nucleus of the solitary tract (NTS) and ventrolateral medulla (VLM), and of descending inputs from the preoptic region (POA) and forebrain circumventricular organs (SFO, OVLT), to the PVH. Vertical bars indicate approximate positions of knife cuts used to assess involvement in IL-1 effects. Right: Histological section through the VLM labeled for IL-1 induced Fos-immunoreactivity (Fos-ir; brown) and IL-1R1 mRNA (blue). Arrows depict a model for prostaglandin-mediated of activation of medullary aminergic neurons. Listed at the right are markers localized to medullary catecholamine neurons and local vascular elements in support of the scheme.

these alternatives is complicated by the fact that certain cytokines and their receptors can be markedly upregulated in brain in response to at least some immune insults [e.g., (22,23)], with expression propagating though the CNS ostensibly in paracrine waves (24), and contribute to the manifestation of acute phase responses (3,25).

3. HPA CONTROL CIRCUITRY

We have used a functional neuroanatomical approach to explore the circuits and mechanisms underlying IL-1 induced activation of the central limb of the HPA axis. The obligatory focus of the analysis is the paraventricular nucleus of the hypothalamus (PVH), which is the seat of parvocellular neurosecretory neurons that express corticotropin-releasing factor (CRF) for the initiation of HPA output (26). The PVH also contains separate populations of cells that project to central autonomic cell groups, including the sympathetic preganglionic column, and magnocellular neurosecretory neurons that release the peptide hormones oxytocin and vasopressin from terminals in the neural lobe of the pituitary (27). Our general approach has been to use immune challenge-induced expression of immediate-early gene markers, such as *c-fos*, to define patterns of cellular activation alone, or in conjunction with histochemical and/or axonal transport methods to define the phenotypes and/or connectivities of activated neurons. Functional dependence of target neuron responses upon the integrity of a candidate afferent mediator is then tested experimentally. Figure 1 depicts the model; key findings in support of it are summarized below:

- Intravenous IL-1 at doses moderately above the threshold for HPA activation provokes a restricted pattern of cellular activation in brain, with a handful of interconnected cell groups comprising a core circuitry for central autonomic and neuroendocrine regulation being prominently activated. Among neurons identified as projecting to the PVH, only functionally related medullary catecholamine neurons in the nucleus of the solitary tract and ventrolateral medulla exhibit IL-1 sensitivity (28).

- Transections of ascending catecholaminergic projections near their origins in the medulla block IL-1-induced upregulation of Fos-ir and CRF mRNA in the PVH (28). A mediating, rather than a permissive, role is indicated by the failure of the same lesion to disrupt emotional stress effects on PVH responses, despite the fact that both challenges activate indistinguishable complements of PVH effector neurons and their medullary aminergic afferents (29).

- A vascular site for the initial transduction of blood-borne IL-1 signals is suggested by the observations that neither ablations of projections from circumventricular organs nor abdominal vagotomy disrupt IL-1-stimulated activational responses in hypothalamus or medulla (18,28). More directly, non-neuronal elements associated with the cerebral vasculature are the only site of type 1 IL-1 receptor (IL-1R1) expression that exhibit sensitivity to our standard iv IL-1 challenge (30).

- Prostaglandin mediation is supported by the ability of systemic or central cyclooxygenase (COX) inhibition to comparably antagonize iv IL-1-induced responses in brainstem and hypothalamus, and the ability of intramedullary microinjections of prostaglandin E2 (PGE2) to discretely activate local aminergic neurons, as well as mimic systemic IL-1 induced responses in the PVH, all in dose related manners (18,31). A link between the postulated local (medullary) release of prostaglandins with catecholaminergic neurons is provided by the localization of IL-1 induced Fos expression to medullary aminergic cells that express the EP3 receptor, the dominant PGE2 receptor subtype expressed in brain (32).

The proposal that the initial transduction step from the periphery to the brain occurs at or near the blood brain interface is in keeping with the fact that under normal

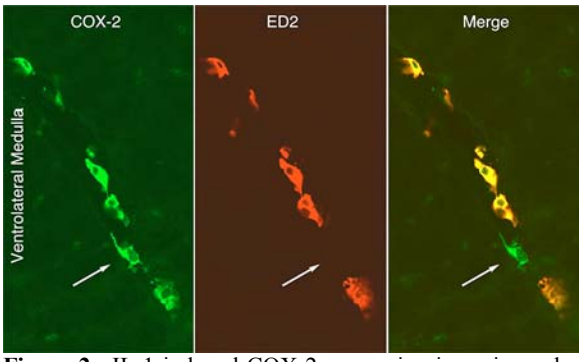

Figure 2. IL-1-induced COX-2 expression in perivascular cells. Scanning confocal laser microscopic images showing dual immunostaining for COX-2 (green) and a marker for perivascular cells/macrophages (ED2; red) associated with a blood vessel in the medulla. The yellow color in the left panel is from merged confocal images, and represents a positive signal for both COX-2- and ED2-irs. Results of dual immunolabeling of material from IL-1-challenged rats revealed that very nearly all COX-2-ir cells also stained positively for the ED2 antigen, suggesting that they may be considered "perivascular cells" as defined by Graeber et al. (49). COX-2-ir was never found to co-localize with endothelial markers, suggesting that endothelial cells do not manifest substantial COX-2 expression in this paradigm. The arrow indicates a single COX-2-positive, ED2-negative cell.

conditions, at least, receptors for proinflammatory cytokines that are capable of independently eliciting HPA responses are expressed mainly at the barriers between the brain and its fluid environments (33-35). This is true of IL-1R1, which mediates the cytokine's biological activities. IL-1R1 in brain is expressed most prominently at the meninges, ependyma, choroid plexus and vasculature. Neuronal expression is sparse, generally lacking from cell groups implicated as key players in HPA control, and, in contrast to vascular receptor expression, unresponsive to systemic immune insults (30).

4. THE PROSTAGLANDIN CONNECTION

A key feature of the HPA and at least some other acute phase responses is their susceptibility to interference by blocking the production of prostaglandins (36-38). PGE2 levels within the brain are elevated following IL-1 or LPS administration (39), and blocking their synthesis with aspirin-like drugs can disrupt cytokine-induced activation of the secretory and biosynthetic activities of HPA control systems (18,40). Furthermore, a recent study involving the use of mice bearing targeted disruption of each of the PGE2 receptors has shown that either the loss of EP1or EP3 receptors can attenuate HPA secretory responses induced by LPS (41). A key enzyme in the initiation of prostaglandin synthesis from arachidonic acid is cyclooxygenase, which exists in at least two isoforms. COX-1 is expressed constitutively in a variety of cell types, including neurons (42), and is generally unresponsive to immune insults (43), while COX-2 exhibits limited neuronal expression, but is readily inducible in the vasculature and meninges (31).

There is debate as to the identity of the vascular cell type(s) that exhibits inducible prostanoid production for the engagement of CNS mechanisms (44). It was initially assumed that this capacity localized to endothelial cells, and subsequent work has established unequivocally their capacity to mount such responses. Most of the data supporting a primary involvement of endothelia have been gathered in paradigms employing relatively high doses of LPS (45-47), and evidence is available to implicate perivascular cells (PVCs), a subset of resident brain macrophages, as playing a significant role in this regard (48). To determine whether this discrepancy might derive from differences in the nature and/or intensity of the stimuli used to model immune insults, COX-2 expression was monitored alone, or in conjunction with endothelial, perivascular and glial cell markers, in brains of rats treated with varying doses of IL-1 or LPS. Vehicle-treated animals displayed weak COX-2 expression in the meninges, choroid plexus and larger blood vessels. Rats challenged intravenously with IL-1 over a range of doses showed a marked increase in the number of vascular cells displaying COX-2-ir. More than 90% stained positively for the ED2 antigen, specifically identifying them as PVCs, while none co-expressed EC or glial cell markers (Figure 2). A subset of ED2-positive cells co-labeled for IL-1R1, identifying them as potential targets for circulating or locally released IL-1. Low doses of LPS (0.1 μg/kg) elicited a similar response profile, but higher ones (2 or 100 μg/kg) provoked COX-2 expression in a progressively greater number of cells exhibiting distinct round versus multipolar morphologies, which corresponded to cells expressing endothelial (RECA-1) or PVC (ED2) markers, respectively. Similarly, ultrastructural analysis localized COX-2-ir to the perinuclear region of endothelial cells of LPS-, but not IL-1-, treated rats. We conclude that PVCs exhibit the lower threshold to COX-2 expression in response to either IL-1 or LPS treatment, and that enzyme expression by endothelial cells requires one or more facets of the more complex stimulus presented by LPS. Because PVCs are unlikely to be accessed directly by circulating cytokines, the results point toward an interaction between vascular macrophages and endothelia in transducing immune signals and manifesting induced prostanoid expression and release.

5. ANATOMY AND PHYSIOLOGY OF PERIVASCULAR CELLS

We follow Graeber et al. (49) in referring to vascular-associated macrophages as *perivascular cells* (PVCs*)*, though this population has also been referred to as perivascular microglia and fluorescent granular perithelial cells, among other designations (50). Along with a similar cell type associated with the meninges, PVCs comprise the brain's principal resident macrophage population. Both are derived from bone marrow progenitors that take up residence in the brain in the early postnatal period and turn over throughout adult life at a relatively low rate (51,52,53). They are not integral components of the vascular wall, situated instead in the perivascular space between the vascular basal lamina and the glia limitans (Figure 3). While they may share a common lineage with parenchymal microglia and pericytes (50,51), PVCs are

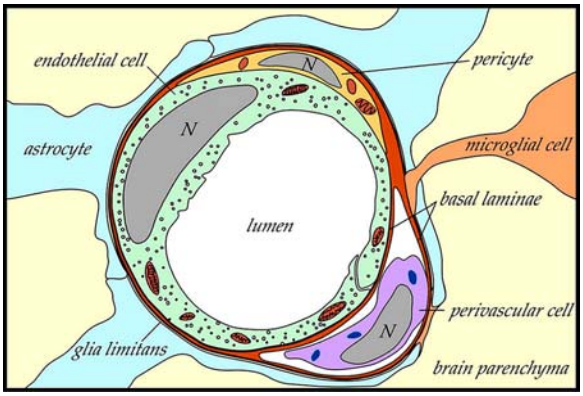

Figure 3. Schematic drawing of a cerebral blood vessel to illustrate the disposition of PVCs in relation to other vascular, glial and parenchymal cell types. PVCs are situated in the perivascular space between the vascular basement membrane and the glia limitans. They are distinct from pericytes and parenchymal microglia.

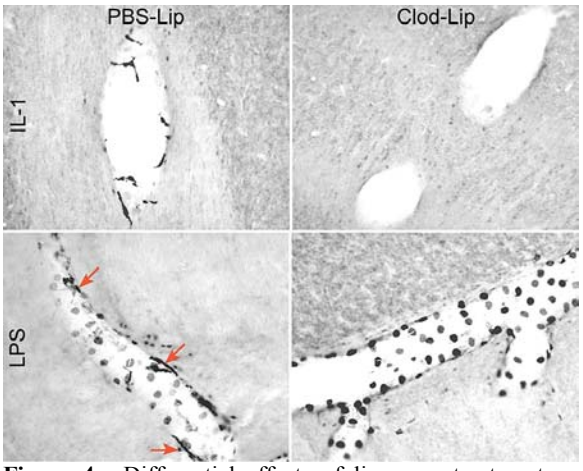

Figure 4. Differential effects of liposome treatment on induced vascular COX-2 induction. IL-1 treatment in control (PBS-Lip) animals induces COX-2 in multipolar cells (PVCs), while LPS activates in enzyme in both PVCs (arrows) and in round profiles that conform to perinuclear regions of endothelia. While clodronate liposome (Clod-Lip) treatment eliminates IL-1-induced COX-2 expression by ablating PVCs, it enhances LPS effects on the number and staining intensity of COX-2-ir in endothelia, suggesting that PVCs serve normally to restrain endothelial cell responsiveness. Note that apparent presence of labeled cells within the lumen of vessels raises questions as to the extent to which circulating monocytes may contribute to and confound the analysis. We believe these profiles to be representative of perivascular cells captured in tangential sections that graze the top or bottom of vessels contained within relatively thick sections used for light-level analysis. In ultrathin sections processed from challenged rats prepared for immunoelectron microscopy, we have never observed either COX-2- or ED2-labeled elements on the luminal side of a vessel (67).

anatomically, functionally and phenotypically distinct. They are phagocytic, and can be labeled by tracer injected either centrally or systemically (52,54). In the rat, they are identified conclusively by immunostaining for the ED2 macrophage differentiation antigen (49,55), which has been recently identified as a macrophage scavenger receptor, designated as CD163. No comparable marker has been identified for murine or human PVCs, although this cell type clearly exists in these species (49,51,56). PVCs exhibit upregulated expression of MHC class II antigens upon stimulation (57), and have been suggested as the major antigen presenting cell type of the CNS (58). They have also been implicated as key participants in the phenomenon of immune surveillance of the nervous system (59,60), and in a host of pathological processes, including the entry of AIDS virus into the nervous system (50,61,62).

The second brain macrophage population, associated with the leptomeninges is commonly referred to as meningeal macrophages. This population shares with PVCs a number of general developmental, morphological, phenotypic (ED2), and functional (capacity for COX-2 induction) characteristics. The extent to which they may be differentiated by factors other than location remains to be determined.

6. PROBING PERIVASCULAR CELL FUNCTION

A means for selectively ablating the PVC/meningeal macrophage population has been described (63) that exploits the phagocytic activity of PVCs and meningeal macrophages to selectively deplete them by intracerebroventricular injection of liposome-encapsulated clodronate. This approach holds potential for ascertaining the role of these cell types, and their capacity to display induced COX-2 expression, in HPA and other acute phase responses. Clodronate is a biphosphonate drug used in the treatment of osteolytic disease, which is not in and of itself toxic, but does cause irreversible metabolic damage and apoptosis at sufficient intracellular concentrations (64). Liposomes may be labeled with a fluorophore, such as the carbocyanine dye, DiI, to enable visualization of cells that have incorporated them. Injection of control liposomes, encapsulating neutral-buffered saline, resulted in selective DiI labeling of meningeal and vascular cells that express the ED2 marker for perivascular and meningeal macrophages (63,65). Following clodronate liposome treatment, ED2 positive cells were frankly reduced in rats sacrificed at 3 days, and virtually absent in animals killed 5-7 days, after central injection. Control rats sacrificed 3 hours after intravenous injection of a moderate dose (2 µg/kg) of IL-1 displayed the expected induction of COX-2 in the meninges and cerebral vasculature; pretreatment with central injection of clodronate liposomes 5 days earlier completely eliminated detectable IL-1-induced COX-2 expression at these loci, without affecting that seen constitutively in neurons (65). By contrast, LPS injections (2 µg/kg) in PBS-Lip rats provoked the expected COX-2 induction in both multipolar (PVC) and round (endothelial cell) vascular profiles (Figure 4). Predictably, LPS challenges of Clod-Lip treated rats elicited COX-2 responses only in endothelia, but this effect was remarkably potentiated, in that COX-2-ir endothelial cells were far greater in number (2.6 fold increase) and staining intensity in macrophage-depleted than control rats (65). This

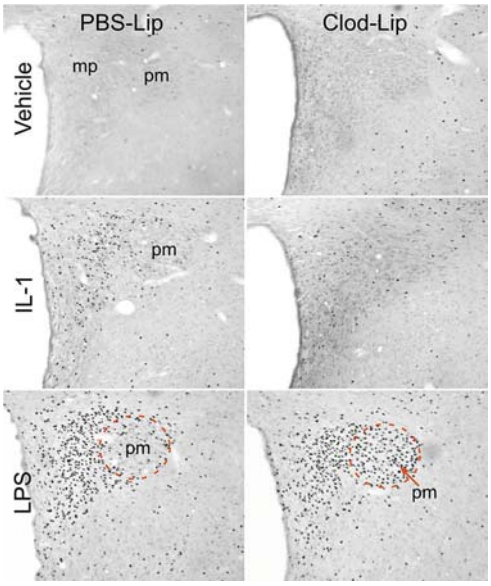

Figure 5. Liposome treatment effects on PVH responses. Fos-immunoreactivity in rats treated with PBS- or Clod-liposomes and subsequently challenged with intravenous injections of vehicle, 2 µg/kg IL-1 or LPS and sacrificed 2 hr later. Control (PBS-Lip) rats show characteristic distributions and strengths of IL-1- and LPS-induced Fos induction, with labeled cells massed in the CRF-rich parvocellular (mp) region. Clod-Lip treatment markedly reduces the IL-1 response, whereas LPS recruits additional cells in the magnocellular (pm) part of PVH. These effects correlate with distinct vascular COX-2 responses observed in this same paradigm (see Figure 4).

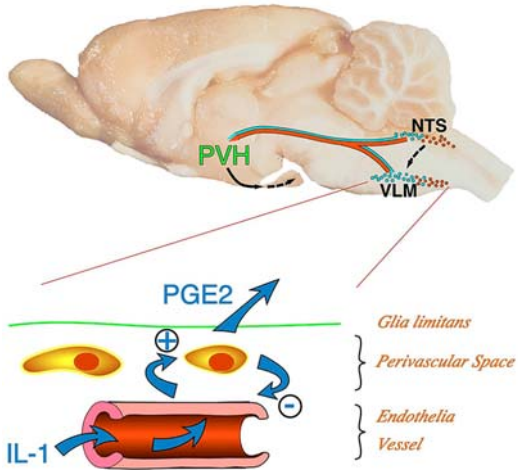

Figure 6. Posited vascular mechanism for the activation of HPA control circuitry by circulating cytokines. Top: Reprise of core circuitry identified as being required for IL-1 mediated activation of the HPA axis, highlighting the role of inputs from catecholamine neurons in the medulla to CRF-expressing neurosecretory cells in the PVH (see Fig. 1). Bottom: Proposed vascular mechanism for the transduction of increased circulating IL-1 signals as involving an initial recording by the vascular endothelium, with subsequent activation of prostanoid (PGE2) synthesis and release by adjoining PVCs (yellow/orange). Factor(s) produced by PVCs feed back to restrain endothelial cell responsiveness. Paracrine factors involved in interactions between the vascular cell types remain to be identified.

enhancement of LPS-induced endothelial cell responses to LPS in the brain macrophage-depleted model suggests that PVCs normally exert a restraining influence on endothelial cell activity, including their capacity to induce COX-2 expression.

Observed differences in IL-1- and LPS-induced vascular COX-2 induction in clodronate liposome-treated animals was mirrored in challenge effects on Fos-ir in PVH (Figure 5). Thus, while Clod-Lip treatment reduced PVH activational responses to IL-1, those elicited by LPS were enhanced. This was not reflected as an increase in the number of responsive cells in the CRF-rich region of the parvocellular division of the PVH, which may be activated in near maximal numbers by LPS treatment, but rather in the recruitment to Fos expression of an additional PVH effector population, magnocellular neurosecretory neurons. The results support a highly significant role for PVCs and meningeal macrophages in the PG-dependent effects of IL-1 on HPA -related endpoints, and endorse the liposome approach as a means by which to assess their role in immune influences on the CNS.

7. SUMMARY AND PERSPECTIVE

Recent findings summarized above inform the current "either-or" debate as to the vascular source of COX-2 induction by indicating that an interplay between at least two key cell types is involved in the transduction and activation of prostanoid synthesis by circulating cytokine signals. These results challenge the widely held view that endothelial cells are the dominant seat of cytokine-induced COX-2 expression in brain (43,45,46,66) by demonstrating a greater sensitivity of PVCs in this regard (67). But because PVCs are not in a position to be accessed directly by circulating macromolecules, and manifest only a limited capacity for IL-1R1 expression, they would appear unlikely to mediate initial transduction events. Moreover, the observation that ablation of PVCs enhances the sensitivity of endothelial cells to COX-2 induction (see Figure 6) suggests a two-way interaction, with PVCs restraining endothelial cell responsiveness. It thus appears likely that both cell types participate in the early steps of the CNS response to systemic immune challenges, with endothelia mediating the initial recording of circulating cytokine signals, as well as the subsequent activation of COX-2 expression in PVCs. It is further suggested that factor(s) derived from PVCs feed back to negatively regulate endothelial sensitivity to circulating immune signals, as reflected in *their* capacity to activate COX-2 expression. The identity of the signaling molecules that mediate endothelial-perivascular cell interactions remain to be determined, though proinflammatory cytokines and nitric oxide may be considered as candidates.

Systemic IL-1- and/or LPS-induced brain and/or vascular upregulation of all major IL-1 signaling components, of IL-6 and its receptor subunits, and of both the endothelial and inducible isoforms of nitric oxide synthase (NOS) have been reported (35,68,69), and these may comprise participants in vascular cell interactions. Endothelial NOS has been directly implicated in inhibiting

TNF-alpha-induced COX-2 expression in endothelial cells (70). Functionally, central antagonism of IL-1 effects, by central administration of neutralizing antibodies or the endogenous IL-1 receptor antagonist, can profoundly disrupt various indices of LPS-elicited HPA activation (3,25). In addition, IL-6-deficient mice have been shown to exhibit impairment of some acute phase responses (71), including LPS-induced HPA secretory activation (72). Where nitric oxide was originally considered as a vascular-derived local mediator of central cytokine effects, the weight of the evidence now supports a restraining role (3), making it a possible mediator of the inhibitory influence exerted by PVCs on endothelial cells suggested by liposome targeting data. It should be noted that brain vascular expression of cytokines is most commonly reported following only very strenuous (>500 µg/kg LPS) insults (22). This will place a premium on using conditions minimally effective for activating either PVCs or endothelial cells, and/or COX-2 induction within them, to identify candidate participants in their interaction. Pursuit of these factors, with special care given to specifying the nature, strength and route of administration of challenges used to model immune insults, may well provide an instructive context for viewing the phenomenon of induced cytokine expression in the brain.

Analyses like those summarized above are aimed at clarifying the circuits and mechanisms that orchestrate CNS responses to immune or inflammatory insults. Although the discrete organization of neurosecretory neurons governing HPA axis function enables one to predict how altered biosynthetic activity of this population may find functional expression in altered secretory output, such predictions require direct empirical testing. Similarly, the available evidence indicates that other centrally-mediated acute phase responses vary substantially in their dependence on COX-2-mediated inducible prostaglandin synthesis in brain. Thus, the extent to which the kind of mechanisms considered here may be relevant to febrile responses and sickness behaviors also demands independent analysis.

Where the brain was once thought to be a site of immune privilege (73), it is now clear that there exists a regular and regulated two-way traffic of immune cells and signals across the blood-brain barrier. Not only does the immune system populate the brain with some of its own, but it also utilizes many of the same peripheral inflammatory mediators (prostaglandins, cytokines) to independently access CNS circuits in a closely regulated manner. Clarifying the nature of this interaction at a basic level should serve to identify points of access relevant to a range of disease states. For example, the brain macrophage ablation technique has already been shown to affect the progression of animal models of multiple sclerosis and meningitis (54,74) and others have established the feasibility of using the stem cells that give rise to this population as vehicles for gene therapy (75,76). Glucocorticoid mediators of the principal system of interest here, the HPA axis, exert profound immunosuppressive and anti-inflammatory effects relevant to a host of pathologies, and, as noted, dysregulation of its central control contributes to the genesis of a wide range of autoimmune disorders in genetically predisposed animal models and in man (10-12).

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