Local Expression of Corticotropin-Releasing Hormone in Inflammatory Arthritis

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Inflammation normally results in enhanced synthesis and secretion of hypothalamic corticotropin-releasing hormone (CRH) which, in turn, exerts antiinflammatory effects by virtue of increased adrenal glucocorticoid production. CRH and CRH binding sites are also expressed in the peripheral nervous and immune systems. In the periphery, CRH has pleiotropic effects on immune/inflammatory systems. We demonstrated CRH expression in the joints of Lewis rats with experimental arthritis and in synovial tissues of patients with rheumatoid arthritis. These data support an autocrine/paracrine immunomodulatory role for CRH.

INTRODUCTION TO CRH AND THE IMMUNE SYSTEM

There is an intricate balance between the immune system and the neuroendocrine system that maintains homeostasis in the face of inflammatory stress. This balance is achieved through shared utilization of peptide mediators and receptors, both in the central nervous system and in the periphery. For example, products of an activated immune system, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), stimulate the production of CRH in the hypothalamus.[1–6] CRH, a 41 amino acid neuropeptide, is a major regulator of the hypothalamic-pituitary-adrenal (HPA) axis, whose release leads to pituitary production of adrenocorticotropic hormone (ACTH), followed by glucocorticoid secretion by the adrenal cortex. Glucocorticoids suppress many components of the inflammatory process, including migration of leukocytes to an inflammatory site, production of proinflammatory cytokines, and production of phospholipid-derived mediators of inflammation. Just as cytokines and their receptors are utilized within the central nervous system, it is increasingly clear that modulatory peptides best characterized by their activity in the nervous system are also critical to the function of the immune system.[7] Many of these "neuropeptides" have been localized to inflamed joints where they may play integral roles in the development or maintenance of inflammation.

A direct role for CRH in immune and inflammatory responses has been postulated since immunoreactive CRH and CRH mRNA are present in human peripheral blood leukocytes, and expression is increased with activation of lymphocytes and monocytes.[8,9] Immunoreactive and bioactive CRH is also present in rat thymus.[10] Human peripheral blood lymphocytes and monocytes[11,12] and resident macrophages of mouse spleen[13] have been reported to bind radiolabeled CRH; however, the mRNA for the recently cloned CRH receptor was not detected in human peripheral blood lymphocytes.[14] A growing body of data support a role for CRH in immunomodulation, although *in vitro* studies have been somewhat contradictory. These data are summarized as follows: (1) stimulation of IL-6 production in peripheral blood monocytes,[15] and enhanced leukocyte IL-1 and IL-2 secretion;[16] (2) down-regulated production of lipopolysaccharide-induced IL-6 and IL-1 in peripheral blood mononuclear cells;[17] (3) stimulation of lymphocyte proliferation and expression of interleukin-2 (IL-2) receptors;[18] (4) inhibition of IL-2 induced splenocyte proliferation;[12] (5) enhancement of NK cell-mediated lysis;[19] and (6) induction of leukocyte-derived ACTH and β-endorphin.[20]

Although it seems clear that CRH can be synthesized by cells of the immune system, CRH and other peripherally expressed neuropeptides can also be derived from the peripheral nervous system. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) causes depletion of neuropeptides from, or destruction of, primary sensory neurons, and significantly reduces inflammation.[21,22] Substance P, a neuropeptide present in capsaicin-sensitive sensory afferent neurons, has been clearly demonstrated to augment inflammatory processes.[23–25] However, many other neuropeptides, including CRH, are also present in capsaicin-sensitive neurons.[26] Additionally, CRH is found in sympathetic components of the peripheral nervous system.[26,27] Levine and colleagues have shown that both sensory afferent and sympathetic innervations contribute to inflammatory processes in the joint,[22,28,29] and CRH could be delivered to the joint by both sensory and sympathetic nerve terminals.

Karalis and colleagues showed that CRH was present locally in the acute inflammatory reaction stimulated by injection of carrageenin, a seaweed-derived polysaccharide, in a subcutaneous airpouch in rats. Immunoneutralization of CRH resulted in a significant decrease of both the volume and cellularity of the inflammatory exudate.[30] The decrease in these inflammatory measures was comparable to that of treatment with antibody to TNF-α.[80] These studies suggest that CRH has predominantly proinflammatory effects in the setting of acute carrageenin-induced inflammation in rats.

Peripheral effects of CRH have been examined by other investigators who have concluded that CRH may also have antiinflammatory activities. Hargreaves *et al.*[31] concluded that in the carrageenin model of footpad inflammation, peripheral effects of CRH were antinociceptive as measured by decreased paw withdrawal latency after treatment with CRH. There is precedent for neuropeptides that are antinociceptive, but proinflammatory such as β-endorphin.[32,33] Wei and colleagues[34,35] reported that large doses of iv administered CRH inhibited neurogenic plasma extravasation, even in hypophysectomized or adrenalectomized rats. Other peptides of the corticoliberin superfamily, such as sauvagine and urotensin I, and structurally unrelated peptides of the neurotensin family also inhibited acute vascular leakage in the system used by these investigators.[35] These observations are not necessarily at odds with studies suggesting a predominantly proinflammatory effect for local CRH in other models of inflammation. The inhibition of plasma extravasation by CRH is measured very early after the stimulus (≤60 min), whereas the decrease in volume and cellularity by immunoneutralization of CRH in the carrageenin-airpouch model was measured at 5 hours after the stimulus.[30] CRH effects on acute plasma extravasation could be due to effects on primary sensory afferent nerve terminals where CRH antagonizes release of substance P,[36] while proinflammatory effects of CRH could be due to direct actions on cells of the immune system. It should be pointed out that peripheral proinflammatory effects of CRH would be antithetical to central CRH effects, where increased hypothalamic CRH activates the HPA axis leading to production of glucocorticoids and suppression of inflammation.

Our group has characterized the expression of CRH in the peripheral joints of rats with experimental inflammatory polyarthritis and patients with rheumatoid arthritis. These studies provide evidence that CRH may be a mediator of immunologically mediated inflammatory processes in animal models and in human disease.

CRH EXPRESSION IN JOINTS OF LEWIS RATS WITH INFLAMMATORY ARTHRITIS

Euthymic LEW/N and congenitally athymic LEW.rnu/rnu, but not relatively arthritis-resistant F344/N rats develop erythema and swelling of peripheral joints within 24 hours after injection of SCW.[37–39] The acute phase of SCW-induced polyarthritis increases to a maximum at 3 days, then gradually subsides. Euthymic LEW/N, but not athymic LEW.rnu/rnu or F344/N rats, go on to develop T-cell-dependent chronic polyarthritis that reaches maximum severity by 28 days after SCW injection. Histologically, the acute polyarthritis is characterized by edema, deposition of fibrin in the synovia and joint space, and infiltration by granulocytes and macrophages. In the chronic phase of SCW-induced arthritis, there is marked synovial cell proliferation

TABLE 1. CRH Expression in Joint Tissues of Rats[43]

	LEW/N	LEW.rnu/rnu	F344/N
SCW Injected			
Day 0	±	±	±
Day 3	3+	3+	±
Day 28	4+	±	±
Adjuvant Injected			
Day 0	±	±	±
Day 4	2+	2+	±
Day 10	2+	1+	±
Day 18	3+	±	±

with villus formation, angiogenesis, infiltration of the synovial tissue by lymphocytes and macrophages, and erosion of marginal cartilage and bone.[38,39] There is also increased expression of biochemical markers, such as class II major histocompatibility antigens, transin/stromelysin, heparin binding growth factor-1 (FGF-1), and cyclooxygenase, that parallels histologic inflammation.[38,40–42]

Hindfoot specimens from uninjected rats showed little CRH immunoreactivity in any rat strain. However, by day 3 after SCW injection, CRH immunostaining increased markedly in euthymic LEW/N and athymic LEW.rnu/rnu rats, but not F344/N rats. iCRH was present in many different sites including the epidermis, synovium (lining cell layer, vascular endothelium, mononuclear inflammatory cells, and stromal fibroblast-like cells), cartilage chondrocytes, subchondral bone, and bone marrow. At day 28 after injection of SCW, euthymic LEW/N rats expressed high levels of iCRH in joints and surrounding tissues, while athymic LEW.rnu/rnu and F344/N rats were not different from control animals (TABLE 1).[43]

To assess the effect of glucocorticoids on expression of peripheral CRH, we treated Lewis rats with several doses of dexamethasone concomitant with the injection of SCW. We observed a dose-dependent decrease in the extent and intensity of CRH immunoreactivity following dexamethasone treatment.[43]

Chronic, destructive polyarthritis also develops in susceptible rat strains following intradermal injection of a suspension of heat-killed mycobacteria in oil. Clinical arthritis appears on day 10-12 after adjuvant injection and is dependent on the activation and recruitment of T lymphocytes. Similar to SCW-induced arthritis, euthymic LEW/N rats are adjuvant arthritis susceptible, while athymic LEW.rnu/rnu do not develop clinically apparent disease. F344/N rats are usually relatively adjuvant arthritis resistant and develop minimal clinically apparent arthritis. Histologically, LEW/N rats develop perivascular mononuclear cell infiltration and edema that progress to more intense cellular infiltration, fibrin deposition, proliferation of synovial fibroblasts, pannus formation, and erosion of articular cartilage and periarticular bone.[40,42,44]

Hindfoot specimens from LEW/N, Lew.rnu/rnu, and F344/N rats on day 0 (uninjected), and on days 4, 10, and 18 after adjuvant injection were also evaluated for CRH immunoreactivity (TABLE 1). LEW/N rats showed up-regulated iCRH expression

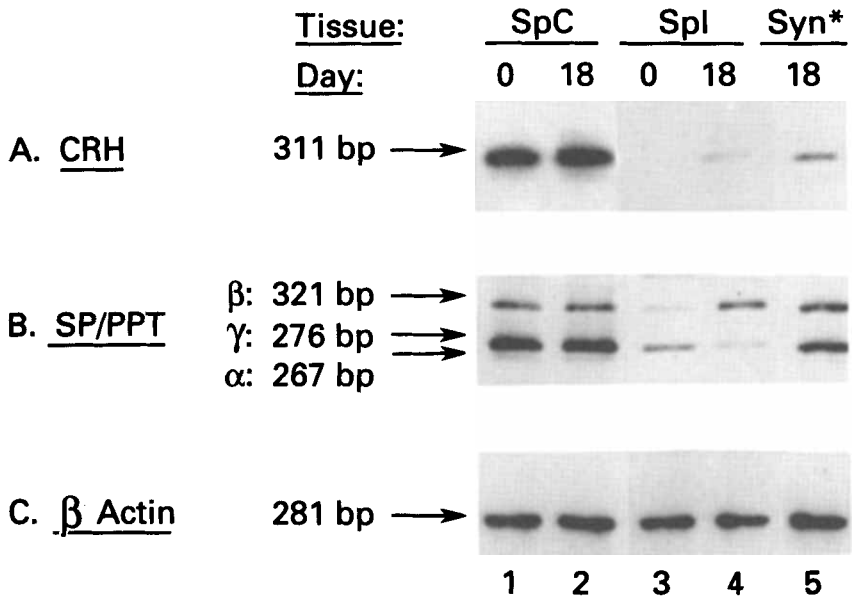

FIGURE 1. PCR evaluation of CRH and SP/preprotachychinin (PPT) mRNA expression in adjuvant-injected LEW/N rats.[43] Total RNA purified from spinal cord (SpC), spleen (Spl), or synovium (Syn) of LEW/N rats either uninjected (day 0) or with adjuvant arthritis (day 18). *RNA from synovia of uninjected rats was not analyzed because sufficient tissue could not be obtained.

beginning on day 4 that was further enhanced on days 10 and 18 after adjuvant injection. Athymic LEw.rnu/rnu rats also showed increased CRH immunoreactivity at day 4 that was not sustained by day 10 after adjuvant injection. Relatively adjuvant arthritis resistant F344/N rats did not increase CRH immunostaining after injection with adjuvant.[43]

We addressed the question of the source of CRH in these tissues by using PCR to evaluate CRH mRNA expression during adjuvant-induced arthritis in LEW/N rats. On day 18 after adjuvant injection, which is the time of maximally severe arthritis, fragments of the predicted size were detected in synovia and spleens, as well as spinal cords (FIG. 1).[43] These data suggest that CRH could be synthesized *in situ* by cells within synovial tissues and/or delivered to the joint from peripheral nervous tissues. For comparison, we examined the same samples for expression of preprotachykinin (PPT) mRNA, the precursor for SP, a well-characterized proinflammatory neuropeptide.[45] PPT mRNA isoforms were easily detectable in control spinal cord and in day 18 adjuvant-injected spinal cord and were also detectable in synovium and spleen from adjuvant arthritic rats.[43]

To determine if CRH expression could be physiologically relevant in the setting of inflammatory arthritis in rats, equilibrium binding of ^{125}I-oCRH was measured in homogenates of rat synovial tissues collected on day 18 after adjuvant injection.

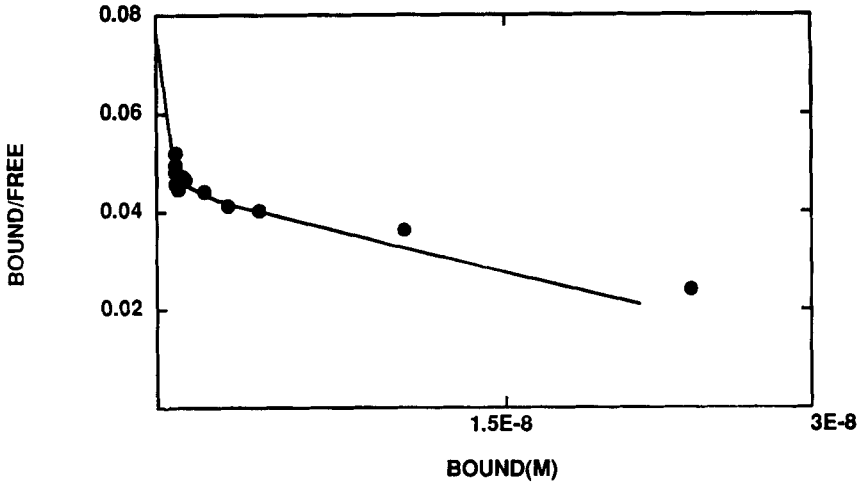

FIGURE 2. Scatchard plot of competitive displacement binding of ^{125}I-oCRH in synovial tissue of adjuvant-arthritic rats.[43] The apparent K_d values were 3.4 and 933 nM corresponding to B_{max} values of 2.7×10^{-11} and 4.1×10^{-8} M, respectively.

Specific binding of radiolabeled oCRH was linear with increasing tissue concentration, and specific binding was saturable. Scatchard plot suggested the presence of two binding sites (FIG. 2).[43]

CRH EXPRESSION IN SYNOVIAL FLUIDS AND TISSUES OF PATIENTS WITH RHEUMATOID ARTHRITIS

RA is a chronic disease characterized by inflammatory synovitis. Histologically, synovia from patients with RA show marked proliferation of synovial fibroblast-like cells, increased vascularity, and infiltration with mononuclear inflammatory cells. RA synovia express many proteins that contribute to the inflammatory process. There is up-regulation of major histocompatibility class II molecules and adhesion molecules, particularly on vascular endothelial cells, which may play a role in inflammatory cell infiltration.[38] Cytokines such as IL-1, IL-6, and TNF-α stimulate a variety of proinflammatory processes including, but not limited to, chemotaxis of inflammatory cells, stimulation of prostaglandin production, and induction of immunoglobulin synthesis.[46] Other peptides also contribute to histologic changes in the inflamed synovia; for example, platelet-derived growth factor stimulates proliferation of fibroblast-like cells[47] and fibroblast growth factor-1 stimulates angiogenesis.[41] Enzymes that degrade cartilage and other matrix proteins, such as transin/stromelysin and collagenase, are also up-regulated.[48]

CRH was also readily detected in the synovial tissue from patients with RA. Synovia from patients with osteoarthritis (OA) revealed CRH immunostaining in the synovial lining cell layer, blood vessel endothelial cells, and scattered infiltrating

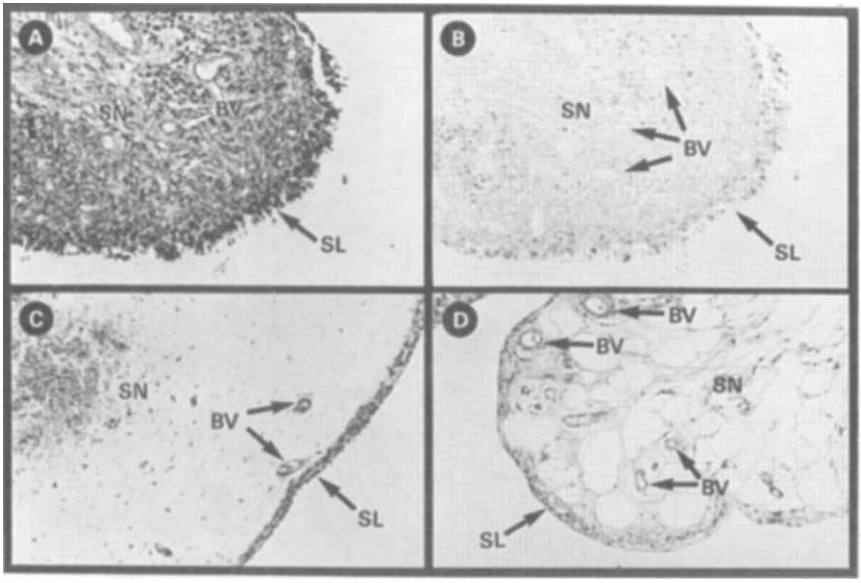

FIGURE 3. CRH immunostaining of synovial tissue sections from patients with RA or OA and normal synovia.[49] A: RA synovium stained with affinity-purified anti-CRH IgG (62x). **B:** RA synovium (section adjacent to that in A) stained with the affinity negative IgG fraction (62x). **C:** OA synovium and **D:** histologically normal synovium stained with affinity-purified anti-CRH IgG (62x). SL, synovial lining cell layer; SN, sublining synovial stroma; BV, blood vessels.

mononuclear inflammatory cells. Histologically normal synovia demonstrated only weak staining with anti-CRH IgG (FIG. 3). The extent and intensity of CRH immunostaining of RA synovia were significantly greater than OA synovia, and correlated with the extent and intensity of mononuclear cell infiltration.[49]

We analyzed synovial fluids from patients with both RA and OA. We found significantly higher concentrations of CRH in RA synovial fluids than in OA synovial fluids.[49] The concentration of CRH in inflamed synovial tissues and synovial fluids was similar to those of hypophyseal portal venous blood[50] and well within the range of what would be expected to be physiologically relevant.

To further document the presence of CRH, we performed reversed-phase HPLC fractionation of peptides extracted from synovial tissues and fluids, then analyzed 1 ml fractions by RIA. The major peak of CRH immunoreactivity coeluted with CRH 1-41 peptide in both synovial tissues and fluids (FIG. 4). Additional peaks most probably represent CRH aggregates and/or metabolites.[49]

We next analyzed synovial tissues collected from patients with RA and OA for CRH mRNA expression. CRH was detected by PCR amplification of a fragment of the predicted size that was also detected in human placenta, a tissue known to express CRH.[49]

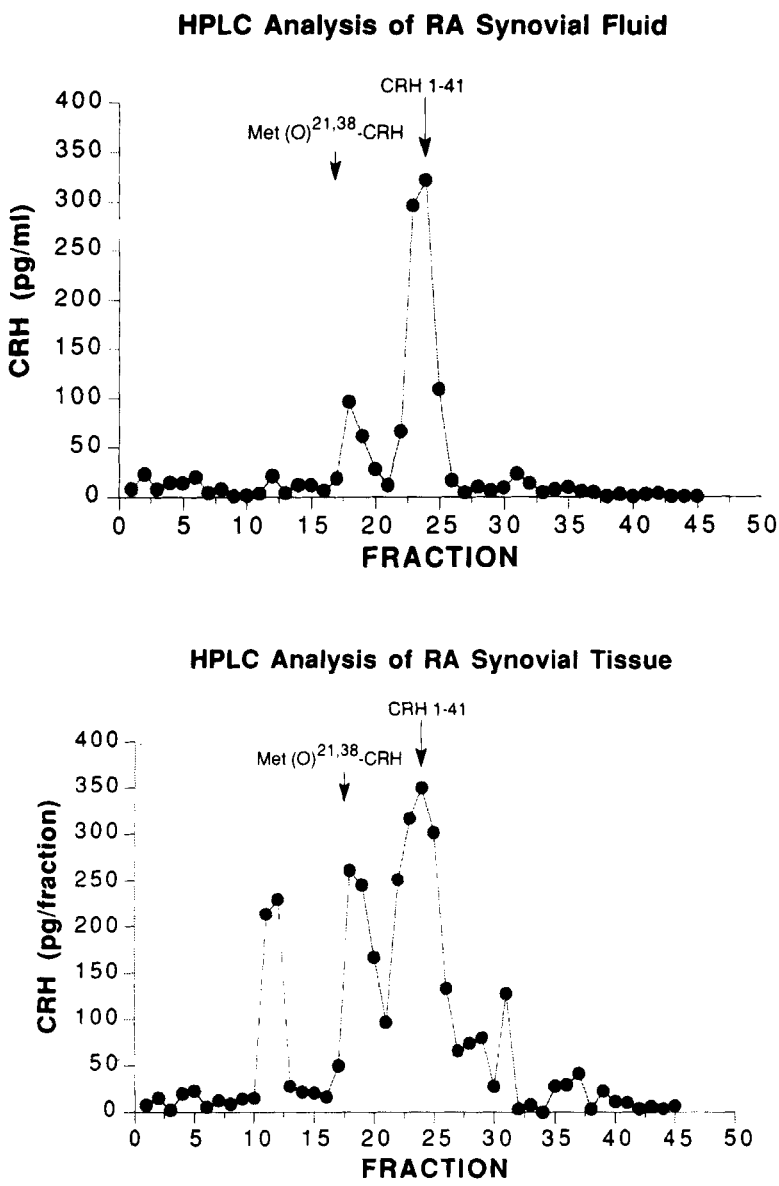

FIGURE 4. HPLC analysis of CRH extracted from RA synovial fluid (top) or tissue (bottom).[49] A standard of CRH 1-41 peptide was subjected to HPLC under identical conditions and eluted as indicated. The retention time for Met $(O)^{21,38}$-CRH is indicated.

DISCUSSION

We have shown that CRH is present in synovial tissues of rats with inflammatory arthritis and patients with RA. However, the effector mechanisms stimulated by CRH remain elusive. Previous *in vivo* data support the notion that local CRH has immunomodulatory effects when expressed in inflammatory sites.[30] It is intriguing that CRH may exert these effects through stimulation of proinflammatory cytokines, as suggested by some *in vivo* data.[15,16] IL-1 and IL-6 increase CRH mRNA in the hypothalamus and proopiomelanocortin (POMC) mRNA in the pituitary, and stimulate circulating ACTH and corticosterone.[1–3,5,6] The possibility that interleukins are utilized as messengers between peripheral and central CRH is intriguing. For example, peripheral CRH could enhance interleukin expression in inflamed tissues and thereby contribute to the inflammatory process, and also stimulate hypothalamic CRH and the HPA axis leading to glucocorticoid production and suppression of inflammation.

It must be noted that acute and chronic inflammation are different in many ways, including their effects on the HPA axis. We have shown that high levels of CRH are expressed locally in the inflamed synovial tissues of chronic arthritis. Studies of HPA axis function in chronic adjuvant-induced arthritis in rats have shown a sustained increase in plasma ACTH and corticosterone levels and increased adrenal weights at both 21 and 28 days after adjuvant injection, suggesting chronic HPA axis activation.[51,52] Harbuz and colleagues, however, also reported that hypothalamic CRH mRNa and hypophyseal portal venous CRH were decreased in these rats with adjuvant-induced arthritis, though levels of pituitary POMC mRNA were increased throughout the course of adjuvant-induced arthritis.[52] The reported decrease in hypothalamic CRH expression could be mediated by down-regulatory effects of chronic glucocorticoid elevation,[53,54] and increased production of hypothalamic arginine vasopressin may have maintained the elevated ACTH secretion in these animals. Taken together, these data suggest that peripheral and central CRH may be regulated differently in chronic inflammation resulting in decreased central CRH, but increased peripheral CRH expression. Glucocorticoids, the end-product of HPA axis activation, suppress inflammation and exert negative feedback to suppress HPA axis function. We showed that treatment with dexamethasone led to decreased local CRH immunostaining within hours in carrageenin-induced acute inflammation[30] and in the joints of Lewis rats on day 3 after injection of SCW.[43] This occurred in parallel with a decrease in inflammation as assessed by infiltration of joint tissues with mononuclear cells in the streptococcal cell wall induced arthritis model.[43] The regulation of peripheral CRH by glucocorticoids remains to be fully elucidated. Additionally, there are many other candidates for regulatory effects on peripheral CRH, including POMC-derived peptides such as ACTH and β-endorphin, the interleukins, β-adrenergic mediators, somatostatin, and platelet activating factor, all of which regulate central CRH expression and may be present in an inflammatory site.[1–6,55–57]

Neuropeptides, such as SP, vasoactive intestinal peptide, calcitonin gene-related peptide, nerve growth factor, somatostatin, and, in this report, CRH, have been demonstrated in inflamed synovial tissues and/or fluids.[58–61] Peripheral nervous system-derived neuropeptides have been postulated in the pathophysiology of RA to explain the symmetry of joint involvement in the disease, the predilection of RA for distal joints, and the observation that some peripheral neurologic disorders, such as

poliomyelitis and hemiplegia, can influence the distribution of joint involvement in RA.[62,63] Our data demonstrating CRH in synovial tissues and fluids provide further support for a role for local neuropeptides in the pathobiology of chronic inflammatory arthritis.

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