The role of the hypothalamic-pituitary-adrenal axis in the susceptibility to adjuvant-induced polyarthritis in the rat

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February 2000

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Doctor of Philosophy

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Contributions of Authors

This thesis includes a published article co-authored by my thesis supervisor, Dr. Ronald Melzack. The idea for the article was mine, prompted by my reading of the literature, which I perceived to neglect the issues discussed in the article. The writing of the manuscript was done entirely by me. Dr. Melzack provided feedback and supervision based on drafts that I wrote. Thus, although Dr. Melzack's teachings and supervision are the basis for the framework in which the literature was analysed, the ideas contained within the article are mine.

Acknowledgements

I would like to thank my friends, family, colleagues, and mentors for their support and praise, which made this endeavour possible and more pleasant. I thank John McKenna, Suntanu Dalal, Lucy Gagliese, and Perry Fuchs for their patience, training, and advice. The technical and clerical help of Janet Raymond, Louise Lebrun, and Chantale Bousquet, the McGill University Animal Resource Centre, and the laboratory of Dr. Shree Mulay which performed the hormone assays, are appreciated. The meticulousness in animal care and performing of experiments of Carmelo Milo, Erin Pooley, and Steve Bors are greatly appreciated. Many thanks to the translators of the abstract, Regis Delage-Mourroux and Karine Bon, and to Rick Mehta for his assistance with the statistical analyses. I thank Professors Keith Franklin, Blaine Ditto, Paul Plotsky, and Jeffrey Mogil for their help in working through the earlier drafts and helping me to form the final ideas of this thesis.

The thesis could not have been completed without the support of my friends and family, Rick Mehta, Francesco Leri, my father and his family, my brothers, Tim and Brian, and especially my mother and my roommate and friend, Luc Thivierge. My mother gave unrelenting support and understanding throughout. Luc not only gave understanding and support at critical times, but his intelligence and interest in pain and science in general encouraged the expression of my ideas, some of which are contained in the thesis.

Finally, I am most grateful to my supervisor, Ron Melzack, for giving me the opportunity to be his very last 'last student'. Ron's continuous praise, encouragement, and faith gave me the confidence and freedom to express and test new ideas, for which I am extremely grateful. He is truly a great man and my most admired mentor. Ron has taught me lessons that I will always remember. Thank you, Ron.

Abstract

The hypothalamic-pituitary-adrenal (HPA) axis, a system activated by stress, is traditionally considered to affect the susceptibility to chronic pain via effects on peripheral processes. This study investigates whether the HPA axis contributes to the development of chronic pain in an animal model via direct effects on central pain mechanisms.

First, correlations between pain processes and the susceptibility to chronic pain in an animal model that is correlated with HPA-axis function were examined. The results show that, in the Fischer rat, the amount of pain suppression observed during the formalin interphase depression is negatively correlated with susceptibility to polyarthritis. Since the formalin interphase depression mechanisms are within the central nervous system, the results suggest a role for central pain mechanisms in the development of polyarthritis.

Hypophysectomy inhibits the development of adjuvant-induced arthritis. To test whether hypophysectomy inhibits adjuvant-induced polyarthritis via central pain mechanisms, the analgesic effect of hypophysectomy was examined in the formalin test. The results show that hypophysectomy specifically prolongs the formalin interphase depression, further supporting that the underlying central pain suppression mechanisms are associated with resistance to adjuvant-induced polyarthritis.

Corticotropin-releasing factor (CRF) was then investigated as a possible underlying mechanism of the effects of hypophysectomy. Peripheral injection of CRF into inflamed tissue affects pain mechanisms unrelated to the susceptibility to adjuvant-induced polyarthritis. However, central and intravenous administration of CRF preferentially affect the formalin interphase depression mechanisms. The observed dose-response relationships indicate that these effects are due to direct actions of CRF within the central nervous system.

In conclusion, the results strongly suggest that the HPA axis modulates the susceptibility to adjuvant-induced polyarthritis via direct effects on supraspinal pain suppression mechanisms. Thus, the HPA axis may contribute to the development of chronic pain syndromes associated with HPA-axis abnormalities, such as rheumatoid arthritis and fibromyalgia, via effects on pain mechanisms within the central nervous system.

Résumé

L'axe hypothalamo-hypophyso-surrenalien (HHS), système activé par le stress, est traditionnellement considéré comme ayant une incidence sur la susceptibilité à la douleur chronique par l'intermédiaire d'effets sur les processus périphériques. Cette étude a pour but de montrer si l'axe HHS contribue au développement de la douleur chronique d'un modèle animal par l'intermédiaire d'effets directs sur les mécanismes centraux de la douleur.

Tout d'abord, les corrélations entre les processus de douleur et la susceptibilité à la douleur chronique sur un modèle animal qui permet d'étudier l'axe HHS furent examinés. Les résultats montrent que, chez le rat Fischer, la quantité de suppression de la douleur observée durant la dépression de l'interphase du test à la formaline est inversement corrélée à la susceptibilité à la polyarthrite. Comme les mécanismes responsables de la dépression de l'interphase du test à la formaline sont en relation avec le système nerveux central, les résultats suggèrent un role des mécanismes centraux de la douleur dans le développement de la polyarthrite.

L'ablation de l'hypophyse inhibe le développement de l'arthrite induite par adjuvant. Pour tester si une hypophysectomie inhibe la polyarthrite par l'intermédiaire des mécanismes centraux de la douleur, l'effet analgésique d'une hypophysectomie fut examiné dans le cas du test à la formaline. Les résultats montrent qu'une hypophysectomie prolonge spécifiquement la dépression de l'interphase du test à la formaline, démontrant ainsi que les mécanismes centraux de suppression de la douleur sous-jacents sont associés à une résistance à la polyarthrite induite par adjuvant.

Le facteur libérant la corticotropine (CRF) fut alors étudié pour essayer d'expliquer les effets d'une hypophysectomie. L'injection périphérique de CRF au niveau de tissus

inflammés affecte les mécanismes de douleur qui n'ont pas de lien avec la susceptibilité à la polyarthrite induite par adjuvant. Cependant, l'administration centrale et intraveineuse de CRF affecte préférentiellement les mécanismes de la dépression de l'interphase du test à la formaline. La relation dose-réponse observée démontre que les effets sont dus à l'action directe du CRF au sein du système nerveux central.

En conclusion, les résultats démontrent que l'axe HHS module la susceptibilité à la polyarthrite induite par adjuvant au travers d'effets directs sur les mécanismes de suppression de la douleur au niveau supraspinal. Ainsi, l'axe HHS pourrait contribuer au développement des syndromes de douleur chronique associés aux anomalies de l'axe HHS, tels que l'arthrite rhumatoïde et la fibromyalgie, au travers d'effets sur les mécanismes de douleur au sein du système nerveux central.

Chapter 1

Introduction

Purpose and Approach

Pain is a multidimensional experience with sensory, affective, and cognitive dimensions (Melzack and Wall 1996). Traditionally, pain has been viewed as a purely sensory phenomenon caused by either injury or pathology of peripheral tissue (Foster 1970; Melzack and Wall 1996). The primary afferents that innervate the tissue were thought to faithfully relay the message of the painful event to spinal cord cells, which project to the brain where the message triggers the sensation of pain.

In contrast to the traditional view, great variability is seen between the intensity of pain experienced and the severity of an injury observed in the clinic or the intensity of experimental stimuli in the laboratory (Melzack and Wall 1996; Mogil 1999). This variability highlights the inadequacy of viewing pain as a peripherally driven sensation, and prompted the proposal of the gate control theory (Melzack and Wall 1965). According to the gate control theory, transmission cells in the spinal cord integrate the activity of afferent fibres and descending inputs from the brain. The inclusion of descending inputs from the brain onto spinal cord cells provides a mechanism for the modulation of ascending input by internal, central factors, such as those related to stress (Melzack 1980, 1999).

It is now well recognized that stress can affect pain. Numerous studies have been published on the phenomenon of stress-induced analgesia and its underlying mechanisms (Tricklebank 1984; Amit and Galina 1986; Kelly 1986). Moreover, the hypothalamic-pituitary-adrenal (HPA) axis, which is activated by exposure to stressors, is recognized as capable of modulating pain (Dunn and Berridge 1990). For example, removal of the pituitary gland has been shown to relieve pain in humans and decrease pain behaviour in animals

(Miles 1994; Lariviere et al. 1995). Furthermore, corticotropin-releasing factor (CRF), a peptide released from the hypothalamus and a mediator of many of the effects of stress, has also been shown to produce analgesia following exogenous administration (Lariviere and Melzack 2000). The effect of hypophysectomy and the role of CRF in pain and analgesia will be reviewed and discussed in detail later in this chapter.

Perhaps due to the view of pain as a peripherally driven sensation, the analgesic effect of CRF has been ascribed to a peripheral mechanism rather than a central mechanism, despite evidence that central mechanisms are involved (Owens and Nemeroff 1991; Schäfer et al. 1997; Lariviere and Melzack 2000). The traditional view of pain is also reflected in our understanding of the relationship between stress and chronic pain. The contribution of stress and the HPA axis to chronic pain such as arthritic pain is poorly understood (Koehler 1985; Huyser and Parker 1998). Contrary to the analgesia often seen following exposure to stressors, chronic stress is commonly thought to lead to events associated with chronic pain. Although the mechanisms are not yet known, the common view derives from Selye's General Adaptation Syndrome (Selye 1975; Cohen 1979; Florence 1981; Asterita 1985). For instance, according to this view, chronic stress results in persistently elevated activity of the HPA axis. This leads to tonically elevated levels of cortisol in humans (or corticosterone in rats), which produce tissue damage in susceptible target organs such as bone or muscle. The tissue damage is expected to produce pain, which, if the stress and tissue damage persist, will become chronic. Although the state of the peripheral tissue certainly contributes to chronic pain, the role of central pain mechanisms in the development of chronic pain is neglected in this model. Thus, the role of the periphery may have been overemphasized and the role of mechanisms within the central nervous system has certainly been neglected in the study of

the relationship between stress and chronic pain.

The purpose of this thesis is to examine whether the HPA axis contributes to chronic pain via direct effects on pain mechanisms within the central nervous system. Specifically, the thesis will: 1) test whether the susceptibility to chronic pain in an animal model is correlated with differences in central pain mechanisms; 2) test whether a manipulation of the HPA axis that inhibits the development of the chronic pain model also affects the same central pain mechanisms; and 3) determine whether CRF affects these pain mechanisms via a central site of action.

The influence of neural pain processes on the susceptibility to chronic pain and inflammation in an animal model, adjuvant-induced polyarthritis, has been demonstrated (Levine et al. 1987; Basbaum and Levine 1991) and will be discussed in the next section. However, the role of the HPA axis in these neural contributions has not been studied. Therefore, to directly test whether pain processes correlated with HPA-axis function also correlate with susceptibility to chronic pain, the study in Chapter 2 compares phasic and tonic pain sensitivity and endogenous pain suppression among groups of rats known to differ in their HPA-axis responsiveness and their susceptibility to adjuvant-induced polyarthritis.

The effect of hypophysectomy, or removal of the pituitary gland, on pain mechanisms is not well understood. Hypophysectomy has been demonstrated to decrease the susceptibility to adjuvant-induced polyarthritis (Neidhart and Flückiger 1992). However, the review of the analgesic effect of hypophysectomy presented later in this chapter indicates that hypophysectomy does not reliably modulate the brief pain evoked in phasic pain tests. In contrast, hypophysectomy is effective against the prolonged pain associated with advanced cancer (Gianasi 1984; Bonica 1990; Miles 1994). Thus, to test whether the HPA axis

selectively modulates pain mechanisms related to prolonged pain, including those associated with adjuvant-induced polyarthritis, the experiments presented in Chapter 3 investigate the analgesic effect of hypophysectomy on phasic and tonic pain sensitivity and endogenous pain suppression in the rat.

To determine the site of action of the HPA axis on pain mechanisms related to adjuvant-induced polyarthritis susceptibility, the final set of experiments presented in Chapter 4 also examines the effect of CRF administration on phasic and tonic pain sensitivity and endogenous pain suppression in the rat. The site of action of CRF is determined by comparing the results of central, systemic, and local CRF administration.

The following section reviews the literature showing the influence of pain mechanisms within the peripheral and central nervous systems on the development of chronic pain and inflammation in the adjuvant-induced polyarthritis model.

The role of pain mechanisms in the susceptibility to adjuvant-induced polyarthritis

Adjuvant-induced polyarthritis is an animal model of rheumatoid arthritis in which a rat is injected with complete Freund's adjuvant, an oil suspension of bacterial material, in the base of the tail (Whitehouse 1988). Several weeks after injection, the susceptible rat shows signs of severe inflammation in the hindlimb joints and often also in the forelimb joints. This model is associated with behaviours indicative of chronic pain, and thus, the model is also a model of chronic pain (De Castro Costa et al. 1981; Colpaert et al. 1982; Colpaert 1987). Compared to non-arthritic control rats, intake of analgesic drugs is increased, weight loss occurs, mobility is decreased, and spontaneous pain-related behaviours such as curling, elevation, shaking of affected paws, and debilitation also occur after injection, peaking in severity approximately three weeks after adjuvant injection (De Castro Costa et al. 1981; Colpaert et al. 1982; Colpaert 1987; Lariviere and Melzack 1997).

This animal model, like rheumatoid arthritis in humans, is typically viewed as an immune-mediated inflammatory disease (Sternberg 1995). Combined with the classical view of pain as a peripherally driven sensation, the pain in this model is typically seen as a passive response to peripheral events. Hence, when neurotransmitters in the central nervous system are implicated, it is their interaction with the immune system and peripheral inflammatory processes that is proposed to be of significance (Harbuz et al. 1994; Sternberg 1995).

However, pain-related mechanisms within both the peripheral and central nervous systems are also involved in the development of adjuvant-induced polyarthritis (Levine et al. 1985a, 1987; Levine and Basbaum 1990; Basbaum and Levine 1991). In the peripheral nervous system, primary afferent fibres have been shown to contribute to adjuvant-induced inflammation. Neonatal treatment with capsaicin on postnatal day 1, or with a subcutaneous

injection of capsaicin in the adult rat 5-14 days prior to adjuvant injection, inhibits the development of inflammation following adjuvant injection (Colpaert et al. 1983; Levine et al. 1985b, 1986; Cruwys et al. 1995; Donaldson et al. 1995). The fact that capsaicin treatment preferentially affects unmyelinated fibres suggests that C-fibres contribute to the inflammation in adjuvant-induced polyarthritis. Dorsal rhizotomy also affects inflammation in the model, increasing the inflammation compared to intact adjuvant-treated rats, which suggests that other afferent fibres, such as large diameter fibres, are also involved in the development of adjuvant-induced arthritis (Levine et al. 1986).

In addition, efferent fibres of the sympathetic nervous system contribute to peripheral inflammation (Levine et al. 1985b). Sympathectomy induced by repeated guanethidine administration has been shown to attenuate inflammation in the adjuvant-induced polyarthritis model (Levine et al. 1985c, 1986). Thus, activity in primary afferent fibres could also contribute to peripheral inflammation through spinal loops connecting with sympathetic efferent fibres in addition to the direct effects of release of inflammatory mediators from their peripheral terminals (Levine et al. 1985b).

Supraspinal mechanisms have also been shown to be involved in the development of adjuvant-induced polyarthritis. Intracerebroventricular administration of 15 μg morphine every 2 hours for 3 days beginning 1 hour prior to adjuvant injection inhibits the development of polyarthritis assessed by radiologic examination 28 days after adjuvant injection (Levine et al. 1985b, 1986).

The influence of the central nervous system on the development of peripheral inflammation is not specific to adjuvant-induced polyarthritis. Other pain models associated with inflammation, including the formalin test and Brewer's yeast-induced pain and

inflammation have been shown to have a central contribution. Down-regulation of the peripheral inflammatory response by supraspinal sites has been demonstrated in the formalin test (Wheeler-Aceto and Cowan 1991a). In addition, intracerebroventricular injection of GABA, serotonin, 5-hydroxytryptophan, histamine, noradrenaline and other noradrenergic drugs, scopolamine, amphetamine, and L-dopa all decrease the inflammation following injection of formalin or Brewer's yeast in the rat hindpaw (Dumka et al. 1996a, 1996b; Hore et al. 1997; Dumka et al. 1998). Conversely, intracerebroventricular injection of L-aspartic acid, PCPA, 6-OHDA, acetylcholine, and haloperidol increase peripheral edema produced by injection of either formalin or Brewer's yeast. Furthermore, an intact neuraxis is required for a negative feedback mechanism of the inflammatory response to bradykinin perfusion. Electrical stimulation of the rat hind paw at intensities that excite C-fibres inhibits bradykinin-induced plasma extravasation in the knee joint (Green et al. 1995). This effect is inhibited by transection of the thoracic spinal cord, suggesting the involvement of supraspinal mechanisms (Green et al. 1995). Moreover, this effect also requires an intact HPA axis since hypophysectomy and adrenalectomy reverse the inhibitory effect of C-fibre stimulation (Green et al. 1995).

Therefore, central mechanisms related to adjuvant-induced polyarthritis susceptibility, such as central serotonin or noradrenaline (Harbuz et al. 1994, 1996), or HPA axis-related pain mechanisms, could exert their effects on central pain mechanisms directly and indirectly on peripheral inflammation via neurogenic inflammation mechanisms. Thus, a comprehensive model of susceptibility to adjuvant-induced polyarthritis should include the contribution of the nervous system as an etiological factor, as represented schematically in Figure 1.

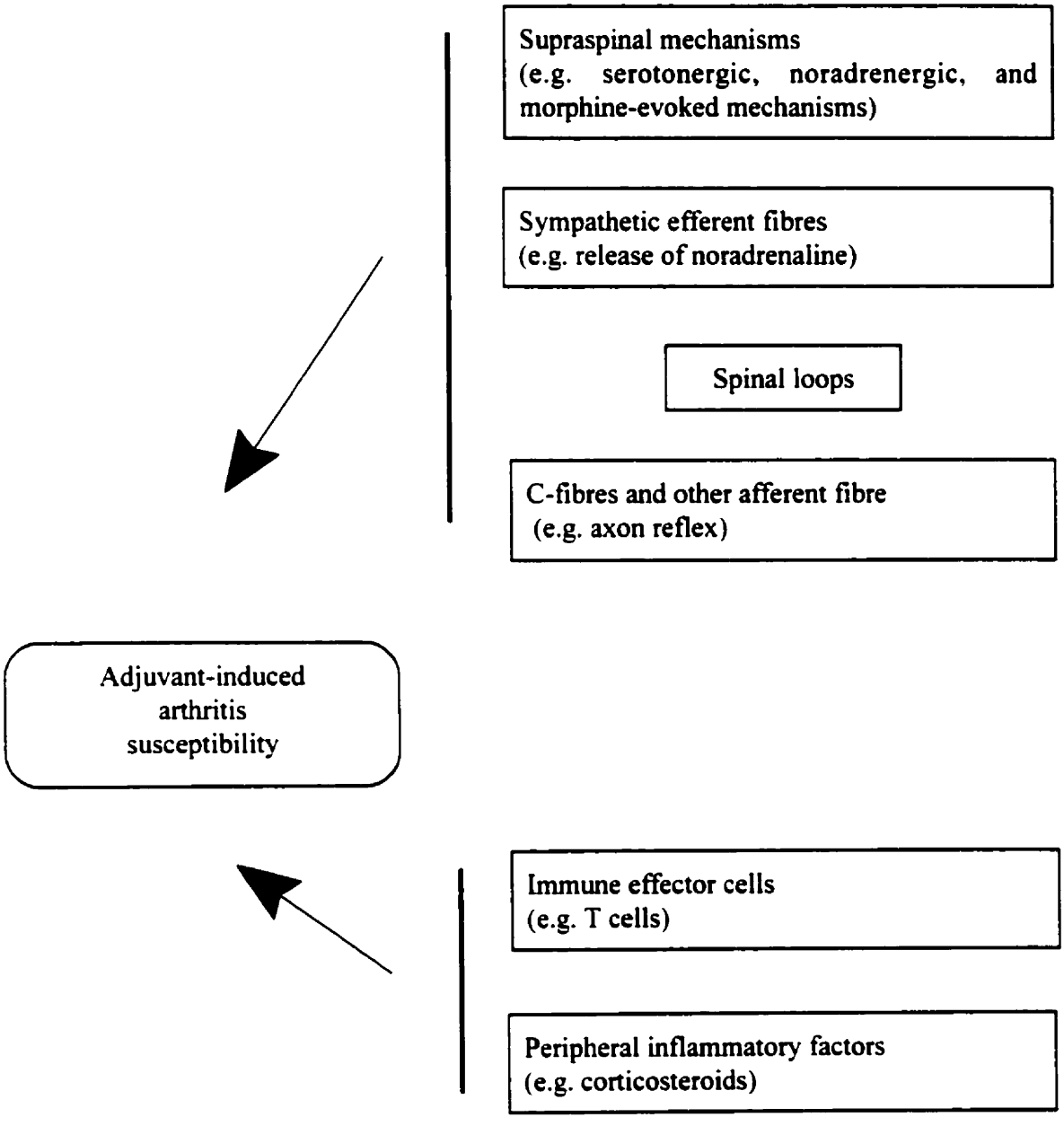


Figure 1. Schematic representation of the contributions of peripheral factors and the nervous system to adjuvant-induced polyarthritis susceptibility.

It should be noted that the present discussion focusses on the factors contributing to the development of the polyarthritis model, not the monoarthritis model, of adjuvant-induced arthritis. In the monoarthritis model, a subcutaneous injection of complete Freund's adjuvant is administered directly to the hind paw either around the ankle joint or under the plantar surface (Iadarola et al. 1988; Donaldson et al. 1993). Unlike the delayed inflammatory response of the polyarthritis model, an acute inflammatory response is observed within one day. Although a delayed response is observed 14 days after adjuvant injection (Donaldson et al. 1993), the study of the susceptibility to the delayed response is confounded by the initial inflammatory response. Furthermore, the polyarthritis model may have a greater neurogenic component to the susceptibility since the bilateral response involves spinal cord circuits and local subcutaneous injection of capsaicin inhibits development of polyarthritis but does not reliably inhibit development of monoarthritis (Levine et al. 1985b; Donaldson et al. 1993; Cruwys et al. 1995).

Susceptibility to adjuvant-induced polyarthritis has been related to HPA axis responsiveness to stress in female Lewis and Fischer rats (Sternberg et al. 1992a, 1995). The Lewis rat is highly susceptible to adjuvant-induced polyarthritis. In response to restraint stress, the female Lewis rat shows a blunted response of CRF mRNA increase in the paraventricular nucleus of the hypothalamus compared to the robust response of the female Fischer rat, which is relatively resistant to adjuvant-induced polyarthritis (Sternberg et al. 1989a, 1989b; Wilder 1993). Since the Lewis rat has also been observed to have a smaller increase of plasma ACTH and plasma corticosterone in response to the inflammatory stress of intraperitoneal injection of streptococcal cell wall polysaccharide, the differential susceptibility has been ascribed to corticosterone-mediated modulations of the immune

system and inflammation (Sternberg et al. 1989a, 1992a).

However, several examples demonstrate that corticosterone responses to stressors do not predict susceptibility to adjuvant-induced polyarthritis (Chover-Gonzalez et al. 1999). For instance, Wistar rats that excreted 6 or more fecal pellets in response to placement in an open field show significantly greater corticosterone responses 30 minutes after exposure to the open field than rats that excreted 2 or less fecal pellets. Despite the differences in corticosterone responses, there was no significant difference in paw volume 14 days after tail base injection of complete Freund's adjuvant (Chover-Gonzalez et al. 1998). Rats that failed to avoid shocks in the learned helplessness paradigm had a significantly lower corticosterone response than rats that rarely failed to avoid a shock within 3 seconds, but showed significantly less inflammation in the polyarthritis model 14 days after adjuvant injection (Chover-Gonzalez et al. 1999). In addition, Piebald-Viral-Glaxo rats show robust corticosterone responses to stressors, but adjuvant-induced arthritis is readily induced in these rats (Harbuz et al. 1994).

Furthermore, basal levels of circulating corticosterone do not predict susceptibility. In the groups of rats divided on the basis of their number of failures to avoid shock, there was no significant difference in basal corticosterone between the groups that differed in their susceptibility to adjuvant-induced inflammation. Moreover, basal corticosterone levels are greater in female Lewis rats than in males, yet females show more inflammation in the arthritis model (Griffin and Whitacre 1991).

Hence, the role of peripheral corticosterone in the susceptibility to adjuvant-induced polyarthritis is questionable, and suggests that other mechanisms are involved. It is known that pain mechanisms within the nervous system are involved in the development of

adjuvant-induced polyarthritis. Furthermore, the HPA axis affects pain mechanisms and it has effects within the central nervous system (Vernikos-Danellis 1972; Lariviere and Melzack 2000). Therefore, it is reasonable that the HPA axis could modulate the susceptibility to adjuvant-induced polyarthritis via effects on mechanisms in the nervous system, including the central nervous system.

To examine this hypothesis, the experiments presented in Chapter 2 investigate pain mechanisms, including pain suppression mechanisms of central origin, in groups of rats known to differ in HPA axis function and adjuvant-induced polyarthritis susceptibility. The next section reviews the effect of hypophysectomy, a manipulation that inhibits adjuvant-induced polyarthritis susceptibility, on pain mechanisms.

Hypophysectomy-induced analgesia and prolonged pain mechanisms

Hypophysectomy is the destruction or removal of the pituitary gland, which is also called the hypophysis. Although hypophysectomy was originally performed on advanced cancer patients to try to control the growth of hormone-dependent cancers, it was discovered that it also produces pain relief that can be quite dramatic. Previously uncontrollable and excruciating pain associated with metastasis of cancer to the bone can be completely relieved for months or even years, an effect that is independent of tumour regression (Katz and Levin 1977; Gianasi 1984; Bonica 1990; Miles 1994).

Hypophysectomy does not affect all forms of pain processing. Phasic pain sensitivity, such as to the brief, sharp pain of a pinprick, is reportedly intact in patients who have undergone the procedure (Misfeldt and Goldstein 1977). Other authors have also reported this specificity for the modulation of prolonged cancer pain without any effect on "ascending nociceptive systems" (Gianasi 1984; Bonica 1990), although no systematic study of phasic and tonic pain sensitivity has been reported in the literature. Further specificity is also seen for the type of prolonged cancer pain. The analgesia after hypophysectomy (or electrical stimulation of the pituitary gland) is more effective for the deep, dull, diffuse pain of bony metastases than for other types of cancer pain (Yanagida et al. 1984). Together, these results demonstrate the specificity of hypophysectomy-induced analgesia for certain prolonged pain mechanisms over others and over brief, phasic pain mechanisms.

Hypophysectomy may also have effects on chronic pain in the rat. Hypophysectomy inhibits the development of inflammation in the chronic pain model of adjuvant-induced polyarthritis (Neidhart and Flückiger 1992), an effect that may be partly due to the effects of the HPA axis on pain mechanisms. Many studies have examined the analgesic effect of

hypophysectomy in the rat and the mouse (see Table 1 for references). As demonstrated in Figure 2, the effect of hypophysectomy on baseline pain responsiveness is dependent on the time from surgery at which the assessment is made. All studies that found significant analgesia tested the animals within 3 weeks from the time of the surgery. All studies that found significant hyperalgesia tested the animals two or more weeks after surgery. A discussion of the effect of time from surgery is beyond the scope of this discussion, which will focus on the analgesic effects of hypophysectomy within 2-3 weeks from surgery.

The most striking feature of Figure 2 is the inconsistency of effects of hypophysectomy. Less than half of the studies found any effect of hypophysectomy, which questions the ability of hypophysectomy to reliably modulate the pain evoked in these studies. Almost all of the animal studies used phasic pain tests in which a brief, high intensity stimulus is terminated by the response of the subject within seconds of the stimulus onset. As listed in Table 1, these tests include the tail flick test, the hot plate test, and the application of a brief electric current. Although Amir and Amit used the formalin test in the rat, which is considered to be a tonic pain test, they tested only the first 15 minutes of the formalin pain response (Amir and Amit 1979). As shown in Figure 3, the formalin response in the rat consists of a first phase of phasic pain that lasts approximately five minutes, followed by a depression in pain responses that lasts approximately 15 minutes and ends when the tonic pain of the second phase begins (Dubuisson and Dennis 1977; Porro and Cavazutti 1993). Therefore, Amir and Amit examined the period of phasic pain in the formalin test, but not the period of tonic pain.

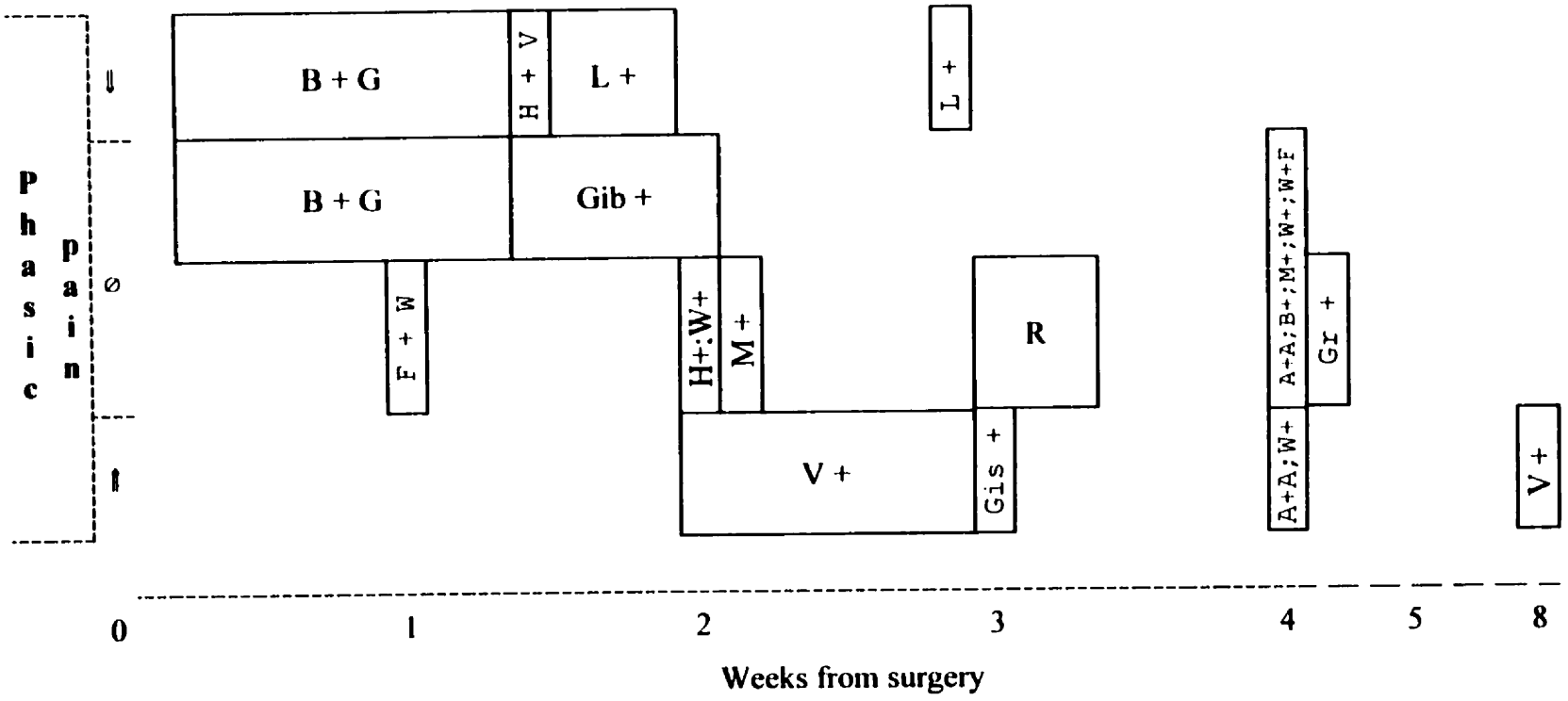
Phasic pain and tonic pain have been shown to involve different neural substrates and to exhibit different pharmacological responsiveness (Abbott et al. 1982b; Dennis and

Table 1. Effects of hypophysectomy on baseline pain responses in animals.

Study	Subjects	Method of Assessment	Time of Testing After Surgery	Results
Amir and Amit 1979	M Wistar rats	Hot plate test, 51 °C, Formalin test, first 15 min	4 weeks	Hyperalgesia (formalin test only)
Baron and Gintzler 1987	Pregnant and non-pregnant SD rats	Foot shock	2, 4, 6, 7, 8, and 9 days	Analgesia (non-pregnant rats only)
Beaton 1981	(?) Rats	Foot shock	?	Analgesia
Bodnar et al 1979	M SD rats	Foot shock	1 month	No effect
Foo and Westbrook 1991	Wistar rats	Hot plate test, 51.5°C	7 days	No effect
Gibbs et al 1973	M SD rats	Foot shock	10-14 days	No effect
Gispen et al 1970	M Wistar rats	Foot shock	3 weeks	Hyperalgesia
Grevert et al 1978	M ICR mice	Hot plate test, 55 °C	29-30 days	No effect
Heybach and Vernikos-Danellis 1978	M SD rats	Hot hollow copper tubes, 55 °C	10 days	Analgesia
Holaday et al 1977	M Long-Evans rats	Tail flick test, light beam	2 weeks	No effect
Lewis et al 1981	M SD rats	Tail flick test, (?)	10-13 days	Analgesia
Lewis et al 1981	M SD rats	Hot plate test, 52.5°C	20 days	Analgesia
Millan et al 1980	M SD rats	Tail flick test, light beam, Tail shock, 15 d only	15 days and 4 weeks	No effect
Ramabadran 1982	M SD rats	Hot plate test, 55°C	21-23 days	No effect
Terman et al 1984	M SD rats	Tail flick test, (?)	?	No effect
Vidal et al 1982	M SD rats	Tail shock	2-3 weeks and 8 weeks	Hyperalgesia
Watkins et al 1982	M SD rats	Tail flick test, (?)	2 and 4 weeks	Hyperalgesia (4 weeks only)
Wesche and Frederickson 1981	M Cox Standard mice, M SD rats	Hot plate test, 52 °C	4 weeks	No effect

Abbreviations F, female, M, male, SD, Sprague-Dawley, ?, information not specified

Figure 2. Effects of hypophysectomy on baseline pain responses as a function of time of testing after surgery.



Symbols: ⇓, analgesia; ⌀, no effect; ⇑, hyperalgesia.
Letters in boxes refer to first letters of the authors' last names as listed in Table 1.

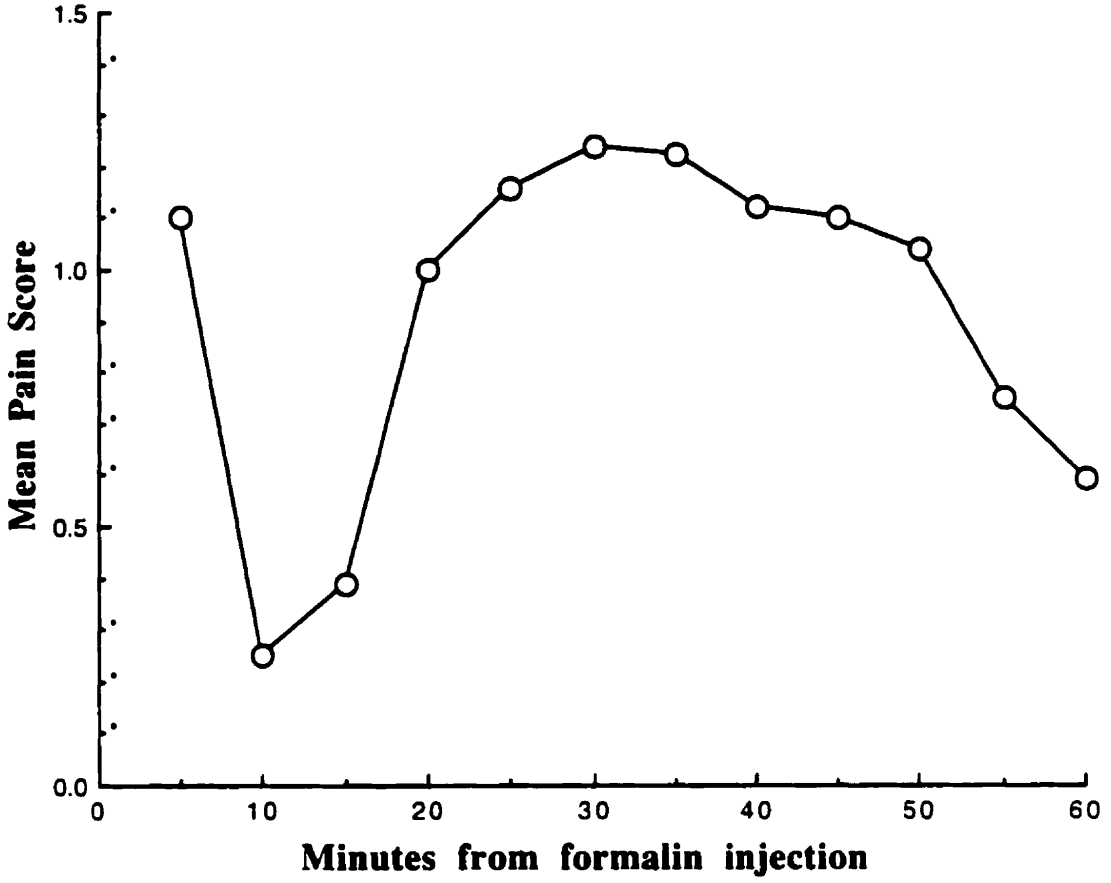


Figure 3. The typical pain response of the rat to intraplantar injection of 2.5 % formalin. The behaviour of the rat is scored as a '2' if the rat licks, bites, or shakes the injected paw; as a '1' if the rat elevates the paw from the floor; and as a '0' if any part of the paw other than the tips of the digits is in contact with the floor. A mean pain score is calculated for each 5-minute period after injection as the sum of the scores divided by the number of scores in the time period.

Melzack 1983; Coderre et al. 1984; Ryan et al. 1985; Vaccarino and Melzack 1989). Furthermore, tonic experimental pain more closely resembles prolonged, clinical pain such as cancer pain due to its duration and the relative lack of tolerance to morphine in situations of tonic pain that are similar to the clinical situation (Mount et al. 1976; Abbott et al. 1981, 1982a; Portenoy 1995). Moreover, the affective component of tonic pain resembles clinical pain more than that of phasic pain (Chen and Treede 1985).

As in humans, the effect of hypophysectomy in rats may show a specificity for prolonged pain mechanisms compared to brief, phasic pain mechanisms. To test the specificity of hypophysectomy-induced analgesia for prolonged pain mechanisms, the effect of hypophysectomy on phasic and tonic pain sensitivity and endogenous pain suppression is examined in Chapter 3. In addition, adjuvant-induced polyarthritis susceptibility and pain mechanisms that are examined in Chapter 2 are examined following hypophysectomy, allowing further assessment of the relationship between arthritis susceptibility and specific pain mechanisms.

The mechanisms underlying hypophysectomy-induced analgesia are still unknown (Miles 1994). Damage to the median eminence or to nuclei of the hypothalamus has been proposed to be responsible, but is unlikely since there is poor correlation of damage due to the spread of the alcohol used to destroy the pituitary in humans with the pain relief reported by patients (Takeda et al. 1978; Miles 1994). Removal of a major source of β-endorphin is not likely responsible since this would be expected to produce hyperalgesia, not analgesia. Furthermore, naloxone does not affect hypophysectomy-induced analgesia (Misfeldt and Goldstein 1977; Takeda et al. 1978; Levin et al. 1980; Yanagida et al. 1984). Enhanced sensitivity to opiates develops after hypophysectomy and may play a role in the analgesic

effects (Holaday et al. 1977, 1979). However, this opiate sensitivity is reversed by ACTH and dexamethasone, demonstrating the interactions of opiate analgesia with the HPA axis (Holaday et al. 1977), and suggesting that the hypersensitivity to opiates may be secondary to changes in the HPA axis.

A role for the components of the HPA axis outside of the central nervous system has been suggested, but not effectively demonstrated. ACTH deficiency due to removal of the pituitary gland has been ruled out by the inability of ACTH to reverse the analgesic effects of hypophysectomy on pain in the rat (Gispen et al. 1970; Amir and Amit 1979). In addition, analgesia is observed following hypophysectomy in humans even when ACTH function is preserved (Miles 1983). Therefore, impaired ACTH function, and by implication, impaired corticosterone release, is not necessary for the analgesic effects of hypophysectomy. However, hypophysectomy also affects the central components of the HPA axis, increasing CRF levels in the hypothalamus and in the cortex (Moldow and Fischman 1982; Yokoe et al. 1988; Frim et al. 1990). It is possible that these central effects of hypophysectomy are responsible for hypophysectomy-induced analgesia. In the next section, the effect of CRF on pain mechanisms is reviewed.

The site of action of the HPA axis on pain mechanisms

This section reviews the effect of CRF on pain mechanisms, focussing on the site of analgesic action and the specificity of effects on prolonged pain mechanisms. The following is a manuscript of an article published in *Pain* (Lariviere and Melzack 2000). Small formatting changes have been made and are indicated with square brackets, [ ].

The Role of Corticotropin-Releasing Factor in Pain and Analgesia

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Key words: corticotropin-releasing factor, endogenous pain suppression, hypothalamic-pituitary-adrenal axis, neuroendocrine, stress

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Abstract

Corticotropin-releasing factor (CRF) is a peptide that is released from the hypothalamus and in widespread areas of the brain following exposure to stressors. It is considered to be a mediator of many of the effects of stress, and its analgesic properties have been demonstrated in many studies. However, for primarily methodological reasons, the effects of CRF in the central nervous system have been neglected whereas the peripheral effects of CRF have been overemphasized. We present evidence that: 1) CRF can act at all levels of the neuraxis to produce analgesia; 2) the release of β-endorphin does not explain the analgesia following intravenous or intracranial CRF administration; 3) inflammation must be present for local CRF to evoke analgesia; and 4) the analgesic effects of CRF show specificity for prolonged pain. These findings suggest that CRF may have a significant role in chronic pain syndromes associated with hypothalamic-pituitary-adrenal axis abnormalities. Furthermore, CRF may represent a new class of analgesics that merits further study. Implications for the relationship between stress and pain are discussed.

Introduction

In recent years, the mechanisms responsible for generating pain are becoming better understood as more researchers focus on how somatosensory input is processed by the central nervous system. Areas of the brain previously thought to be unrelated to pain processing, such as the limbic system, have been shown to play a major role in the experience of pain in animals and humans (Bouckoms 1994). In addition, classes of drugs not normally used as analgesics are being discovered to have powerful effects on pain, especially chronic pain. For example, antidepressants such as amitriptyline and anti-epilepsy drugs such as carbamazapine have been demonstrated to be effective in alleviating chronic pain (Monks 1994). Research on the effects of these drugs on the central nervous system has led to a greater understanding of the basic mechanisms of pain and analgesia.

Corticotropin-releasing factor (CRF) is a peptide involved in the activation of the hypothalamic-pituitary-adrenal (HPA) axis. It is released by the hypothalamus and stimulates the anterior pituitary gland to release adrenocorticotropic hormone, which then activates the adrenal gland to release corticosteroids (Chrousos and Gold 1992). Stress evokes the release of CRF into areas throughout the brain (Chappell et al. 1986), and the administration of exogenous CRF mimics many of the effects of stress (Dunn and Berridge 1990). Therefore, CRF is considered to be a mediator of the effects of stress, including stress-induced analgesia. Indeed, the analgesic effect of cold water swim stress on hyperalgesia induced by complete Freund's adjuvant is antagonized by intraplantar injection of the CRF receptor antagonist, α-helical CRF (Schäfer et al. 1996).

CRF has been recognized for its ability to produce analgesia, but the possibility that it represents a new class of analgesics has been overlooked. This may be because CRF is

generally considered to have a primarily peripheral effect, and the possible central mechanisms of CRF-induced analgesia have been neglected. However, we present evidence which indicates that CRF may act on a large number of brain structures involved in pain processing. This review will show that the central mechanisms of CRF-induced analgesia warrant further study, and that such an investigation should lead to a greater understanding of the interaction between stress and pain mechanisms.

Since stress has been shown to produce analgesia (Amit and Galina 1986), the analgesic effect of CRF has been studied extensively. Dunn and Berridge (1990) and Owens and Nemeroff (1991) argue that CRF produces analgesia following intravenous, intradermal, and subcutaneous administration, but not after intracerebroventricular administration. The mechanism is said to be due primarily to the release of β-endorphin (Dunn and Berridge 1990) with some exceptions (Owens and Nemeroff 1991). Furthermore, the analgesia is also said to involve an anti-inflammatory action of CRF (Owens and Nemeroff 1991). However, possible mechanisms within the central nervous system have been virtually ignored (by Owens and Nemeroff 1991; Schäfer et al. 1997).

In fact, analgesia has been demonstrated following the administration of CRF by all routes attempted, including intracerebroventricular, intracisternal, and intrathecal routes (see Tables [2-4] for references). This underscores the possibility that CRF acts at all levels of the neuraxis as well as in the periphery. Nonetheless, the notion that CRF causes analgesia via a peripheral mechanism, but not a central mechanism, is still suggested by the current emphasis on peripheral mechanisms and the neglect of central mechanisms (by Schäfer et al. 1997, for example).

The idea that CRF produces analgesia by a peripheral mechanism can be easily

Table [2]. Effects of intravenous administration of CRF on responses to noxious stimulation.

Study	Doses Tested[a]	Subjects	Method of Assessment	Results[b]	Antagonists
Ayesta and Nikolarakis, 1989	1-32 µg (4-114 µg/kg)	M Wistar rats	Tail flick test	Analgesia (4-32 µg)	✘ Naloxone, SC ✘ Morphine tolerance, SC
Hargreaves et al., 1987	1 µg/kg	M humans	Post-operative dental pain	Analgesia	
Hargreaves et al., 1987	25.2 nmol/kg (118 µg/kg)	M SD rats	Hot plate test	Analgesia	
Hargreaves et al., 1990	5-25.2 nmol/kg (23-118 µg/kg)	M SD rats	Hot plate test	Analgesia (ED_{50}=10 nmol/kg)	✔ Anti-ß-endorphin, IV ✔ Dexamethasone, IV ✔ Hypophysectomy (with corticosterone replacement) ✔ Naltrexone, IV ✔ Naltrexone methyl bromide, IV
Kiang and Wei, 1987	2-14 µg (8-58 µg/kg)	Anaesthetized M albino rats	Paw flick, 48°C water, 5 min	Antinociception (4-14 µg)	
Kita et al., 1993	5-40 µg/kg	F Std:ddy mice	Phenylquinone injection, IP	Analgesia (10-40 µg/kg)	✘ α-helical CRF, IC ✔ α-helical CRF, IV ✘ Mr2266, SC ✘ Naloxone, SC
Poree et al., 1989	6-18 nmol/kg (29-86 µg/kg)	Anaesthetized M SD rats	Trigeminal neuron activity, noxious heat application	Antinociception (ED_{50}=2.3-7 nmol/kg)	✘ Adrenalectomy ✘ Chlorisondamine, IP ✔ α-helical CRF, IV ✘ Hypophysectomy ✘ Naloxone, IV
Schäfer et al., 1994	Equivalent to (?) 0.1-1.5 ng, I.pl.	M Wistar rats	CFA-induced hyperalgesia	No effect	
Schäfer et al., 1996	10 ng (50 ng/kg)	M Wistar rats	CFA-induced hyperalgesia	No effect	
Wei et al., 1986	1, 10, 100 µg/kg	M mice	Phenylbenzoquinone injection, IP	Analgesia (ED_{50}=5.84 nmol/kg)	

Abbreviations: CFA, complete Freund's adjuvant; F, female; IP, intraperitoneal; I.pl., intraplantar; IV, intravenous; M, male; SC, subcutaneous; SD, Sprague-Dawley; ✔, effective antagonism; ✘, ineffective antagonism;?, information not specified.
[a]Doses in parentheses are calculated from molecular weights and the average weight of the subjects. [b]Doses in parentheses are reported effective doses.

Table [3]. Effects of central administration of CRF on responses to noxious stimulation.

Study	Doses Tested	Subjects	Method of Assessment	Results[b]	Antagonists
Intracerebroventricular (unless otherwise specified)					
Ayesta and Nikolarakis, 1989	3-30 µg	M Wistar rats	Tail flick test	No effect	
Bianchi et al., 1991	500 ng	M SD rats	Hot plate test	Analgesia	✘ Naloxone, IP
Bianchi and Panerai, 1995	500 ng	M SD rats	Hot plate test	Analgesia	✔ 6-hydroxydopamine, ICV ✔ Prazosin, IP
Borsody and Weiss, 1996	ICV: 250 ng, 1 µg, 3 µg; LC micro-injection: 90 ng	Anaesthetized M and F SD rats (microinjection: M only)	Locus coeruleus neuron activity, paw pinch	No effect	
Britton et al., 1985	1 µg	M Wistar rats	Hot plate test; Tail flick test	No effect	
Kita et al., 1993	Intracisternal: 50, 100, 200 ng	F Std:ddy mice	Phenylquinone injection, IP	Analgesia (100, 200 ng)	✔ α-helical CRF, IC ✔ Mr2266, SC ✔ Naloxone, SC
Poree et al., 1989	2 nmol (9.5 µg)	Anaesthetized M SD rats	Trigeminal neuron response, noxious heat application	No effect	
Sherman and Kalin, 1986	0.3 (Hot plate only), 3.0 µg	M SD rats	Hot plate test; Tail flick test	No effect	
Sherman and Kalin, 1987	0.03, 0.3, 3.0 µg	M SD rats	Hot plate test	No effect	
Sherman and Kalin, 1988	300 ng	SD rats	Hot plate test	No effect	
Valentino and Foote, 1987	0.3, 1.0, 3.0 µg	Anaesthetized M SD rats	Locus coeruleus neuron activity, sciatic nerve stimulation	Antinociception (1.0, 3.0 µg)	
Wei et al., 1986	10 µg	M SD rats	Tail flick test	No effect	
Williams et al., 1986	0.25, 0.5, 1.0, 2.0 µg	M New Zealand white rabbit	Ear withdrawal, radiant heat	Hyperalgesia (0.5, 1.0 µg)	
Intrathecal					
Song and Takemori, 1990	12.5, 25, 50 pmol	M Swiss-Webster mice	Acetic acid injection, IP	Analgesia (ED_{50}=22.1 pmol)	✘ ß-funaltrexamine, I.t. ✔ α-helical CRF, I.t. ✔ Naloxone, SC ✘ Naltrindole, I.t. ✔ Nor-binaltorphimine, I.t.
Song and Takemori, 1991	?-0.5 nmol	M Swiss-Webster mice	Tail flick test	No consistent analgesia	

Abbreviations: F, female; IC, intracisternal; ICV, intracerebroventricular; IP, intraperitoneal; I.t., intrathecal; LC, locus coeruleus; M, male; SC, subcutaneous; SD, Sprague-Dawley; ?, information not specified; ✔, effective antagonism; ✘, ineffective antagonism.
[b]Doses in parentheses are reported effective doses.

Table [4]. Effects of subcutaneous and intradermal injection of CRF on responses to noxious stimulation.

Study	Site of Admin.	Doses Tested[a]	Subjects	Method of Assessment	Results[b]	Antagonists
Cabot et al., 1997	Hindpaw, I.pl.	0.1-1.5 ng	M Wistar rats	CFA-induced hyperalgesia	Reduced hyperalgesia (ED_{50}=1.59ng), inflamed paw only	✔ Anti-ß-endorphin, I.pl.
Hargreaves et al., 1989	Neck, SC	20 nmol/kg (25 µg/kg)	M SD rats	Carageenan-induced hyperalgesia	Reduced hyperalgesia	✘ Adrenalectomy ✘ Hypophysectomy
Hargreaves et al., 1989	Hindpaw, I.pl.	0.25 nmol/kg (0.3 µg)	M SD rats	Carageenan-induced hyperalgesia, both hindpaws	Reduced hyperalgesia, CRF-injected paw only	
Kiang and Wei, 1987	Hindpaw, intradermal	0.2-2.5 µg	Anaesthetized M Albino rats	Paw flick, 48°C water, 5 min	Analgesia (0.4-2.5 µg)	
Schäfer et al., 1994	Hindpaw, I.pl.	0.1-1.5 ng	M Wistar rats	CFA-induced hyperalgesia	Reduced hyperalgesia (doses?), inflamed paw only	✘ Anti-dynorphin A, I.pl. ✔ Anti-ß-endorphin, I.pl. ✔ Anti-[Met]-enkephalin, I.pl. ✔ Cyclosporin A, I.pl. ✔ α-helical CRF, I.pl.
Sherman and Kalin, 1986	Neck, SC	0.3, 3.0 µg (1, 10 µg/kg)	M SD rats	Hot plate test	No effect	
Zadina and Kastin, 1986	Neck, SC	1-50 µg/day, D1-D7	M Holtzman rat pups	ß-endorphin analgesia, tail flick test	No effect	

Abbreviations: CFA, complete Freund's adjuvant; D1-D7, first 7 days of life; I.pl., intraplantar; M, male; SC, subcutaneous; SD, Sprague-Dawley; ✔, effective antagonism; ✘, ineffective antagonism; ?, information not specified.

[a]Doses in parentheses are calculated from molecular weights and the average weight of the subjects. [b]Doses in parentheses are reported effective doses

traced. First, the majority of studies in which CRF is administered intravenously demonstrate analgesia (see Table [2]). CRF is a peptide and the blood-brain barrier was traditionally considered to be impermeable to peptides that do not have a specific transport system (Banks et al. 1991). Thus, several authors have concluded that CRF must act outside the central nervous system following intravenous administration (Wei et al. 1986; Ayesta and Nikolarakis 1989; Poree et al. 1989). However, extremely high doses of CRF are necessary to produce analgesia with intravenous administration. It will be argued later that with extremely high intravenous doses, a significant amount of CRF can cross the blood-brain barrier and have central effects. Furthermore, it will be shown that the inability to explain the analgesia following intravenous administration in several studies using the known CRF-induced peripheral mechanisms questions the exclusion of a central mechanism.

Secondly, the ability of local administration of low doses of CRF to produce analgesia (Kiang and Wei 1987; Hargreaves et al. 1989; Schäfer et al. 1994; Cabot et al. 1997); see Table [4]) has provided further support for a peripheral mechanism mediating the effects. However, it will be demonstrated that inflammation in the area is necessary for local CRF to produce analgesia and therefore a local mechanism cannot explain analgesia in conditions that do not involve inflammation.

Finally, the failure of most of the early studies (published before 1991) to demonstrate a significant effect of intracerebroventricular administration of CRF on pain (see Table [3]) further supported the exclusion of a central mechanism in CRF-induced analgesia. Nonetheless, Valentino and Foote (1987) showed that the electrophysiological response of locus coeruleus neurons to high intensity stimulation of the sciatic nerve in the anaesthetized rat is disrupted by intracerebroventricular administration of CRF. In addition, Williams et

al. (1986) also showed that intracerebroventricular administration of CRF affected pain behaviour, *decreasing* the latency of rabbits to withdraw the ear from a radiant heat source. Yet, reviews of the effects of CRF (Dunn and Berridge 1990; Owens and Nemeroff 1991) fail to mention these findings in the context of analgesia, perhaps due to the difficulty of interpreting them.

This paper will review the studies that examine the analgesic effects of CRF following systemic and local administration, and will address the hypothesized underlying mechanisms. It will also examine the findings of more recent studies that demonstrate analgesia following intracranial administration of CRF, and discuss the underlying mechanisms with special emphasis on possible brain mechanisms.

The evidence indicates that: 1) CRF can act at all levels of the neuraxis to produce analgesia; 2) the release of β-endorphin does not explain the analgesia following intravenous or intracranial CRF administration; 3) inflammation must be present for local CRF to evoke analgesia; and 4) the analgesic effects of CRF show specificity for prolonged pain.

Studies of CRF-Induced Analgesia

Intracranial administration studies

Recent studies show that intracerebroventricular (Bianchi et al. 1991; Bianchi and Panerai 1995; Lariviere et al., in preparation) and intracisternal (Kita et al. 1993) administration of CRF can produce analgesia. It appears that the early studies failed to show significant analgesia because the doses of CRF that were used skip over the narrow effective dose range for intracranial administration.

Although Williams et al. (1986) evoked *hyper*algesia following

intracerebroventricular administration of CRF in the rabbit, their results illustrate the narrow effective dose range. They tested doses of 0.25, 0.5, 1.0, and 2.0 μg, and found significant effects of the two middle doses. Their effective and ineffective doses differ merely by a factor of two. Kita et al. (1993) demonstrated the lower end of the dose-response curve in the mouse, producing significant analgesia with intracisternal administration of 100 and 200 ng, but not with 50 ng. Again, the effective and ineffective doses differ by a factor of two. Thus, doses that differ by a factor of ten may easily skip over the effective dose range, as Sherman and Kalin (1986, 1987, 1988) demonstrate in the rat, failing to find significant effects with 0.03, 0.3, and 3.0 μg of CRF.

Bianchi and colleagues (Bianchi et al. 1991; Bianchi and Panerai 1995) further confirm the ability of intracerebroventricular CRF to affect pain responding. In both studies, they evoked analgesia with 500 ng, a dose not previously tested in the rat. Although the effective doses differ slightly among species, the mouse, rat, and rabbit demonstrate effective doses in the nanogram range.

Data recently collected in our laboratory also demonstrate the narrow effective dose range of intracerebroventricular CRF in the formalin test (Lariviere et al., in preparation). We found that of four doses tested (0.3, 0.5, 0.7, and 0.9 μg) only 0.7 μg produces significant analgesia for the pain following intraplantar injection of 50 μl of 2.5% formalin in the rat.

Further support for the narrow range of effective doses is provided by observations from other behavioural studies. With very low doses of CRF, the effect is simply not detectable. However, as highlighted by Dunn and Berridge (1990), differential effects on rats of low ($\leq$ 0.2 μg) versus high ($\geq$ 1 μg) doses of intracerebroventricular CRF have been observed on locomotor activity in a novel environment, feeding in food-deprived rats, and

shock-induced boxing and fighting. Low doses increase locomotor activity, feeding, and shock-induced boxing and fighting, whereas high doses of CRF decrease locomotor activity and feeding, and disrupt behavioural responding following shock. It may be that the analgesic effects are similarly disrupted with doses equal to, or greater than, 1 μg. This would explain the lack of effect of doses of intracerebroventricular CRF from 1 μg to as high as 30 μg (see Table [3]).

Also, intracerebroventricular doses of 10 and 25 μg have been shown to produce both electroencephalographic and behavioural signs of seizure activity in the rat (Ehlers et al. 1983). Despite this, three studies used doses of 10 μg or more (Wei et al. 1986; Ayesta and Nikolarakis 1989; Poree et al. 1989).

It should be noted that the effective doses in the anaesthetized rats of the study of Valentino and Foote (1987) were 1.0 and 3.0 μg, which are higher than the nanogram range of the above mentioned studies. However, direct comparisons cannot be made between the results in the anaesthetized and the unanaesthetized rat. Valentino and Foote (1988) reexamined the effect of CRF on the electrophysiological activity of locus coeruleus neurons in unanaesthetized rats using an auditory stimulus that evokes a discharge similar to that evoked by the sciatic nerve stimulation used in their 1987 study. They found that tonic electrophysiological activity was increased but discharge rates following the auditory stimulus were not significantly affected. Hence, the effects of CRF on the sensory-evoked activity of the locus coeruleus seen in the anaesthetized rat do not transfer to the awake, freely behaving rat and such comparisons should be made with caution.

In summary, several studies have demonstrated significant analgesia following intracranial administration of CRF. Moreover, a dose-response analysis demonstrates that

the effective dose range is in the nanogram range and is very narrow, which explains the failure of the majority of studies that examine intracerebroventricular administration. Therefore, the conclusion that central administration of CRF does not affect pain or pain-related electrophysiological activity and behaviour is not valid.

In addition to intracranial administration, intrathecal administration of CRF has also been shown to produce analgesia in the acetic acid writhing test in the mouse (Song and Takemori 1990). The analgesia seems to involve kappa opioid receptors, but not mu or delta opioid receptors, since it is inhibited by intrathecally administered naloxone and nor-binaltorphimine, but not by ß-funaltrexamine nor naltrindole. Paradoxically, intrathecal CRF antagonizes the analgesic effect of subcutaneous morphine (Song and Takemori 1991), an effect also seen with intracerebroventricular CRF, which antagonizes the analgesic effect of intracerebroventricular β-endorphin (Williams et al. 1986).

Since CRF causes the release of ß-endorphin from the pituitary gland (Guillemin et al. 1977), this has been examined as the mechanism of analgesia following intracranial administration of CRF. Kita et al. (1993) provide some supporting evidence, showing antagonism of the analgesic effects of intracisternal CRF in the mouse phenylquinone writhing test by subcutaneous administration of two opiate antagonists, Mr2266 and naloxone. Conflicting results were found by Bianchi et al. (1991) who showed that intraperitoneal naloxone prolongs the analgesia seen in the rat tested with the hot plate test. It is not possible at present to determine the source of this discrepancy since the parameters of species and pain test differ between these studies, and neither is predictive of naloxone-reversibility. Subcutaneous naloxone did not reverse the analgesia in the mouse phenylquinone writhing test following intravenous administration of CRF (Kita et al. 1993).

In addition, Hargreaves et al. (1990) found that the pituitary gland and endogenous opioids were involved in analgesia seen in the rat tested with the hot plate test. Thus, although endogenous opioids are involved in CRF-induced analgesia, they are not involved all of the time.

Intracranial administration of CRF may also affect brain mechanisms involved in pain processing. It has already been noted that CRF has effects on the tonic electrophysiological activity of the locus coeruleus (Valentino and Foote 1987; Borsody and Weiss 1996), which is involved in the tonic descending inhibitory control of spinal cord circuits (Besson and Chaouch 1987). CRF also induces electrophysiological activity in the hippocampus, specifically in the CA1 and CA3 areas (Aldenhoff et al. 1983; Siggins et al. 1985). This effect may modulate pain since processing in the hippocampus has been shown to be involved in pain behaviour in the formalin test (McKenna and Melzack 1992). CRF also has excitatory actions in the amygdala, the cortex, and the hypothalamus (Ehlers et al. 1983; Siggins et al. 1985), all of which have been shown to be involved in pain processing (Bouckoms 1994; Melzack and Wall 1996). Furthermore, CRF has predominantly inhibitory actions on the electrophysiological activity of the thalamus, and the paraventricular nucleus of the hypothalamus (Siggins et al. 1985), areas also shown to be involved in pain processing, and especially in stress-induced analgesia for the latter structure (Truesdell and Bodnar 1987).

In addition to the above sites at which CRF has electrophysiological effects, the distribution of CRF immunoreactivity (the binding of antibodies that recognize CRF) and of CRF receptors throughout the brain (Chappell et al. 1986; De Souza 1987; Dunn and Berridge 1990; Chalmers et al. 1996), suggests that there are potentially many other sites in

the brain at which CRF could modulate pain processing (see Table [5]). CRF and CRF-immunoreactive areas are found in cerebrocortical areas as well as in the limbic system, diencephalon, and brainstem (Chappell et al. 1986; Dunn and Berridge 1990). All of these areas contain nuclei which have been shown to be involved in pain processing.

Although a sensory function has been given to the CRF1 receptor subtype based on its anatomical distribution in classical sensory relay structures, there is overlap in the distribution of receptor subtypes and only very high densities of receptor subtypes are considered in the analysis (Potter et al. 1994; Chalmers et al. 1996). The present analysis considers pain to be a multidimensional experience whose neural substrates are distributed throughout the brain (Melzack 1989; Melzack 1990).

Areas within the limbic system subserve the affective dimension of pain (Melzack and Casey 1968; Bouckoms 1994). CRF has been shown to be present in these areas and CRF concentrations have been shown to be modulated by acute and chronic stress in many of them (Chappell et al. 1986). These areas include the arcuate nucleus, amygdala, cingulate cortex, hippocampus, lateral habenula, lateral hypothalamus, median eminence, and the paraventricular nucleus of the hypothalamus, each of which has been shown to be involved in pain perception (Fuchs and Cox 1993; Bouckoms 1994; Fuchs and Melzack 1995; Hsieh et al. 1995).

The cerebral cortex, which plays a role in the evaluative dimension of pain (Melzack and Casey 1968), also exhibits changes in CRF levels in its medial prefrontal areas in response to acute and chronic stress (Chappell et al. 1986). Moreover, the ventrobasal thalamus and its cortical projections, which subserve the sensory dimension of pain (Melzack and Wall 1996), also have the potential to be affected by stress-induced changes in CRF.

Table [5]. Potential pain modulation sites of CRF.

Site	Reference	Site	Reference
Within the CNS			
Amygdaloid nuclei	2, 3, 4, 6	Median eminence	3, 4
Anterior hypothalamic nucleus	4, 6	Paraventricular nucleus of the hypothalamus	3, 4, 8
Arcuate nucleus	3, 4	Periaqueductal gray	4, 6
Cingulate cortex	6	Prefrontal cortex	6
Hippocampus	1, 4, 5	Raphe nuclei	4
Insular cortex	6	Spinal cord	5, 8
Lateral habenula	4	Ventrobasal thalamus	7
Lateral hypothalamus	3	Ventromedial nucleus of the hypothalamus	4
Locus coeruleus	3, 4, 6, 9	Zona incerta	3
Medial prefrontal cortex	4		
Outside the CNS			
Adrenal medulla	6, 8	Sensory ganglia	8
Anterior pituitary gland	5, 8	Sympathetic ganglia	6
Immune cells	8		

References: 1, Aldenhoff et al., 1983; 2, Beaulieu et al., 1987; 3, Brown, 1986; 4, Chappell et al., 1986; 5, De Souza, 1987; 6, Dunn and Berridge, 1990; 7, Merchenthaler et al., 1984; 8, Schäfer et al., 1997; 9, Valentino and Foote, 1988.

Areas of the thalamus, including the ventromedial nucleus and other nuclei of the posteromedial complex (Merchenthaler et al. 1984), as well as the zona incerta (Brown 1986) contain CRF-like peptides or show responses to microinjection of CRF.

Furthermore, systems involved in the tonic descending inhibitory control of pain-related signals in the spinal cord also contain CRF-immunoreactive areas or cause an increase in plasma norepinephrine in response to microinjection of CRF. These areas include the locus coeruleus, midbrain periaqueductal gray, and the raphe nuclei (Brown 1986; Chappell et al. 1986; Valentino and Foote 1988; Dunn and Berridge 1990). These descending inhibitory mechanisms are likely to be involved in CRF-induced analgesia since they are partly noradrenergic (Basbaum and Fields 1984), and the analgesia following intracerebroventricular administration of CRF is antagonized by 6-hydroxydopamine and prazosin (Bianchi and Panerai 1995).

In addition to intracranial sites of action, the substantia gelatinosa in the spinal cord, a part of the dorsal horn that receives pain-related afferent signals, also contains receptors for CRF throughout its length (Skofitsch et al. 1985). The analgesia following intrathecal administration of CRF appears to be due to the action of CRF at these receptors, since the analgesia is antagonized by intrathecal administration of the CRF receptor antagonist, α-helical CRF (Song and Takemori 1990).

Taken together, these data indicate that centrally-administered CRF has effects on pain, and that the inability of earlier studies to demonstrate this is due to the use of doses outside the narrow effective dose range of intracranially-administered CRF. Furthermore, the distribution of CRF and CRF receptors suggests that CRF could act at a number of sites throughout the central nervous system to affect pain processing.

Since CRF is transported across the blood-brain barrier from brain to blood (Martins et al. 1996), central administration of CRF may also act via peripheral mechanisms. However, our discussion below on analgesia following intravenous administration of CRF indicates that certain conditions are necessary to say with confidence that CRF is producing analgesia by a peripheral mechanism.

Local administration studies

CRF also acts outside of the central nervous system, at the site of inflammation in peripheral tissue (see Table [4]). CRF has been shown to have anti-inflammatory effects (Wei et al. 1986; Schäfer et al. 1997), which could indirectly decrease nociception. Schäfer et al. (1997) have described an additional mechanism by which local CRF can have analgesic effects. In inflammatory conditions, immune cells migrate to the inflamed area. Peripheral CRF in inflamed tissue acts at receptors on the immune cells to evoke the release of opioid molecules. These opioid molecules then act at opioid receptors on peripheral sensory afferent neurons and inhibit their activity.

Support for the involvement of this mechanism in analgesia following intraplantar administration of CRF is provided by Schäfer and Stein and their colleagues. They demonstrated that local intraplantar injection of antibodies to ß-endorphin, antibodies to [Met]-enkephalin, and the immunosuppressant cyclosporin A inhibited the CRF-induced reduction of complete Freund's adjuvant-induced hyperalgesia in the rat (Schäfer et al. 1994; Cabot et al. 1997). Hargreaves et al. (1989) and Kiang and Wei (1987) also provide support for the involvement of a peripheral antinociceptive mechanism following local

administration of CRF in two other inflammatory conditions, carageenan-induced hyperalgesia and immersion of the rat hindpaw in 48°C water for 5 minutes.

These local mechanisms appear to be involved only in inflammatory conditions since local injection of CRF fails to produce analgesia when administered in a noninflamed area. The same doses which increase paw pressure thresholds in an inflamed paw of the rat, are ineffective at altering paw pressure thresholds when injected in the contralateral, noninflamed paw (Schäfer et al. 1994; Cabot et al. 1997).

According to the known mechanisms and findings, there must be inflammation at the site for local CRF in peripheral tissue to induce analgesia. Therefore, studies in which CRF evokes analgesia in noninflammatory pain tests must explain the analgesia by using mechanisms that are not related to inflammation.

Intravenous administration studies

The majority of studies that administer CRF by intravenous injection demonstrate analgesia (see Table [2]). This, however, does not prove whether CRF is producing analgesia via a peripheral site or a central site of action.

Intravenous administration of CRF could produce analgesia via direct anti-inflammatory actions (Wei et al. 1986) or via the immune cell-mediated mechanism. This is probably the case in the Kiang and Wei (1987) study in which they invoke a thermal injury associated with inflammation. But these mechanisms are involved only when inflammation is present. As such, they cannot explain the results when analgesia is assessed with pain tests that do not involve inflammation. For example, intravenous administration of CRF produces analgesia in the tail flick test (Ayesta and Nikolarakis 1989) and in the hot plate test

(Hargreaves et al. 1987, 1990), both of which do not involve inflammation at the time of the response by the animal. Therefore, non-inflammatory mechanisms must be involved in these studies.

Since CRF causes the release of ß-endorphin from the anterior pituitary gland (Guillemin et al. 1977), this is another possible mechanism of analgesia following intravenous administration. However, the majority of studies that examine this possibility did not reverse the effects of CRF by systemic administration of the opiate antagonists naloxone and Mr2266, by morphine tolerance, or by the removal of the pituitary gland (Ayesta and Nikolarakis 1989; Poree et al. 1989; Kita et al. 1993). Thus, alternate mechanisms must underlie the evoked analgesia.

Nevertheless, systemic opiate antagonists and hypophysectomy did counter the CRF-induced analgesia in one study (Hargreaves et al. 1990). This study used doses comparable to the doses used by Poree et al. (1989) and the same strain of rat, but unlike any other intravenous administration study, they assessed the analgesia with the hot plate test. This would suggest that the involvement of the pituitary gland and endogenous opioids may be specific to the hot plate test. However, systemic naloxone was not able to reverse the analgesia in the hot plate test following intracerebroventricular administration of CRF (Bianchi et al. 1991), precluding the use of the hot plate test as the determining factor.

The strongest support for the involvement of underlying mechanisms not related to inflammation or endogenous opioids is provided by the results of Ayesta and Nikolarakis (1989). The method of analgesia assessment used is the tail flick test, which excludes the involvement of inflammation-dependent mechanisms of CRF-induced analgesia. Furthermore, endogenous opioid involvement is ruled out since the analgesia is not reversed

by systemic naloxone or morphine tolerance. This provides direct evidence that mechanisms not related to inflammation or endogenous opioids must be involved.

Although it has not yet been demonstrated, systemically circulating CRF could modulate afferent input that contributes to central pain processing. Receptors for CRF are found on sensory (Schäfer et al. 1997) and sympathetic ganglia (Dunn and Berridge 1990). Therefore, it is possible that systemic CRF could act at these sites to affect afferent input directly. Receptors are not present on peripheral nerve endings of subcutaneous sensory neurons (Mousa et al. 1996), precluding a direct effect of CRF on peripheral nerve endings.

In addition, there is reason to believe that peripherally administered CRF can cross the blood-brain barrier, where it could then affect central mechanisms. Although there is a specific transport mechanism for CRF that transports CRF out of the brain to the blood very effectively (Martins et al. 1996), there is also reason to believe that CRF goes from the blood to the brain, albeit much less effectively. Martins and colleagues discovered that following intracerebroventricular administration of [I^{125}-]labelled CRF [half-life of 60 days], brain radioactivity decreased by half in approximately 11 minutes and continued to decrease for the entire 30-minute observation period. Following intravenous administration, the brain acquired a small amount of radioactivity (brain/serum ratio approximately 0.035), but the brain radioactivity did not increase over time relative to the serum. The conclusion drawn was that CRF does not effectively cross the blood-brain barrier from the blood to the brain.

Despite their conclusion, they also state that "we cannot exclude the possibility that CRH could be rapidly transported into the brain and then rapidly returned to the circulation so that no net transport would be apparent" (p.346) and that "the lack of a measurable influx of CRH into the brain over time after peripheral administration was not expected from its

high lipid solubility" (p.346). Thus, it appears that the expected movement of at least small amounts of CRF from the blood to the brain cannot be ruled out since its measurement is confounded by the very effective mechanism of CRF transport out of the brain.

Thus, due to the lipophilicity of CRF, it is expected that with the doses of intravenous CRF necessary to evoke significant analgesia (approximately 10 μg/kg), a significant amount of CRF would cross the blood-brain barrier. For comparison, a 10 μg/kg intravenous dose of CRF given to a rat with approximately 50 ml of blood/kg of rat (Canadian Council on Animal Care 1993) would produce an initial concentration of 0.2 μg CRF/ml blood, or approximately 0.4 μg CRF/ml plasma. This is approximately 15 000-200 000 times as high as plasma levels of 5-13 pg/ml in the unstressed rat (Sumitomo et al. 1987; Hashimoto et al. 1989; Nishioka et al. 1993, 1994; Tojo et al. 1996)) and 2-28 pg/ml in the unstressed human (Linton et al. 1987; Wittert et al. 1992). Following ether stress or water immersion with restraint, plasma levels rise to 19-30 pg/ml in the rat (Hashimoto et al. 1989; Nishioka et al. 1993, 1994), which are approximately 15 000-20 000 times less than 0.4 μg CRF/ml plasma.

Thus, the amount that would enter the brain could be highly significant, perhaps even in the nanogram range that is effective with intracranial administration. Alternatively, the CRF that does enter could have an effect on the proposed ultrashort positive feedback loop of CRF release (Ono et al. 1985). Having reached significant levels in the central nervous system, CRF could act at sites in the spinal cord and throughout the brain.

Therefore, intravenous administration does not necessarily mean that only peripheral sites of action are involved, especially when extremely high doses are administered. That is, intravenous administration of CRF does not preclude the involvement of central mechanisms.

Specificity of CRF-Induced Analgesia for Tonic Pain

Despite the action of CRF at all levels of the neuraxis, the analgesic effects of CRF maintain some specificity for tonic pain compared to phasic pain. Comparisons among the available studies of CRF-induced analgesia which are based solely on the pain test used do not provide conclusive information. All of these comparisons are confounded by differences in species or doses administered, by the administration of extremely high doses of intravenous CRF, or by the very narrow effective dose range of intracranial CRF. The comparison of studies that use identical parameters, except for the type of pain test used, provide the most reliable evidence and support the specificity of CRF effects for tonic pain.

Song and Takemori (1990) provide evidence for the modulation of the tonic pain after intraperitoneal injection of acetic acid in mice. However, with the same route of administration, and equal doses or greater, they (1991) were unable to produce consistent analgesia for the phasic pain of the tail flick test.

Schäfer and Stein and colleagues (Schäfer et al. 1994; Cabot et al. 1997) provide further evidence of this specificity. They showed that intraplantar injection of CRF increases paw pressure thresholds in the tonically hyperalgesic, inflamed rat paw. However, in the noninflamed paw, intraplantar CRF has no effect on the purely phasic pain of paw pressure threshold assessment. Superficially, the inflammation-induced hyperalgesia paradigm used appears to have components of both tonic pain (due to the inflammation) and phasic pain (since the hyperalgesia is assessed with a phasic pain test). However, inflammation-induced hyperalgesia should be considered to be tonic since its duration is prolonged compared to phasic pain tests and the phasic pain is only a component of the assessment of the hyperalgesia in the laboratory.

Electrophysiological findings in the awake rat correlate with the behavioural findings. The tonic electrophysiological activity of locus coeruleus neurons is increased by intracerebroventricular CRF in the awake rat, but in the same preparation, the electrophysiological response to a brief auditory stimulus is not significantly affected (Valentino and Foote 1988). Similarly, in the anaesthetized rat, the response of locus coeruleus neurons to a brief paw pinch is not affected by microinjection of CRF directly onto locus coeruleus neurons, whereas the tonic activity is significantly affected (Borsody and Weiss 1996).

The most interesting results on the specificity of the effects of CRF are those of humans given intravenous CRF for post-operative dental pain. These patients report significant overall analgesia and analgesia on an affective scale, but not on a sensory scale (Hargreaves et al. 1987). Since tonic pain has a greater affective component than phasic pain (Chen and Treede 1985), this may be related to the specificity for tonic pain seen in the animal studies.

Conclusion

CRF has been shown to produce analgesia by all routes of administration attempted, including local, systemic, and central routes, highlighting the fact that CRF can affect pain processing at all levels of the neuraxis. We have shown that the belief that centrally administered CRF is unable to affect pain processing is not supported by the data. In addition, the inability to explain some findings of CRF-induced analgesia with the known peripheral mechanisms supports the involvement of central mechanisms.

Although endogenous opioids have been implicated in the analgesia following

intrathecal and local administration of CRF, pituitary activation and the release of β-endorphin does not explain the analgesia following intracranial or intravenous administration. In fact, the majority of studies find that the pituitary gland or endogenous opioids are not necessary for the analgesia following intracranial or intravenous administration of CRF. Therefore, other mechanisms must be involved.

It is possible that CRF could produce analgesia via the release of corticosteroids from the adrenal cortex. Corticosteroids have anti-inflammatory effects which could indirectly produce analgesia (Cato and Wade 1996). However, corticosteroids also have direct effects on the central nervous system as demonstrated by the excitation of raphe neurons following microelectrophoretic application of corticosterone (Avanzino et al. 1984). In addition, corticosterone has been shown to be necessary in a form of stress-induced analgesia, since blocking of an opioid form of stress-induced analgesia by adrenalectomy is reversed by corticosterone replacement (MacLennan et al. 1982). Furthermore, this effect is seen in the tail-flick test, which demonstrates that corticosterone has pain modulating effects other than its anti-inflammatory effect. However, adrenalectomy only tended to antagonize the effect of CRF (Hargreaves et al. 1989), or had no effect (Poree et al. 1989), which suggests that corticosterone does not mediate the analgesic effects of CRF.

Although Schäfer and colleagues provide evidence that inflammation must be present for local CRF to produce analgesia, more studies are needed to confirm their findings in other inflammatory conditions. We have tested the hypothesis by administering an intraplantar injection of 1.0 μg of CRF ten minutes prior to 2.5% formalin injection in the rat (Lariviere et al., in preparation). If inflammation must be present, then analgesia should occur in the second phase, which is thought to be due in part to inflammatory mechanisms,

but not in the first phase, which is attributed to the direct effects of formalin on peripheral nerve afferents (Porro and Cavazutti 1993). Our study showed no effect ($P > 0.90$) of CRF for the entire 60-minute observation period following formalin injection, suggesting that inflammation alone is not sufficient for CRF to induce analgesia.

We are presently repeating the experiment using the bee venom test, which produces edema approximately three to four times greater than the edema seen in the formalin test (Lariviere and Melzack 1996). In addition, we are examining the response in the formalin test four days after injection of complete Freund's adjuvant in the hindpaw, replicating the design of Schäfer and colleagues who measured paw pressure thresholds (Schäfer et al. 1994; Cabot et al. 1997). This will produce significant infiltration of immune cells from which CRF can release β-endorphin (Cabot et al. 1997). Together these studies will test whether the presence of marked inflammation is sufficient, or if immune cell infiltration is necessary, for local CRF to induce analgesia.

The specificity of CRF's effects on tonic pain suggests that CRF may preferentially play a role in prolonged clinical pain. In fact, altered CRF release and neurotransmission is likely to be involved in certain chronic pain syndromes in humans. For instance, irregularities of HPA-axis function have been associated with pain syndromes that show little or no evidence of pathology in the painful tissue, such as fibromyalgia (Clauw and Chrousos 1997). The effects of CRF within the central nervous system may play a role in these pain syndromes.

Consequently, CRF may represent a new class of analgesic drugs that has been overlooked due to an overemphasis on the peripheral effects of CRF. CRF-related drugs administered at multiple sites of pain modulation may have therapeutic value, especially for

pain syndromes associated with irregularities of HPA-axis function. Furthermore, since CRF can act at all levels of the neuraxis and in the periphery, side-effects may be minimized by the administration of drugs directly at the site of inflammation or at the spinal cord, for example. The evidence suggests that further investigation of the therapeutic value of CRF for the treatment of prolonged pain is warranted and promising.

The role of CRF in pain and analgesia has implications for how stress and pain are related. Studies predominantly demonstrate an analgesic effect of CRF, and CRF is a major mediator of the effects of stress (Dunn and Berridge 1990). Thus, this would suggest that stress has predominantly analgesic effects. However, the effects of acute stress exposure are often the opposite of the effects of chronic stress exposure. Hence, chronic exposure to stress may result in hyperalgesia instead of analgesia. Indeed, students undergoing the chronic stress of an examination period lasting over a month displayed decreased latencies to remove their finger from 55°C water (Cristea et al. 1994). This hyperalgesia may be related to changes in CRF neurotransmission.

Differential effects of acute and chronic stress have been demonstrated for the excitatory effect of CRF on the tonic activity of locus coeruleus neurons. In rats exposed to five daily 30-minute sessions of footshock, the CRF dose-response curve was shifted to the left compared to controls that were not shocked; in contrast, the dose-response curve was shifted to the right in rats exposed for only one day (Curtis et al. 1995). Furthermore, differences in the reduction of CRF content in the median eminence have been demonstrated between acute and chronic immobilization stress (Culman et al. 1991). Repeatedly stressed rats do not show a significant reduction of CRF in the median eminence, whereas acutely stressed rats show a marked, significant reduction in response to immobilization. Therefore,

chronic stress may have the opposite effect of acute stress on pain and, if so, the effect may be mediated through central changes in the release of CRF and in the central response to CRF release.

Acknowledgments

We would like to thank Luc Thivièrge, Marie-Claire Albanese, and Drs. Keith Franklin, Blain Ditto, and Paul Plotsky for their useful criticisms of drafts, not all of which could be addressed here.

References

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The preceding review demonstrates that CRF can act at all levels of the neuraxis and in peripheral tissue to produce analgesia. Furthermore, the specificity of CRF analgesia for tonic pain compared to phasic pain suggests that CRF may preferentially play a role in prolonged pain mechanisms more relevant to prolonged clinical pain.

To test the specificity of effects of CRF for prolonged pain mechanisms, the effect of CRF on phasic and tonic pain sensitivity and on endogenous pain suppression is examined in Chapter 4. To determine the site of action of CRF on pain mechanisms related to adjuvant-induced polyarthritis susceptibility, the effects of central, systemic, and local administration of CRF are compared to the effects of hypophysectomy and the correlations of susceptibility with pain processes found in Chapter 2.

In conclusion, this thesis will examine which pain mechanisms, including central pain mechanisms, are correlated with the susceptibility to adjuvant-induced polyarthritis among groups of rats that differ in HPA axis function. Secondly, it will be determined whether a manipulation of the HPA axis that reduces susceptibility to adjuvant-induced polyarthritis also affects the same pain mechanisms. And third, the effect and site of action of CRF on these pain mechanisms will be investigated. The following three chapters investigate these issues in turn and lead to the conclusion that the HPA axis modulates the susceptibility to the chronic pain model of adjuvant-induced polyarthritis via direct actions of CRF on central pain mechanisms.

Chapter 2

Study of Pain Mechanisms and Susceptibility to Adjuvant-Induced Polyarthritis

This study investigates whether susceptibility to chronic pain that is associated with HPA axis function is also associated with differences in pain processes prior to the onset of chronic pain. Pain sensitivity was examined in groups of rats known to differ in HPA axis function and adjuvant-induced polyarthritis susceptibility. Several weeks prior to polyarthritis induction, phasic and tonic pain sensitivity and endogenous pain suppression in the tail flick and formalin tests were assessed in males and females of two strains of rat. In addition, the effect of postnatal maternal separation on pain processes and adjuvant-induced polyarthritis susceptibility was also examined since maternal separation has been shown to affect HPA axis responsiveness (Meaney et al., 1996).

Methods

Subjects

Male and female Fisher and Lewis rats (10 per group) were used. Pregnant dams arrived from the supplier (Charles River, St.Constant) 4-6 days before giving birth to pups that were tested as adults. The animals were given free access to rat chow and water, and were maintained on a 12 h light/dark cycle with the lights on from 7:00AM. The experimental protocol is summarized in Figure 4 and is described in detail below.

Early Postnatal Treatments

The effects of maternal separation were investigated since early postnatal treatments can produce permanent effects on HPA-axis regulation in adulthood in the rat (Meaney et al.,

Figure 4. Experimental protocol of study of pain mechanisms and susceptibility to adjuvant-induced polyarthritis.

	Dams arrive	Pups born	Maternal separation	Weaned to 2-3/cage	Moved to 1/cage	Tail flick testing	Formalin testing	Airpuff startle	Arthritis induction	Pain behaviour and edema measured
Age	- (4-6 d)	0 d	1-21 d	22 d	3 mo. less 1 wk.	3 mo.	3 mo. and 1 wk.	4 mo. less 1 wk.	4 mo.	4 mo. and 3 wks.

1996). On the second day of life, pups of the same strain were equally distributed among the available dams of the same strain, each dam receiving 7-11 pups. Beginning the same day, rat pups were exposed to one of three treatments for 21 days: maternal separation for 15 minutes (MS15), maternal separation for 180 minutes (MS180), or no daily handling by the experimenter (Control). Maternal separation consists of removing the dam from the home cage after which the pups are placed as a group into a similar cage with bedding. The dam is then returned to her home cage for the period of separation. The pups are taken to another room, where the cage is placed on a towel over a heating pad set at low temperature. The reverse procedure is followed to return the pups to their home cage, where they are rolled in bedding prior to returning the dam. Control rats were handled only to change dirty cages for clean ones every three to four days. At 22 days of age, the pups were weaned from the dams and housed 2 to 3 rats of the same sex per cage until 3 months old less one week when they were housed alone for the remainder of the experiment.

Estrous cycle determination

To control for the effect of estrous cycle, female rats were tested in the diestrous phase of the cycle. At least two hours prior to testing, a vaginal smear was performed in which the tip of a 1 ml syringe is inserted into the vagina and 0.3 ml saline is injected and immediately withdrawn. The fluid is then viewed under a light microscope and the presence of mainly leukocytes determines that the rat is in diestrous (Fox and Laird 1970). The procedure was repeated daily until the rat was in diestrous. Each male underwent from one to four mock smear procedures on consecutive days in which the tip of a syringe was pressed

against the anogenital region for 10 seconds. Pairing of males with females on test days was done as much as possible.

Tail Flick Test

At 3 months of age, phasic pain sensitivity was assessed in the tail flick test. Rats were handled for several minutes and habituated to the testing room on two occasions prior to the day of testing and again on the day of testing. The rat was removed from its home cage, gently restrained in a towel, and its entire tail was immersed in 54°C water. The latency to flick the tail was recorded three times, each time separated by 10 seconds, and the average of the three measures was calculated. To prevent tissue damage, a maximum response latency of 15 seconds was permitted, after which the tail would be withdrawn from the water by the experimenter. However, all rats responded within 5 seconds. All tail flick testing was performed between 9:00AM and 1:00PM.

Formalin Test

At least seven days later, the formalin test was administered to examine phasic and tonic pain sensitivity in the first and second phases, and endogenous pain suppression responsible for the interphase depression in pain responding. Tail flick testing one week prior is not expected to affect formalin pain responses since there is no effect of repeated formalin testing at one week intervals (Rosland et al. 1990; Matthies and Franklin 1992, 1995). The rats were habituated to the 30cm x 30cm x 30cm transparent plexiglass observation box for 30 minutes on two occasions prior to the day of testing and immediately prior to testing. The rat was removed from the observation box, restrained in a towel, and

50 µl of 1.5% formal saline was injected into the plantar surface of the hind paw. The rats were then placed in the observation box and the pain behaviour was scored for 60 minutes. Below the floor of the box, a mirror at a 45° angle facilitated viewing of the injected paw. Pain behaviour was recorded for 60 minutes after injection using a previously validated 3-point scoring method similar to the method of Dubuisson and Dennis (Dubuisson and Dennis 1977; Abbott et al. 1995; Watson et al. 1997). The behaviour was scored as a '2' if the rat licked, bit, or shook the injected paw; as a '1' if the rat elevated the paw from the floor; and as a '0' if any part of the paw other than the tips of the digits was in contact with the box. The score was entered into a computer that recorded the score once every half second. A mean pain score was calculated for each 5-minute period after injection as the sum of the scores divided by the number of scores in the time period. All formalin testing was performed between 9:00AM and 2:00PM.

Airpuff Startle

To assess HPA-axis responsiveness, the plasma corticosterone response to airpuff startle was measured at least 14 days after formalin testing. While in their home cage, the rats were habituated to a room other than the pain testing room for 30 minutes on two occasions prior to the sampling day and immediately before sampling. To obtain a blood sample, each rat was removed from its home cage, restrained in a transparent plastic restraining cone, and brought to the adjacent room. After warming the tail in 40 °C water for 1 minute, the distal 2 mm of the tail was excised with a scalpel. The tail was then milked and 0.3 ml of blood was collected. The procedure was repeated 30 and 120 minutes after airpuff without further excision of the tail. All blood samples were collected between 9:00

AM and 12:30 PM.

Airpuff startle was administered immediately after the first sampling of blood. The unrestrained rat was placed in an empty 48 x 25 x 20 cm shoe box cage without a lid. Three sets of airpuffs were directed towards the side of the head of the rat from approximately 15 cm. Each set consisted of three 5-second air blasts from a pressurized air can (Kensington Dust Blaster©), and each air blast was separated by a 10-second interval. A 1-minute interval separated each set of three airpuffs. The rat was then returned to their home cage.

Blood samples were collected directly into a microcentrifuge tube containing 5 µl of heparin (1000 IU/ml). The tube was then centrifuged at 2000 rpm for 15 min at 4°C. The plasma was drawn off, immediately frozen on dry ice, and stored at -70 °C until sent to the laboratory where the corticosterone assay was performed. Corticosterone assays were performed by radioimmunoassay (see Appendix 1 for the complete protocol) on 5 plasma samples per group. The intra-assay coefficient of variance was 5.6 % and the inter-assay coefficient of variance was 7.4 %.

Adjuvant-Induced Polyarthritis

At least seven days after airpuff startle, the susceptibility to adjuvant-induced polyarthritis was assessed. Complete Freund's adjuvant (1.0 mg Mycobacterium butyricum/300 g rat; 10 mg/ml paraffin oil; M. butyricum purchased from Difco) was injected intradermally at the base of the tail of rats anaesthetized with 2.5 mg/kg acepromazine and 75 mg/kg ketamine. The Female Control Fischer group contains 9 rats instead of 10, since one rat died immediately prior to adjuvant injection, likely due to an adverse reaction to the anaesthetic.

Since the polyarthritis model was used as a model of chronic pain, at 21 days following adjuvant injection, pain and disability behaviour was scored as a measure of symptom severity using the ten-point rating scale shown in Table 6. The rating scale was developed in pilot studies by observing the behaviours that developed as the disease progressed. This scale includes behaviours indicative of pain such as curling, elevation, and shaking of the hind paws, and disability behaviours such as dragging of the affected hind limbs. The scale has been shown to be sensitive enough to detect the effect of rat strain on adjuvant-induced polyarthritis susceptibility, and to be more sensitive to strain effects than measuring the ankle diameter with precision calipers (Lariviere and Melzack, 1997; see data in Appendix 2). Each rat was removed from its home cage, placed on a metal carrier, and observed for 5 minutes. Rats were observed in groups of 2 or 3, since in pilot studies, they explored more in the company of other rats than when alone, allowing for a full range of behaviours to be observed. When more than one behaviour in the scale was observed, the behaviour with the highest score was used in the analysis.

To assess edema produced by adjuvant injection, the mediolateral dimension of the tibiotarsal joint of both hind paw ankles was measured with precision calipers immediately prior to injection and on the 21st day after injection. Since sex differences in ankle diameter before adjuvant injection are expected, the percent increase in diameter was calculated and compared among groups. The percent increase in ankle diameter was calculated as: [(Diameter on day 21 - Diameter before injection) / Diameter before injection] x 100.

Statistical Analyses

Analyses of variance (ANOVAs) were performed to test for significant interactions

Table 6. Scale used to score pain and disability associated with adjuvant-induced polyarthritis.

Behaviour	**Score**
Never *moves; doesn't* explore	10
Drags hindquarters to move	9
Drags one hind limb, using opposite hind limb	8
Shows signs of debilitation, but not always	7
Paw shaking	6
Elevation of both hind paws, excluding digit tips	5
Elevation of one hind paw, excluding digit tips	4
Elevation of at least one hind paw, but not always	3
Curling of a hind paw at all times	2
Curling of a hind paw, but not always	1
None of the above behaviours	0

and main effects of the independent variables. Where appropriate, repeated measures ANOVAs were performed. An effect was determined to be significant if the p value was less than $\alpha = 0.05$. Post-hoc ANOVAs and Tukey HSD tests were performed when necessary to examine significant three-way and four-way interactions or to identify which group means were significantly different. Conservative α levels were used for post hoc analyses, calculated by dividing 0.05 by the number of post hoc analyses performed.

Since the 3-point scale used to score the formalin-induced pain behaviour has been shown to have interval properties (Coderre et al. 1993; Watson et al. 1997), formalin data were analysed as parametric data. Although the pain and disability scale used to measure the response to adjuvant injection has not yet been shown to have interval properties, parametric analyses were performed for several reasons. The lower end of the scale includes the behaviours observed in the formalin pain behaviour scale in the same order, and thus, the scale is expected to have some interval properties. Secondly, parametric analyses are necessary to investigate the interactions of the independent variables. And thirdly, while all categorical data theoretically require nonparametric analyses, the use of parametric analyses has little or no practical consequence due to the robustness of the parametric analyses (Harris, 1995). Nonetheless, to assess whether there was any gain in power from the use of parametric analyses, post hoc nonparametric Kruskal-Wallis analyses were also performed and the results compared to the results obtained with parametric analyses.

Results

Tail Flick Test

The mean tail flick latencies for each group are shown in Figures 5 and 6. A three-

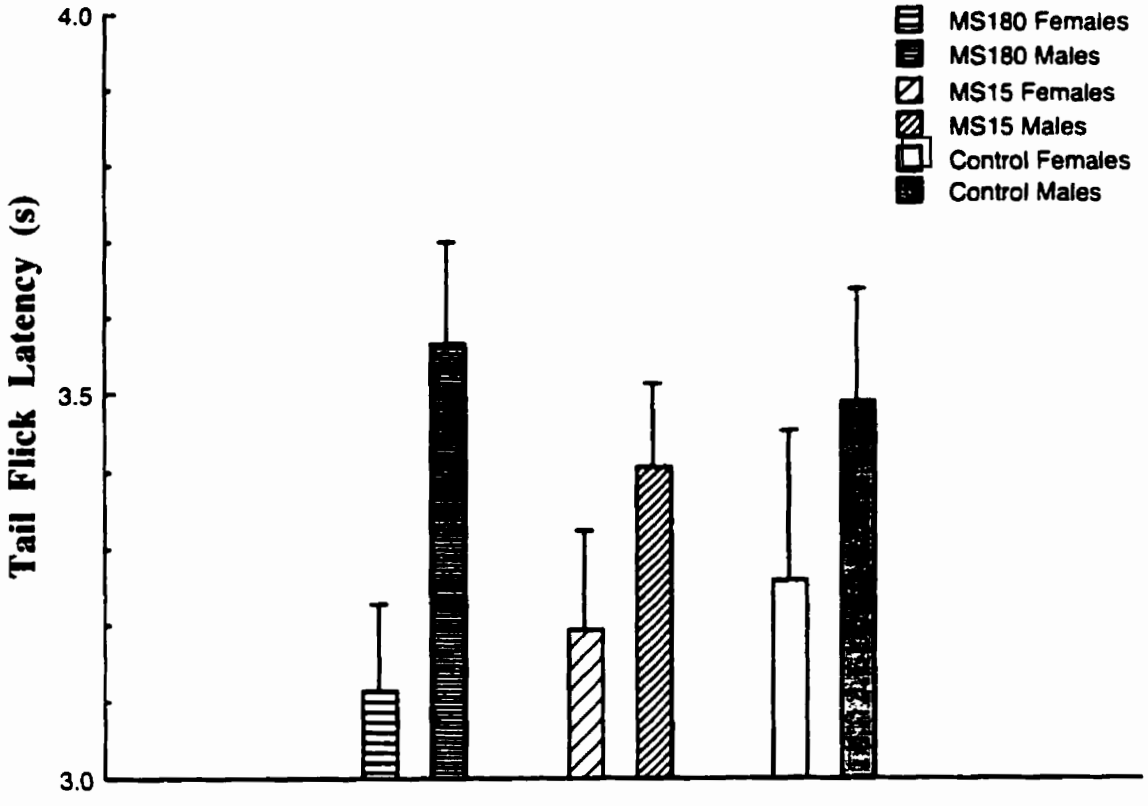

Figure 5. Mean tail flick latencies of Fischer rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 10). Error bars indicate SEM. There is no significant interaction of Maternal Separation x Sex and no effect of Maternal Separation ($p > 0.05$). There is a significant effect of Sex ($p = 0.01$) before correction of the α level for the number of post hoc tests ($p > 0.05/10$ post hoc tests).

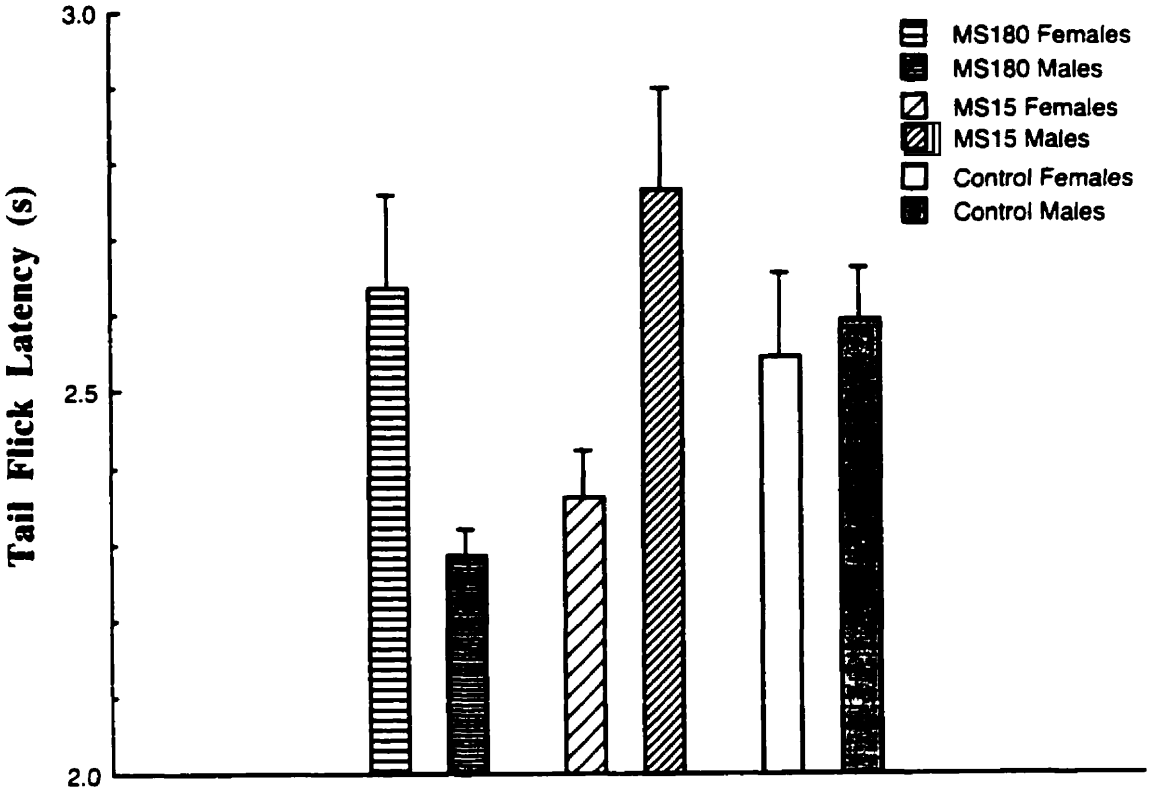

Figure 6. Mean tail flick latencies of Lewis rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 10). Error bars indicate SEM. There is a significant interaction of Sex x Maternal Separation ($p < 0.005$), but no significant pairwise comparisons within Sex or Maternal Separation groups ($p > 0.05/ 10$ post hoc tests). There is no significant effect of Maternal Separation or Sex ($p > 0.05$).

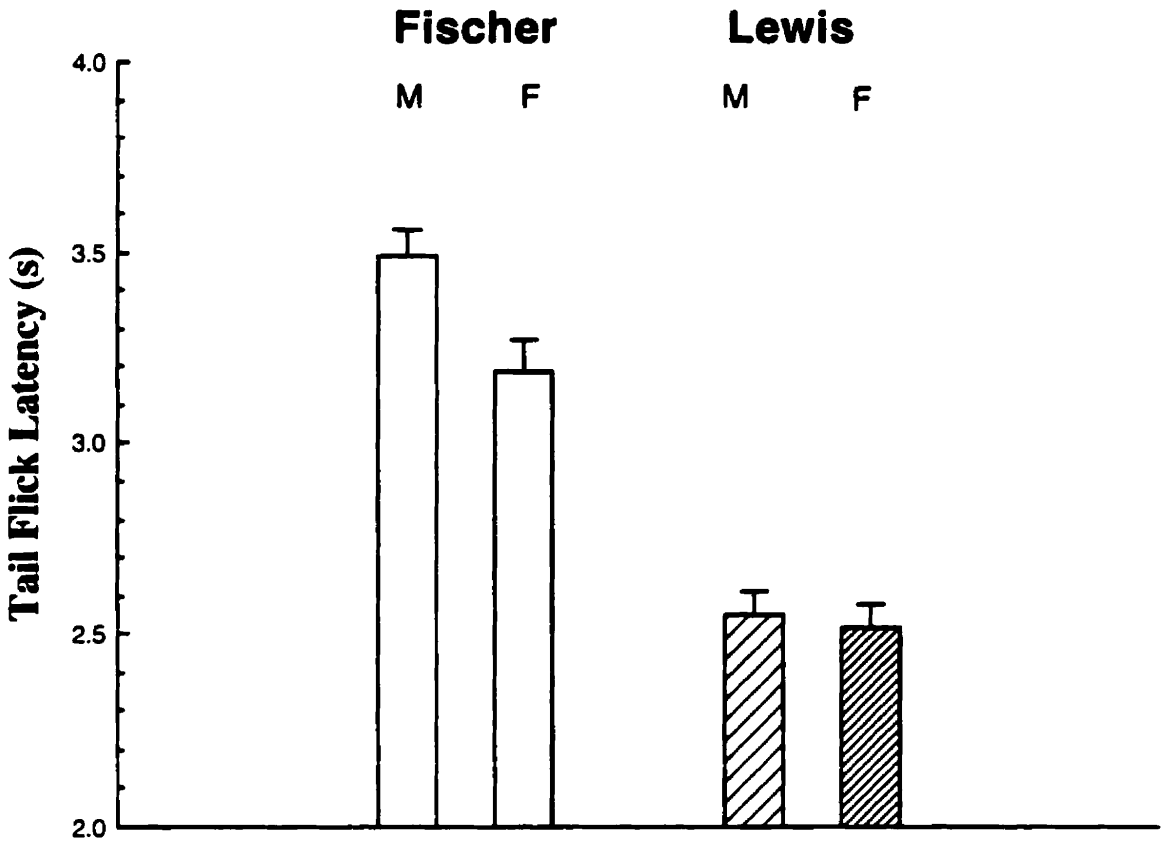

Figure 7. Mean tail flick latencies of Fischer and Lewis rats with Maternal Separation groups collapsed ($n = 30$). Error bars indicate SEM. There is a significant effect of Strain within both sexes ($p < 0.001$), a tendency for an effect of Sex within the Fischer strain ($p = 0.01$; NS: $p > 0.05/10$ post hoc tests), and no effect of Sex within the Lewis strain ($p > 0.05$).

way ANOVA was performed on the mean tail flick latency with factors of Sex (male or female), Maternal Separation (MS180, MS15, or Control), and Strain (Fischer or Lewis). The three-way interaction was significant (F (2, 108) = 4.26). Hence, post hoc 2-way ANOVAs were performed within each sex and each strain. Within the Fischer strain, there is no significant interaction of Sex x Maternal Separation (F (2, 54) = 0.44), no effect of Maternal Separation (F (2, 54) = 0.15), and an effect of Sex (F (1, 54) = 6.76, p = 0.01) that is significant only before correction of the α level for the number of post hoc comparisons (0.05/10 = 0.005). Within the Lewis strain, there is a significant interaction of Sex x Maternal Separation (F (2, 54) = 7.54), but no significant pairwise comparisons within Sex or Maternal Separation groups ($p > 0.005$; post hoc Tukey HSD). The main effects of Sex and Maternal Separation were not significant (F (1, 54) = 0.19; F (2, 54) = 0.79). Within both sexes, there is a significant effect of Strain (females: F (1, 54) = 40.8; males: F (1, 54) = 104.0) and no significant effect of Maternal Separation (F (2, 54) = 0.52, 1.08). Since there is no effect of Maternal Separation, the same data are presented with groups collapsed in Figure 7. This figure illustrates clearly the shorter latencies of the Lewis rat compared to the Fischer rat, the lack of significant sex differences in the Lewis rat, and the tendency for an effect of Sex within the Fischer strain.

Formalin Test

The formalin pain responses are shown in Figures 8 and 9. A four-way repeated measures ANOVA was performed with the factors Sex, Maternal Separation, Strain, and the repeated measures factor Time from formalin injection. The four-way interaction was not significant (F (22, 1188) = 0.90), and only the three-way interaction of Time x Sex x Strain

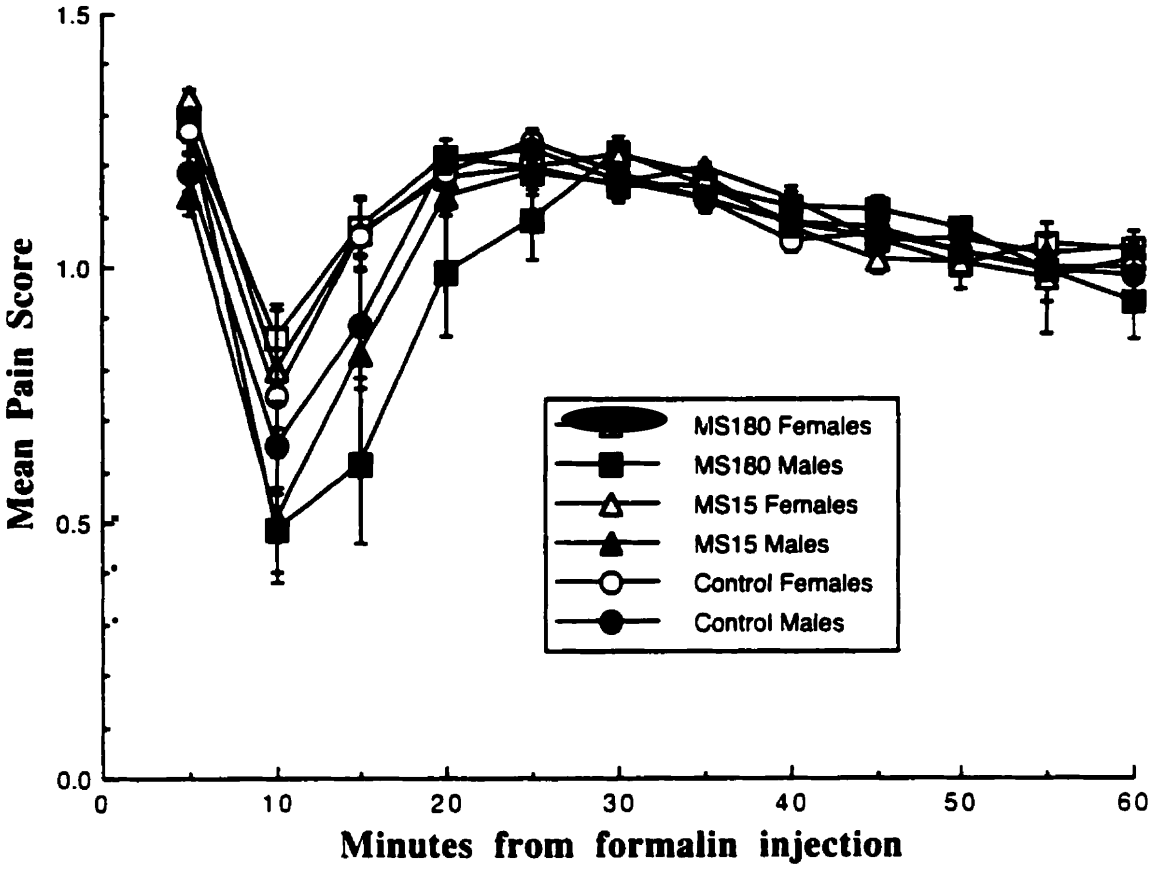

Figure 8. Mean pain scores of Fischer rats maternally separated for 180 or 15 minutes (MS180, MS15) following intraplantar injection of formalin (n = 10). Error bars indicate SEM. There is no interaction with, or effect of, Maternal Separation ($p > 0.05$). There is a significant Time x Sex interaction ($p < 0.001$). Females have significantly greater mean pain scores 10 and 15 minutes following formalin injection ($p < 0.05/20$ post hoc tests).

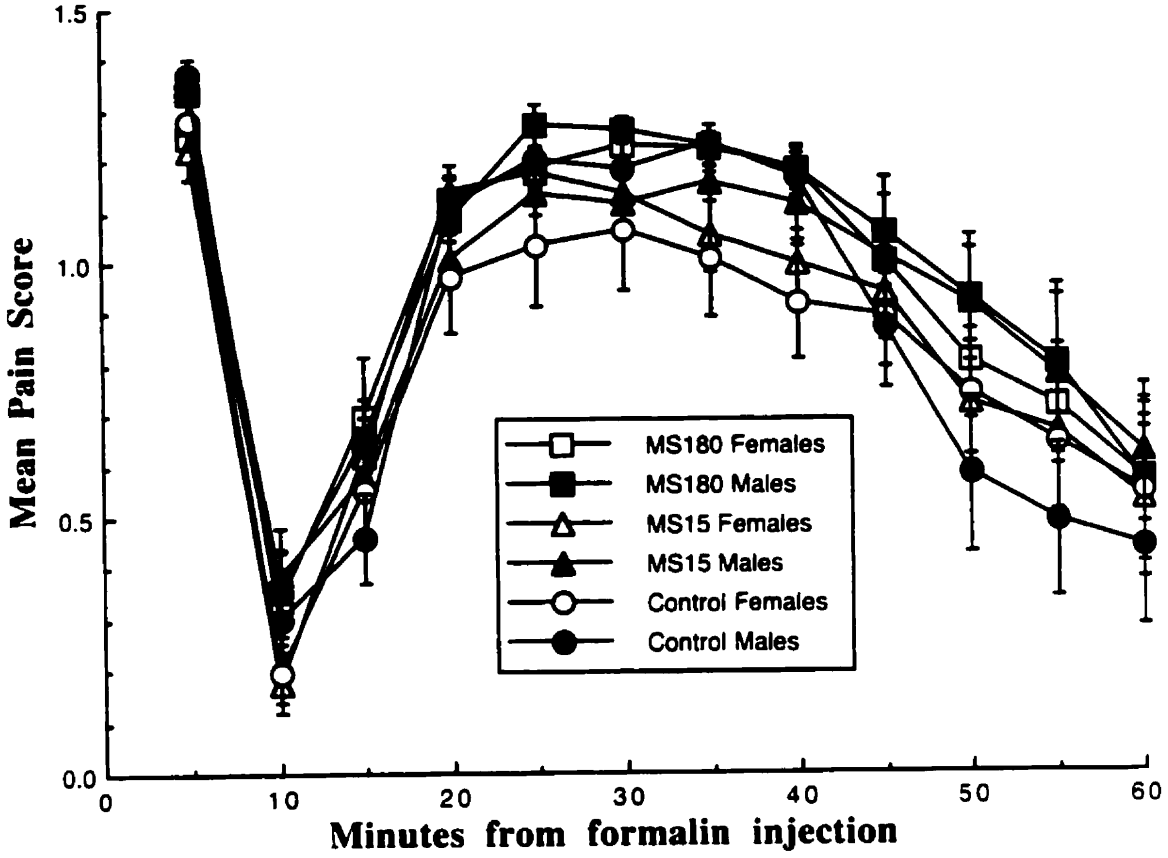

Figure 9. Mean pain scores of Lewis rats maternally separated for 180 or 15 minutes (MS180, MS15) following intraplantar injection of formalin ($n = 10$). Error bars indicate SEM. There is no significant interaction with, or main effect of, Maternal Separation or Sex ($p > 0.05$). There is a significant effect of Time ($p < 0.001$).

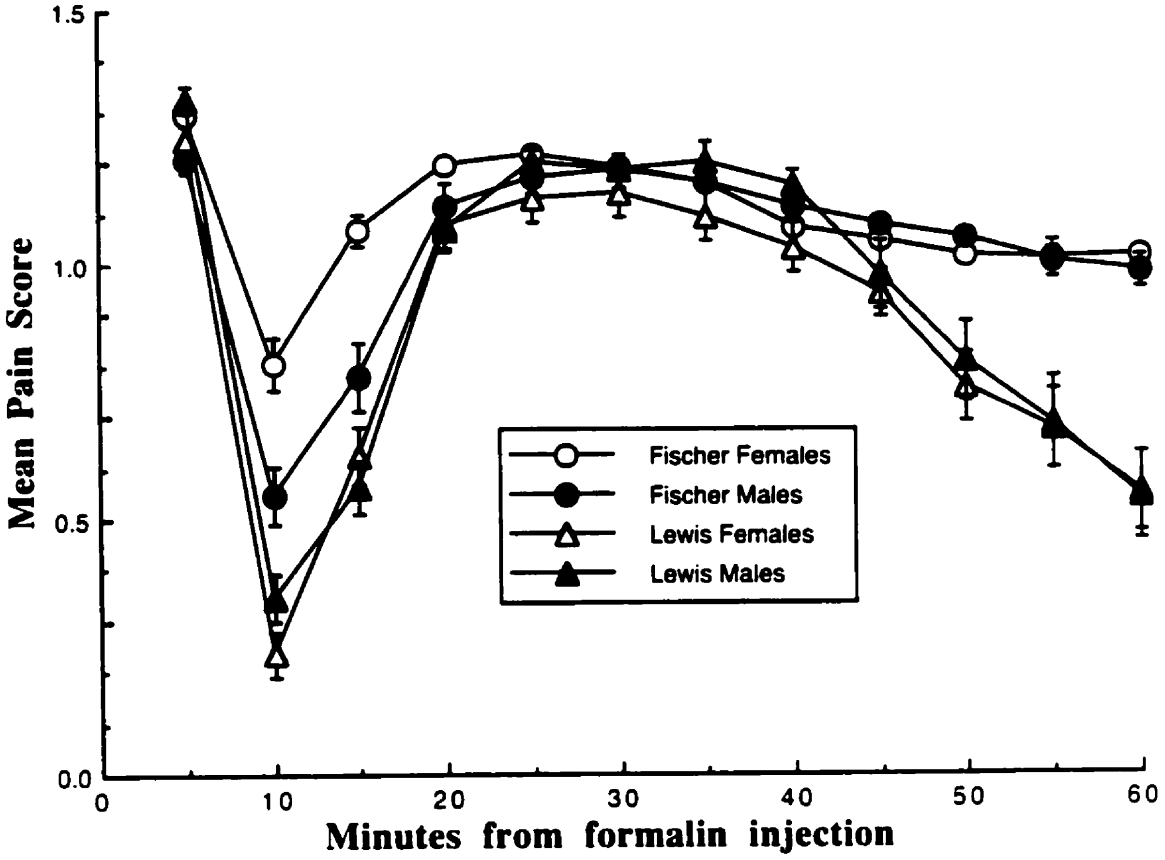

Figure 10. Mean formalin pain scores of Fischer and Lewis rats with Maternal Separation groups collapsed (n = 30). Error bars indicate SEM. Female Fischer rats have greater mean pain scores than male Fischer rats and Lewis rats 10 and 15 minutes after formalin injection ($p < 0.05/20$ post hoc tests). Lewis rats have lower mean pain scores than Fischer rats 50, 55, and 60 minutes after formalin injection ($p < 0.003$). Male Lewis rats have greater mean pain scores than male Fischer rats 5 minutes after formalin injection ($p < 0.003$). Within the Lewis rat, there is no effect of Sex ($p > 0.05$).

was significant (F(11, 1188) = 1.87). There was no significant effect of Maternal Separation (F (2, 108) = 1.65). Post hoc repeated measures ANOVAs show that, within the Fischer strain, there is a significant Time x Sex interaction (F (11, 594) = 7.20), since females show significantly greater mean pain scores at 10 and 15 minutes after formalin injection ($p <$ 0.05/20 post hoc tests). Within the Lewis strain, there is no significant interaction of Time x Sex (F (11, 594) = 0.70), no effect of Sex (F (1, 54) = 1.12), and a significant effect of Time (F (11, 594) = 86.9) due to the nature of the formalin test. Within females, there is a significant Time x Strain interaction (F (11, 594) = 13.2), since Fischer females show greater mean pain scores than Lewis females at 10, 15, 50, 55, and 60 minutes after injection. Within males, there is also a significant Time x Strain interaction (F (11, 594) = 8.68), since Fischer males show greater mean pain scores at 50, 55, and 60 minutes after injection, and significantly lower mean pain scores at 5 minutes. For clarity, the same data are presented in Figure 10 with groups collapsed since there is no effect of Maternal Separation. In this figure, it is clear that there are no significant sex differences within the Lewis strain, and that the Lewis rat shows less pain behaviour than the Fischer rat late in the second phase. In addition, the female Fischer rat shows more pain behaviour than the other groups during the interphase depression in pain responding. In addition, the male Lewis rat shows significantly more pain behaviour during the first phase at 5 minutes after formalin injection, although the effect size is small.

Airpuff Startle

Baseline plasma corticosterone and responses to airpuff startle are shown in Figures 11 and 12. There is a significant three-way interaction of Sex x Maternal Separation x Strain

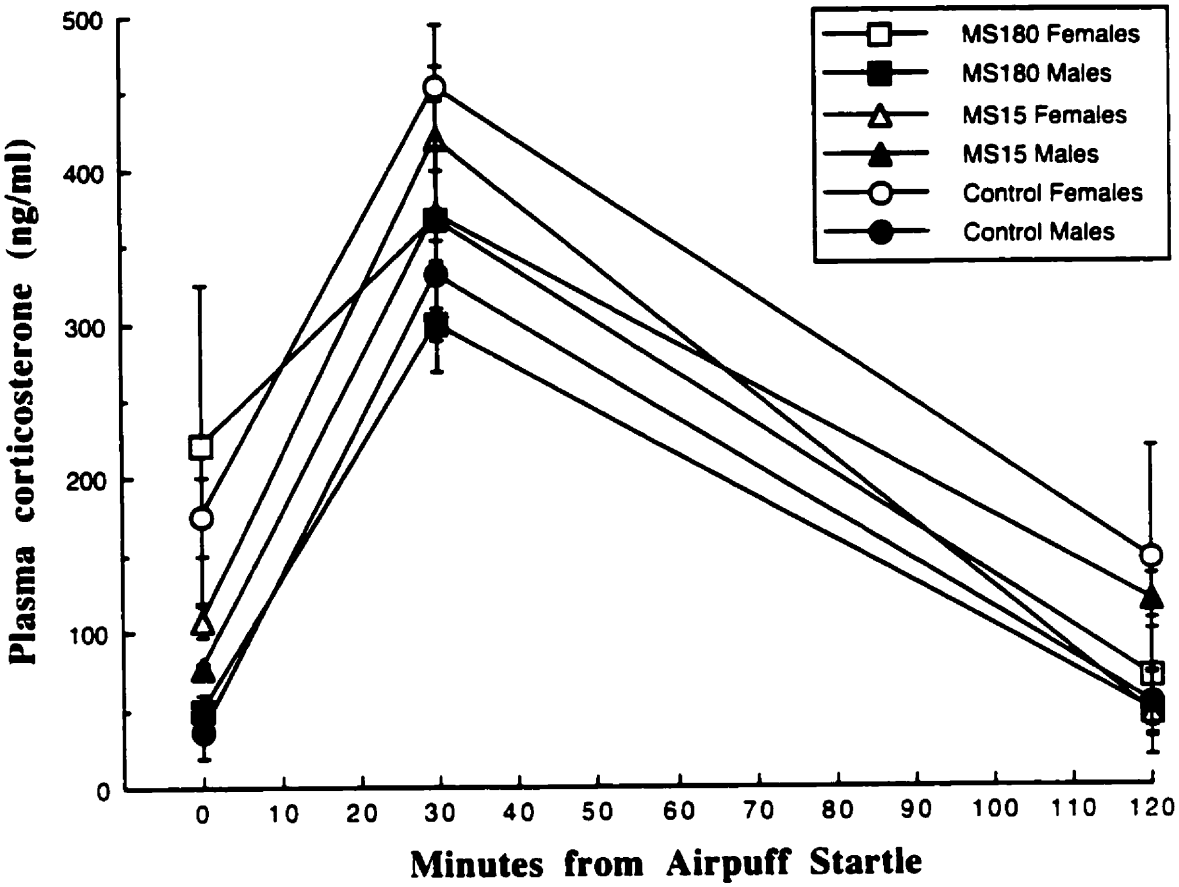

Figure 11. Baseline plasma corticosterone and corticosterone responses to airpuff startle of Fischer rats maternally separated for 180 or 15 minutes (MS180, MS15) ($n = 5$). Error bars indicate SEM. On baseline plasma corticosterone, there is a significant effect of Sex ($p < 0.01$), and no interaction with, or effect of, Maternal Separation ($p > 0.05$). On the area under the curve, there is no significant interaction, or effect of, Maternal Separation or Sex ($p > 0.05/3$ post hoc tests).

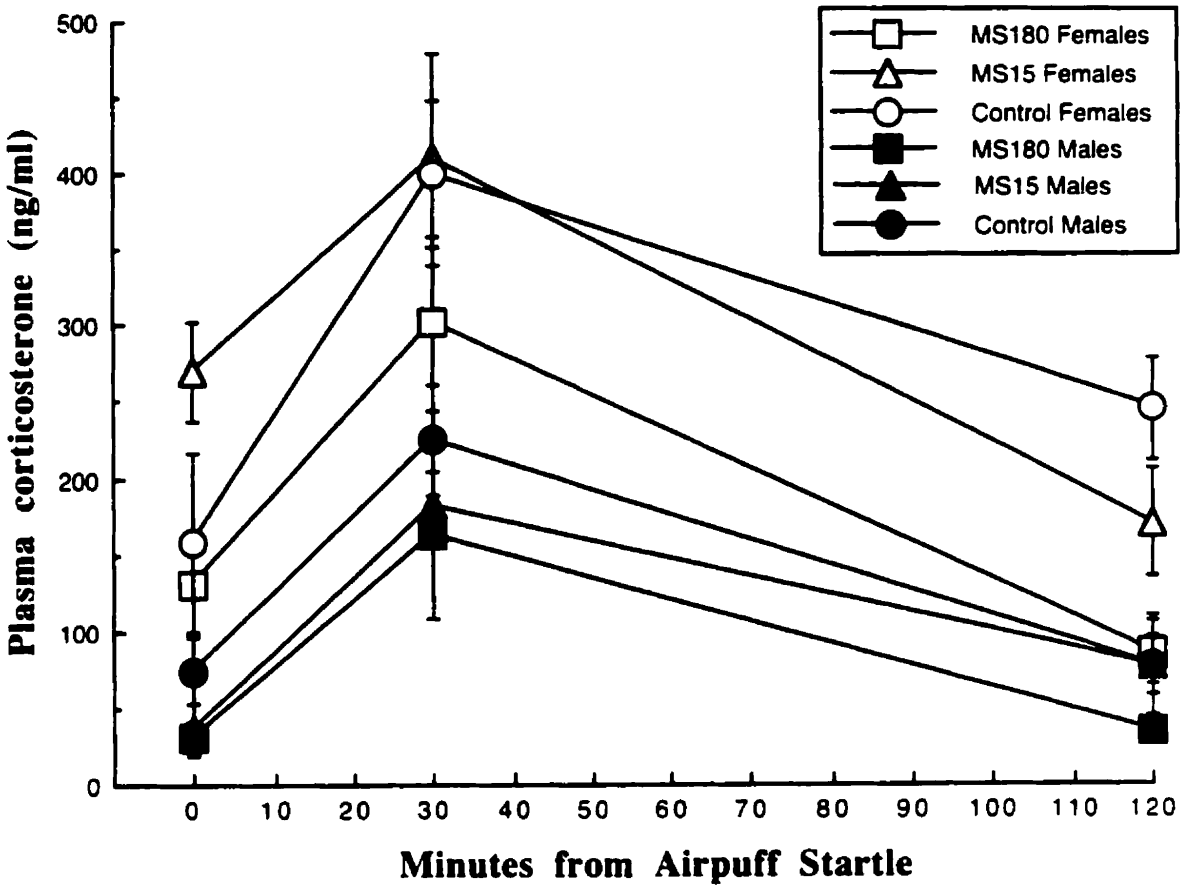

Figure 12. Baseline plasma corticosterone and corticosterone responses to airpuff startle of Lewis rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 5). Error bars indicate SEM. On baseline plasma corticosterone and the area under the curve, there is a significant effect of Sex ($p < 0.001$), and no interaction with, or effect of, Maternal Separation ($p > 0.05/3$ post hoc tests).

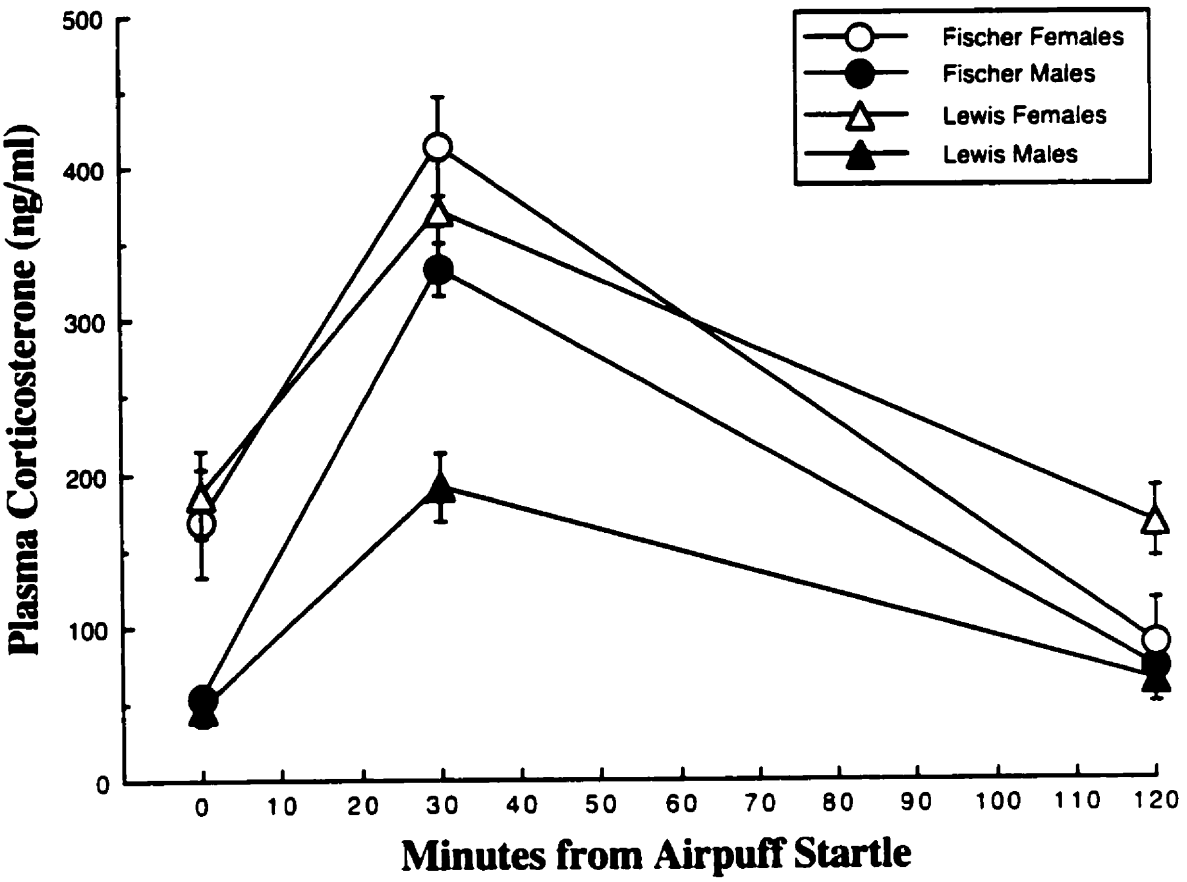

Figure 13. Baseline plasma corticosterone and corticosterone responses to airpuff startle of Fischer and Lewis rats with Maternal Separation groups collapsed (n = 15). Error bars indicate SEM. Females have greater baseline plasma corticosterone than males, and Lewis males have a lower area under the curve compared to Fischer males and Lewis females ($p <$ 0.05/4 post hoc tests).

on baseline plasma corticosterone levels (F (2, 48) = 3.87). Post hoc ANOVAs determined that there is no significant interaction of Maternal Separation x Strain within females (F (2, 24) = 2.94) and no significant main effects (F (2, 24) = 0.08; F (1, 24) = 0.18). Within males, there is a significant interaction of Maternal Separation x Strain (F (2, 24) = 3.95), however, no pairwise comparison was significant ($p \geq 0.26$). Within both Fischer and Lewis strains, there is a no significant Sex x Maternal Separation interaction (F (2, 24) = 1.36, 3.17), a significant effect of Sex (F (1, 24) = 9.77, 27.4), and no effect of Maternal Separation (F (2, 24) = 0.48, 2.42).

The area under the curve, a measure of the integrated HPA axis response, shows no significant Sex x Maternal Separation x Strain interaction (F (2, 48) = 1.53), and no significant Maternal Separation x Strain or Sex x Maternal Separation interactions (F (2, 48) = 0.45, 0.92). There is a significant effect of Maternal Separation (F (2, 48) = 4.37), with MS180 rats displaying lower corticosterone responses than Control rats before adjustment of the α level ($p = 0.02$), but not after ($p > 0.05/3$ post hoc tests). There is a significant Sex x Strain interaction (F (1, 48) = 5.76), since there are no significant differences within females or within Fischer rats, but there is a significant effect of Strain within males and a significant effect of Sex within Lewis rats ($p < 0.05/4$ post hoc tests). Figure 13 shows the same data with Maternal Separation groups collapsed, demonstrating the significantly greater baseline plasma corticosterone of females compared to males, and the significantly lower area under the curve of Lewis males compared to both Fischer males and Lewis Females.

Adjuvant-Induced Polyarthritis

The symptom severity (pain and disability) 21 days after adjuvant injection is shown

in Figures 14 and 15. A three-way ANOVA shows a significant interaction of Sex x Maternal Separation x Strain ($F(2, 107) = 4.34$). Post hoc ANOVAs demonstrate that within each sex, there is a significant effect of Strain (females: $F(1, 53) = 10.7$; males: $F(1, 54) = 20.8$) and no significant interaction with, or effect of, Maternal Separation ($F(2, 53) = 1.43$, $F(1, 53) = 0.03$; $F(2, 54) = 3.556$, $F(1, 54) = 0.51$; $p > 0.05/4$ post hoc tests). Within the Fischer strain, there is no significant Sex x Maternal Separation interaction ($F(2, 53) = 2.41$), a significant effect of Sex ($F(1, 53) = 7.76$), and no effect of Maternal Separation ($F(2, 53) = 0.91$). Within the Lewis strain, there is no significant interaction ($F(1, 54) = 2.03$) or main effects ($F(1, 54) = 2.59$; $F(2, 54) = 0.06$). Nonparametric Kruskal-Wallis analyses also found a significant effect of Sex within the Fischer rat and not within the Lewis rat, and a significant effect of Strain within both sexes ($p < 0.05/4$ post hoc tests). Since there are no significant effects of Maternal Separation, the same data are presented with Maternal Separation groups collapsed in Figure 16. This figure demonstrates the significantly greater symptom severity in the Lewis rat compared to the Fischer rat, the greater symptom severity in the female Fischer rat compared to the male Fischer rat, and the lack of significant sex differences in the Lewis rat.

Percent increase in ankle diameter is shown in Figures 17 and 18. The interactions of Sex x Maternal Separation x Strain (left: $F(2, 107) = 1.30$; right: $F(2, 107) = 1.06$), all two way interactions ($F(2, 107) \leq 0.69$; $F(1, 107) \leq 2.35$), and the effect of Maternal Separation ($F(2, 107) = 0.06, 0.14$) are not significant. For both left and right hind paws there is a significant effect of Strain ($F(1, 107) = 20.7, 28.1$) and of Sex ($F(1, 107) = 17.7, 22.1$). The same data are presented with Maternal Separation groups collapsed in Figure 19. The graph illustrates that the Lewis rat shows more swelling of the hind paws compared to

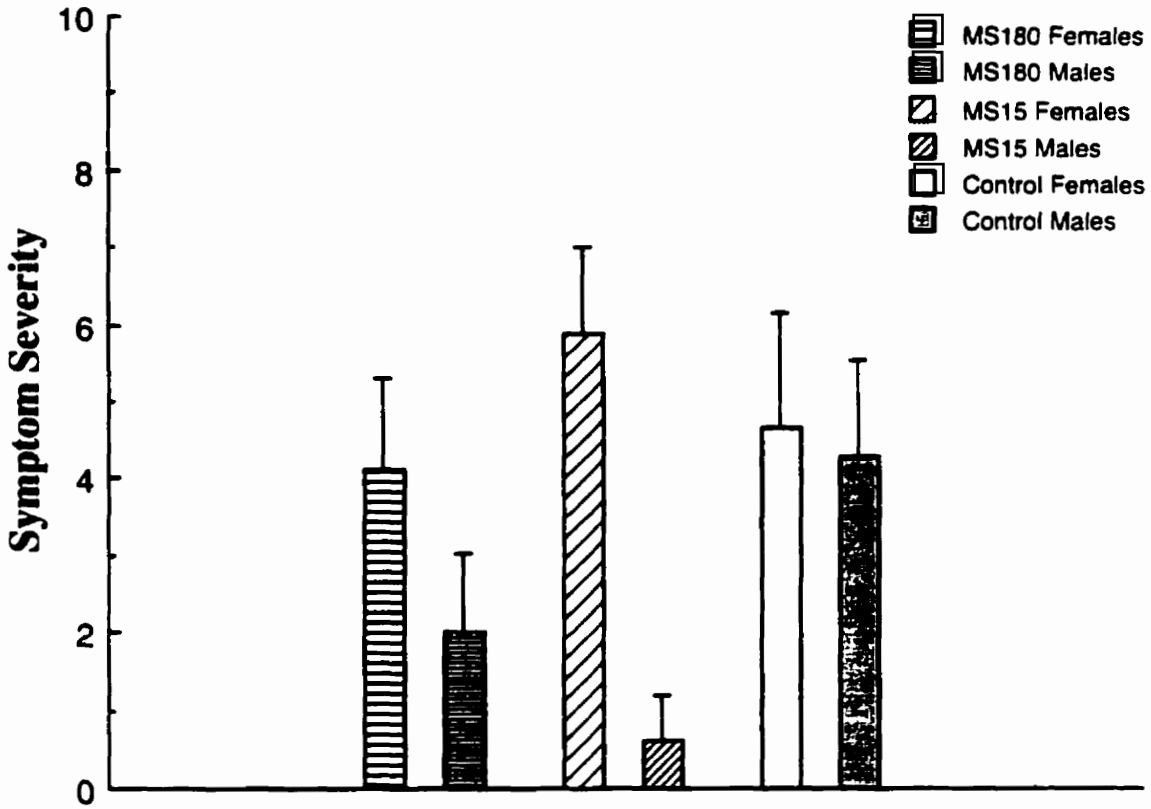

Figure 14. Pain-related symptom severity of Fischer rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 9-10). Symptoms were measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base. Error bars indicate SEM. There is a significant effect of Sex ($p < 0.01$), and no interaction with, or effect of, Maternal Separation ($p > 0.05$).

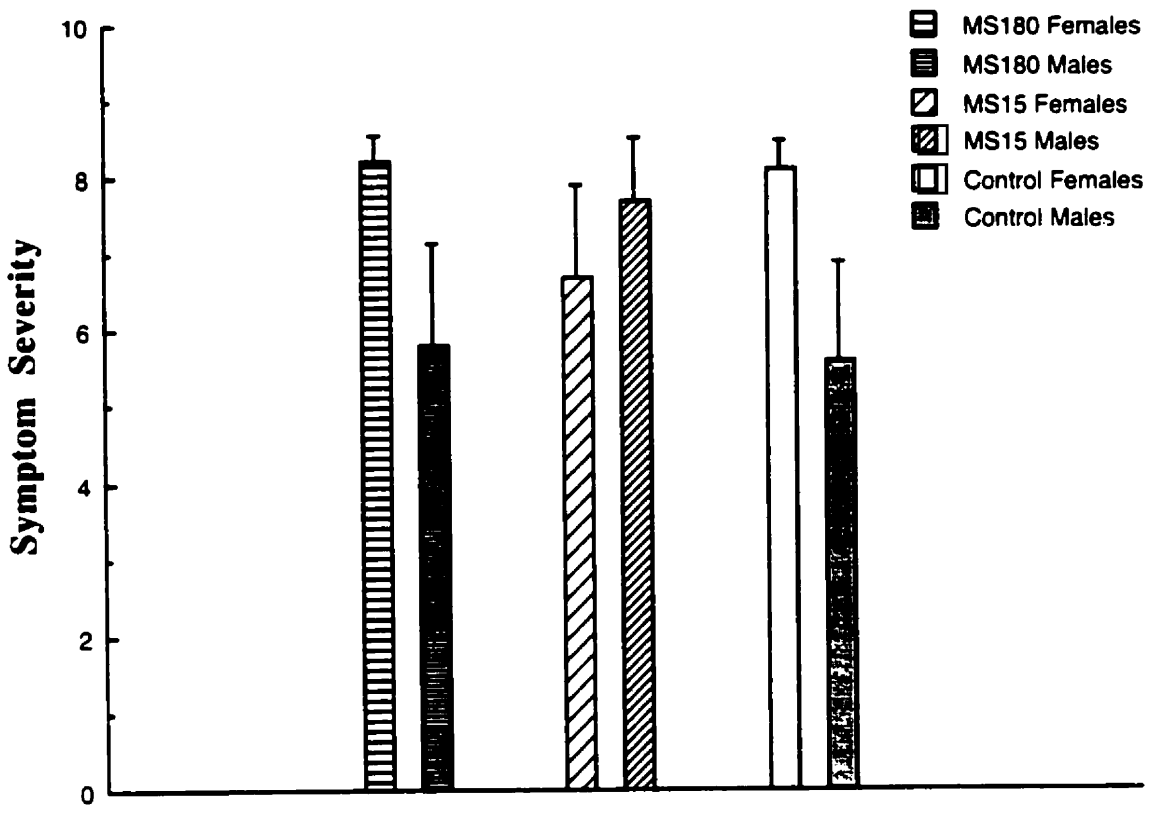

Figure 15. Pain-related symptom severity of Lewis rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 10). Symptoms were measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base. Error bars indicate SEM. There is no significant interaction between, or effects of, Sex and Maternal Separation ($p > 0.05$).

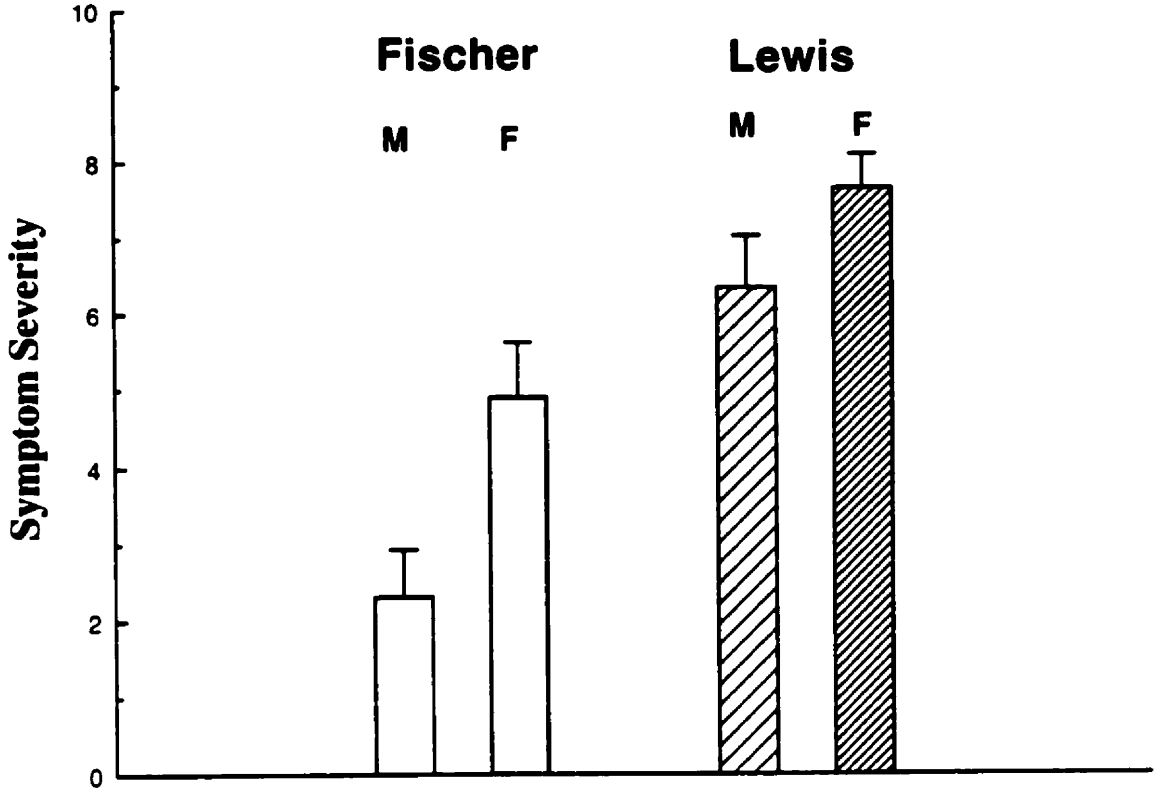

Figure 16. Pain-related symptom severity of Fischer and Lewis rats with Maternal Separation groups collapsed (n = 29-30). Symptoms were measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base. Error bars indicate SEM. Lewis rats shows greater symptom severity than Fischer rats, and female Fischer rats show greater symptom severity than male Fischer rats ($p < 0.05/4$ post hoc tests). There is no effect of Sex within the Lewis strain ($p > 0.05$).

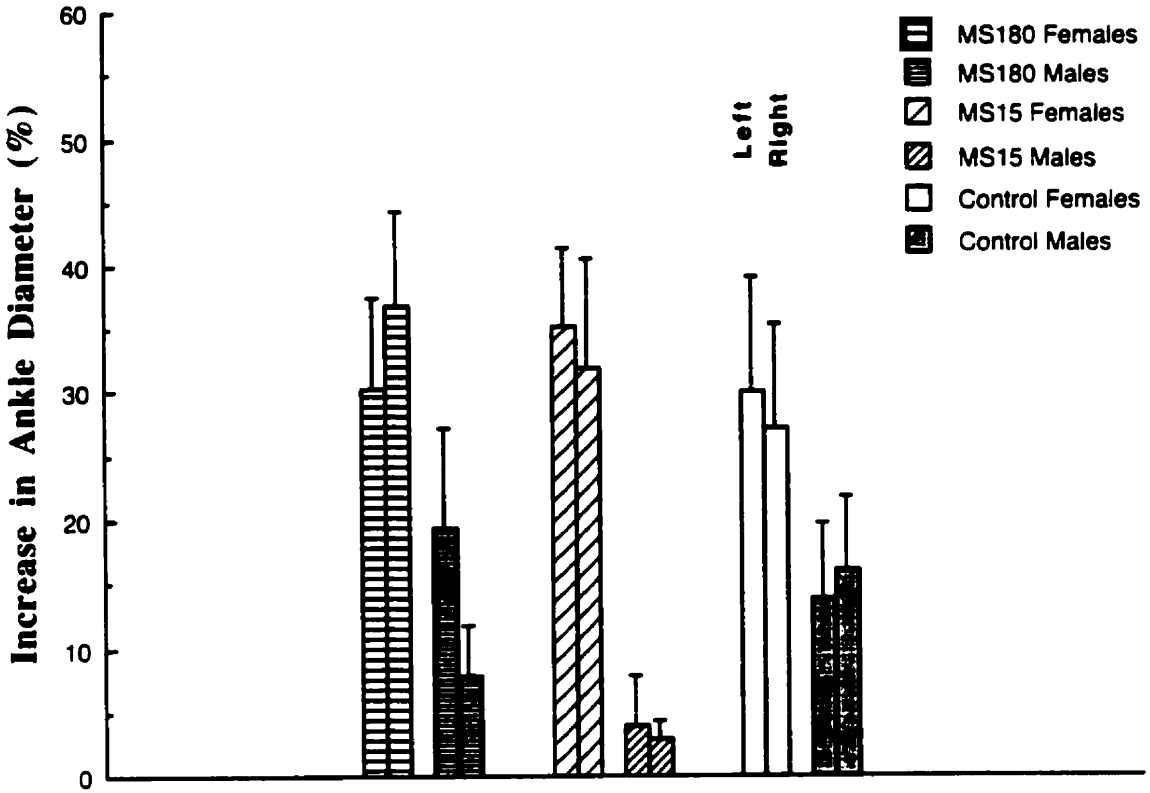

Figure 17. Increase in ankle diameter of Fischer rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 9-10). Ankle swelling was measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base. Error bars indicate SEM. For both left and right hind paws, there is a significant effect of Sex ($p < 0.001$), and no interaction with, or effect of, Maternal Separation ($p > 0.05$).

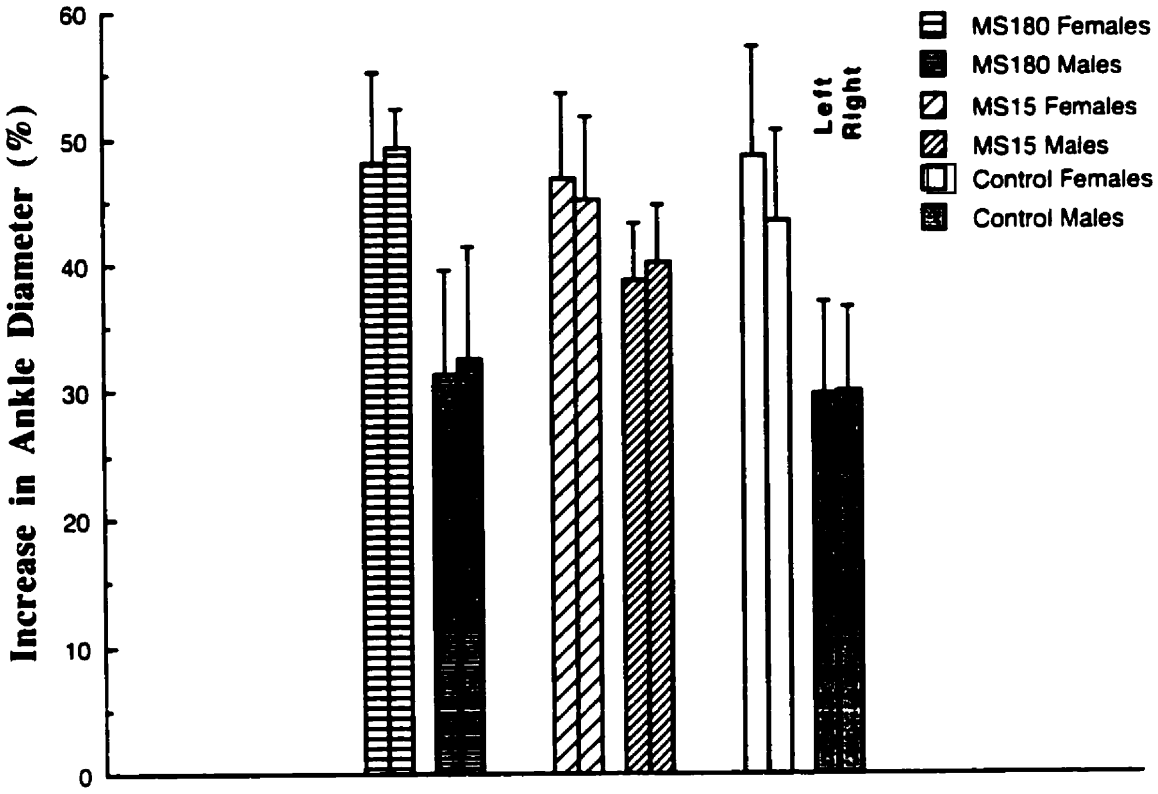

Figure 18. Increase in ankle diameter of Lewis rats maternally separated for 180 or 15 minutes (MS180, MS15) (n = 10). Ankle swelling was measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base. Error bars indicate SEM. For both left and right hind paws, there is a significant effect of Sex ($p < 0.001$), and no interaction with, or effect of, Maternal Separation ($p > 0.05$).

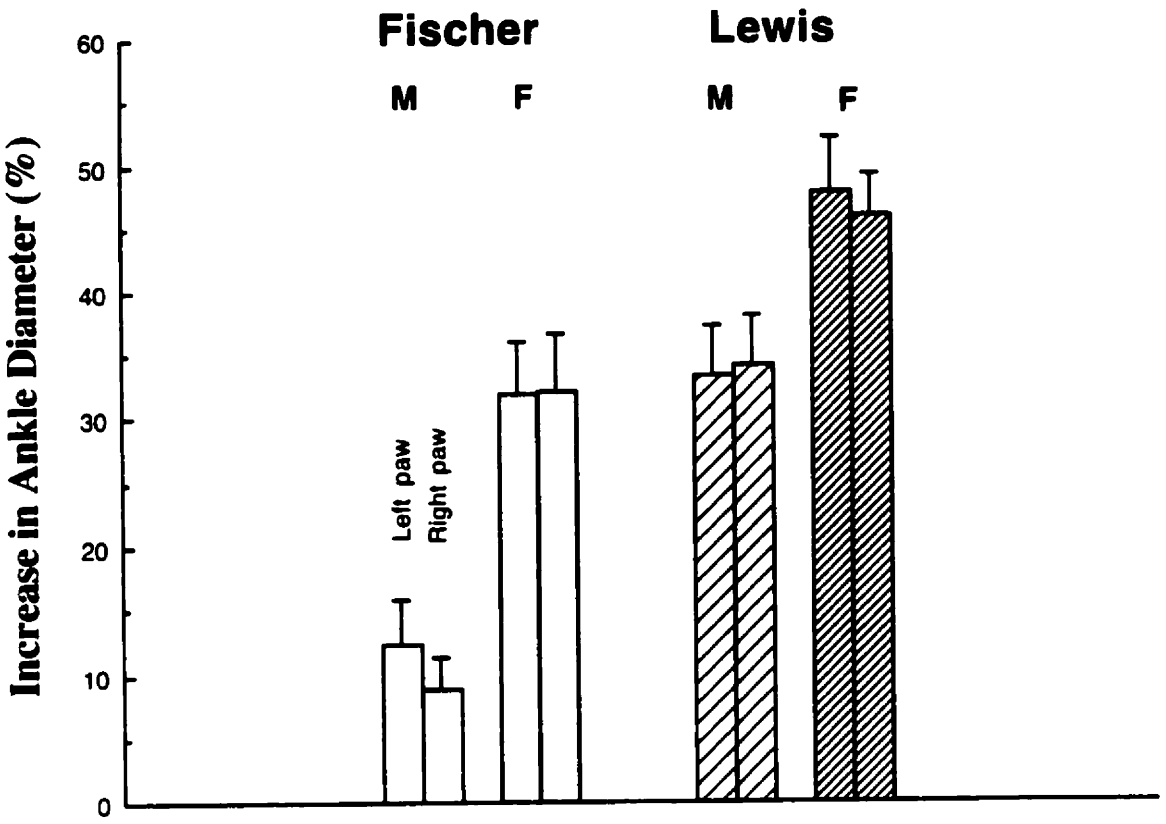

Figure 19. Increase in ankle diameter of Fischer and Lewis rats with Maternal Separation groups collapsed (n = 29-30). Ankle swelling was measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base. Error bars indicate SEM. For both left and right hind paws, there is a significant effect of Strain and of Sex ($p < 0.001$).

the Fischer rat, and that in both strains, females show significantly more swelling compared to males.

Discussion

The observed pattern of adjuvant-induced pain and disability in Figure 16 is expected from previous reports of the relative susceptibility of rats to adjuvant-induced polyarthritis and other chronic inflammation models. The Lewis rat is more susceptible than the Fischer rat to several inflammation models, including adjuvant-induced arthritis, and the female Fischer rat is more susceptible to streptococcal cell-wall induced polyarthritis than the male Fischer rat (Wilder et al. 1982; Sternberg et al. 1989a; Wilder 1993; Karalis et al. 1995). In contrast to a previous report of sex differences in arthritis susceptibility of the Lewis rat (Holmdahl 1995; Misiewicz et al. 1996), in the present study, pain-related symptom severity is not significantly different between Lewis females and males. However, sex differences were found with the measure of adjuvant-induced edema. This dissociation between inflammation and pain behaviour will be discussed below.

Early postnatal maternal separation did not have an effect on adjuvant-induced polyarthritis susceptibility. In fact, maternal separation did not have an effect throughout the study. This was not expected since maternal separation has an effect on HPA axis responsiveness in adulthood in the Long-Evans strain of rat (Plotsky and Meaney 1993); Huot et al., in press). A previous report also found that adjuvant-induced polyarthritis susceptibility is not affected by maternal separation for 3 minutes per day in the first three weeks of life in the Fischer strain of rat (Amkraut et al. 1971). Thus, it is possible that the Fischer and Lewis strains used in this study are resistant to the effects of maternal separation

on HPA axis responsiveness, since these two strains do not show any effect of prenatal maternal immobilization stress on basal or stress-induced corticosterone release in adulthood (Stohr et al. 1998). Although prenatal stress does not have an effect on corticosterone release in Lewis and Fischer rats, their response thresholds in the hot plate test are increased by prenatal stress (Stohr et al. 1998). Thus, it is possible that in the present study transportation of the pregnant dams during the last week of gestation may have had an effect on pain processes making it difficult to detect an effect of maternal separation. However, this is unlikely since the long term effect of maternal separation for 15 to 20 minutes on paw lick latencies in the hot plate test is more pronounced in prenatally stressed male rats compared to rats not prenatally stressed (Smythe et al. 1994). Thus, it is most likely that the genetic contribution of the strain of rat prevented any significant effects of maternal separation.

The key findings of the present study are the results of pain testing prior to adjuvant injection. The results show a pattern of pain sensitivity and endogenous pain suppression that strongly suggests a relationship between pain mechanisms and the susceptibility to adjuvant-induced polyarthritis. The pattern of polyarthritis susceptibility matches the pattern of sensitivity in the tail flick test. The more susceptible Lewis rat is more sensitive to pain evoked in the tail flick test than the Fischer rat, showing shorter latencies to respond. In addition, the lack of significant sex differences in tail flick test sensitivity of the Lewis rat corresponds with the lack of sex differences in adjuvant-induced pain and disability. Furthermore, the female Fischer rat, which is more susceptible to polyarthritis than the male Fischer rat, is slightly more sensitive in the tail flick test.

Previous studies have found similar results. Female rats are more sensitive than male rats in numerous studies of experimental phasic pain sensitivity (Bodnar et al. 1988),

although the overall effect is small if all studies are considered (Berkley 1997; Riley et al. 1998). Studies recently reviewed by Mogil have also found differences in pain processes between the Lewis and Fischer strains (Mogil 1999). In phasic pain tests, including the hot plate test and the tail flick test, the Lewis rat has lower thresholds compared to the Fischer rat (Woolfolk and Holtzman 1995; Stohr et al. 1998). In addition, female Lewis rats shows less pain suppressive effects of morphine in the tail flick test than female Fischer rats, and they show tolerance to morphine even when morphine is paired with prolonged pain, unlike Fischer rats (Vaccarino and Couret 1995).

The present study shows for the first time that there are strain differences between the Lewis and Fischer rat in the formalin test. Paradoxically, the more polyarthritis-susceptible Lewis rat shows less pain behaviour than the Fischer rat during the late second phase, which is associated with significant inflammation (Wheeler-Aceto and Cowan 1991a; Tjølsen et al. 1992; Lariviere and Melzack 1996; Yashpal and Coderre 1998). Moreover, there are no differences in the second phase of the formalin pain response between male and female Fischer rats despite significantly different polyarthritis susceptibility. This demonstrates that the differences in pain-related behaviour following adjuvant injection are not simply due to a nonspecific sensitivity to pain associated with inflammation.

In fact, a dissociation exists between the pain-related behaviour and the inflammation evoked by adjuvant injection in the Lewis rat. There are no significant sex differences in pain and disability in the Lewis rat despite significant sex differences in the degree of ankle swelling. This dissociation is not due to an insensitivity of the pain and disability scale since it has been shown to discriminate polyarthritis susceptibility among rat strains more effectively than the measurement of swelling (Lariviere and Melzack 1997). Recent

evidence has shown that pain and inflammation can be dissociated in two other pain models associated with inflammation, the formalin and bee venom tests. Two strains of mice, the A/J and C57BL/6J strains, exhibit very different amounts of pain behaviour after intraplantar injection of formalin or bee venom. The A/J strain licks the injected hind paw for less than 100 seconds during the 60 minutes following injection of formalin or bee venom. In contrast, the C57BL/6J strain licks the injected paw for more than 500 seconds after formalin injection and more than 700 seconds after bee venom injection in the 60 minute postinjection period. They do not, however, show any difference in paw edema measured with precision calipers (Mogil et al. 1998). Thus, although inflammation in the arthritis model is expected to contribute to pain and disability, there are also genetic contributions to pain processing that modulate the response to a similar peripheral inflammatory event.

As discussed in Chapter 1, there is poor correspondence of adjuvant-induced polyarthritis susceptibility with basal corticosterone levels or with corticosterone responses to acute stress (Chover-Gonzalez et al. 1998, 1999). The results of the present study confirm this conclusion. Female rats had higher basal corticosterone levels than male rats, precluding the anti-inflammatory effects of peripheral corticosterone as a predictor of polyarthritis susceptibility. In addition, the integrated corticosterone response to airpuff startle does not correspond with polyarthritis susceptibility since within the Fischer strain there are no sex differences in the corticosterone response but there are in the adjuvant-induced pain and disability, and vice versa within the Lewis strain. Only within males was there a correlation of increased corticosterone response with decreased adjuvant-induced polyarthritis in the Fischer rat compared to the Lewis rat. These results demonstrate that the anti-inflammatory effects of peripheral corticosterone are not responsible for the observed pattern of adjuvant-

induced pain and disability. Therefore, although exogenous administration of corticosteroids inhibit, and corticosteroid inhibitors enhance, the development of carrageenin-induced inflammation (Karalis et al. 1995), endogenous peripheral basal corticosteroid levels and corticosterone responses to acute stress are not a predisposing factor to the development of adjuvant-induced polyarthritis pain-related behaviour. The poor correlation between the peripheral components of the HPA axis and the development of pain-related behaviour in adjuvant-induced polyarthritis also suggests that the central components of the HPA axis may be responsible for modulating the susceptibility.

The greatest contribution of the present study is the novel finding that the female Fischer rat exhibits more pain behaviour during the interphase depression in pain responding of the formalin test than the less susceptible, male Fischer rat. Previous studies have investigated sex differences in the formalin test, but have not found an effect of sex on the interphase depression. Female Wistar rats have greater durations of licking and of flexing than male Wistar rats during the 60 minutes after injection of 10% formalin (Aloisi et al. 1994, 1995, 1996). However, their statistical analysis was performed without the repeated measures factor of time within the 60-minute observation period, precluding the assessment of whether the Wistar rat shows sex differences in the interphase depression in responding as seen in the Fischer rat. In the C57BL/6J mouse, there is an effect of sex on a third phase of pain reported to occur in the mouse after the second phase, but no effect of sex on the biphasic response to injection of 5% formalin (Kim et al. 1999). It is possible that the use of a moderate dose of 1.5 % formalin contributed to the detection of the sex differences in the present study. Since it is now recognized that the formalin interphase depression is mediated by pain suppression mechanisms in the central nervous system (Matthies and

Franklin 1992, 1995; Henry et al. 1999), these results show that inherent differences in central pain suppression mechanisms are correlated with the susceptibility to adjuvant-induced polyarthritis.

In conclusion, the present study shows that there is a pattern of pain sensitivity in the tail flick test and endogenous pain suppression in the formalin test that matches the pattern of adjuvant-induced polyarthritis susceptibility in the rat. The fact that the present study examines pain mechanisms several weeks prior to arthritis induction suggests that there is a possible causal relationship between the inherent pain mechanisms and adjuvant-induced polyarthritis susceptibility. The mechanisms underlying pain sensitivity in the tail flick test have been proposed to be peripheral, mediated more by spinal mechanisms and peripheral afferent fibres (Carstens 1996). In contrast, the mechanisms underlying the formalin interphase depression are within the central nervous system (Matthies and Franklin 1992; Henry et al. 1999). Therefore, the formalin interphase depression mechanisms can be used to study the relationship between the HPA axis, central pain mechanisms, and the susceptibility to adjuvant-induced polyarthritis. In the next chapter, the effect of disruption of the HPA axis by hypophysectomy is investigated in the formalin test. Since hypophysectomy inhibits the development of adjuvant-induced polyarthritis (Neidhart and Flückiger 1992), the study in the next chapter examines the role of the pain mechanisms underlying the formalin interphase depression in adjuvant-induced polyarthritis susceptibility.

Chapter 3

Study of Hypophysectomy-Induced Analgesia

The data presented in the previous chapter demonstrate a correlation between certain pain mechanisms and adjuvant-induced polyarthritis susceptibility among groups of rats that differ in HPA-axis function. If these pain mechanisms are integrally related to adjuvant-induced polyarthritis susceptibility, then a manipulation of the HPA axis that inhibits adjuvant-induced polyarthritis should also affect these pain mechanisms.

Hypophysectomy inhibits the development of adjuvant-induced polyarthritis in very young (65-75 g) male Sprague-Dawley rats, in which arthritis develops in all of the injected rats (Neidhart and Flückiger 1992). However, the adult male Sprague-Dawley rats are not as susceptible, showing a susceptibility between that of the Lewis and Fischer rat (Lariviere and Melzack 1997). Furthermore, the development of arthritis in the young rats was assessed by the measurement of change in hind paw thickness. As shown in the previous chapter, changes in inflammation are not necessarily paired with changes in pain behavior. Thus, the first experiment of this study examines the effect of hypophysectomy on the development of chronic pain-related behaviour and inflammation of the hind paws after adjuvant injection in the adult rat.

To test whether the inhibition of adjuvant-induced arthritis is due to an effect on pain mechanisms associated with adjuvant-induced polyarthritis susceptibility, the effect of hypophysectomy on phasic and tonic pain sensitivity and on endogenous pain suppression is examined. Phasic pain is evoked in the first phase of the formalin test, and tonic pain is evoked in the second phase of the formalin test and after injection of bee venom in a new tonic pain test, the bee venom test (Lariviere and Melzack 1996). Endogenous pain suppression is examined in the interphase depression in pain responding of the formalin test,

which has been shown to be due to active endogenous pain suppression mechanisms (Matthies and Franklin 1992; Franklin and Abbott 1993; Henry et al. 1999).

Hypophysectomy is expected to not have an effect on phasic pain sensitivity since previous animal studies demonstrate inconsistent effects of hypophysectomy in phasic pain tests (see Figure 2 and Table 1). In contrast, hypophysectomy is expected to significantly decrease sensitivity to prolonged, tonic pain since hypophysectomy decreases severe, prolonged cancer pain in humans. In addition, since certain types of cancer pain are affected more than others by hypophysectomy, the effects may show a preference for the tonic pain evoked in either the formalin or bee venom tests.

Methods

Subjects

Adult male hypophysectomized (Hypox) rats and sham hypophysectomized (Sham Hypox) rats, weighing 180-325 g at the time of testing, were purchased from Charles River, St. Constant, Quebec. Rats were hypophysectomized by a transpharyngeal approach. Sham hypophysectomized rats underwent the same surgical procedure in which the pituitary gland was exposed but not aspirated. All surgeries were performed by the supplier's surgical technician, who verified the completeness of pituitary removal by visual inspection after aspiration. The completeness of hypophysectomy was also confirmed by significantly less body weight gain of hypophysectomized rats compared to sham hypophysectomized rats several weeks after surgery, and by postmortem intracranial examination of the pituitary space. The animals were given free access to standard rat chow and 5% sucrose water, and were maintained on a 12 h light/dark cycle with the lights on from 7:00AM. All formalin

and bee venom pain testing was performed during the light phase.

Adjuvant-Induced Polyarthritis

Seven Hypox and 9 Sham Hypox Lewis rats were used to assess the inhibitory effect of hypophysectomy on adjuvant-induced polyarthritis susceptibility. The Lewis strain was chosen to promote the detection of inhibitory effects that may be missed by the use of a less susceptible strain such as the Sprague-Dawley or Fischer strains. Arthritis was induced and assessed by the same procedure used in Chapter 2. Briefly, complete Freund's adjuvant (1.0 mg Mycobacterium butyricum/300 g rat; in 10 mg/ml paraffin oil) was injected intradermally at the base of the tail of anaesthetized rats 12 days after surgery. At 21 days following adjuvant injection, pain and disability behaviour was scored using the ten-point rating scale used in Chapter 2 (see Table 6). To assess inflammation produced by adjuvant injection, the mediolateral dimension of the tibiotarsal joint of both hind paw ankles was measured with precision calipers immediately prior to injection and on the 21st day after injection. The percent increase in ankle diameter was calculated as:

[(Diameter on day 21 - Diameter before injection) / Diameter before injection] x 100.

Formalin Test

In a separate group of 8 Hypox and 9 Sham Hypox Long-Evans rats, the formalin test was administered 10-14 days after surgery using the same procedure as in Chapter 2, except that 2.5% formalin was used instead of 1.5% formalin. Briefly, after habituation, the rats were injected with 50 µl of 2.5% formalin into the plantar surface of the hind paw, and the pain behaviour was scored for 60 minutes using the same method and 3-point scale as in

Chapter 2. A mean pain score was calculated for the entire 60-minute observation period and for each 5-minute period after injection as the sum of the scores divided by the number of scores in the time period. During testing, the experimenter was blind to the group to which the rats belonged since the rats appeared to behave normally and the group mean weights differed by only 33 g at the time of testing.

Bee Venom Test

In another group of rats (9 Hypox and 9 Sham Hypox), the bee venom test was administered 6-9 days after surgery. The procedure is the same as in the formalin test, except that 0.2 mg of honey bee venom (of *Apis mellifera*, purchased from Sigma) in 50 µl of saline is injected into the hind paw instead of formalin. Bee venom injection evokes the same individual pain behaviours that are evoked by formalin injection, and thus, the same scoring method is used. Unlike the biphasic formalin pain response, the pain response to 0.2 mg bee venom injection is monophasic, peaking within 5 minutes and continually decreasing, producing significantly greater pain behaviour than saline-injected animals for 50 minutes (Lariviere and Melzack 1996). The pain behaviour was scored for 45 minutes.

Results

Adjuvant-Induced Polyarthritis

The response to adjuvant injection is shown in Figures 20 and 21. Hypox rats display significantly lower pain-related symptom severity scores (t (14) = 2.11) and left and right hind paw swelling (t (14) = 2.10, 2.73) than Sham Hypox rats (one-tailed Student's t test; $p < 0.05$) . Nonparametric Kruskall-Wallis analysis also confirms a significant effect of

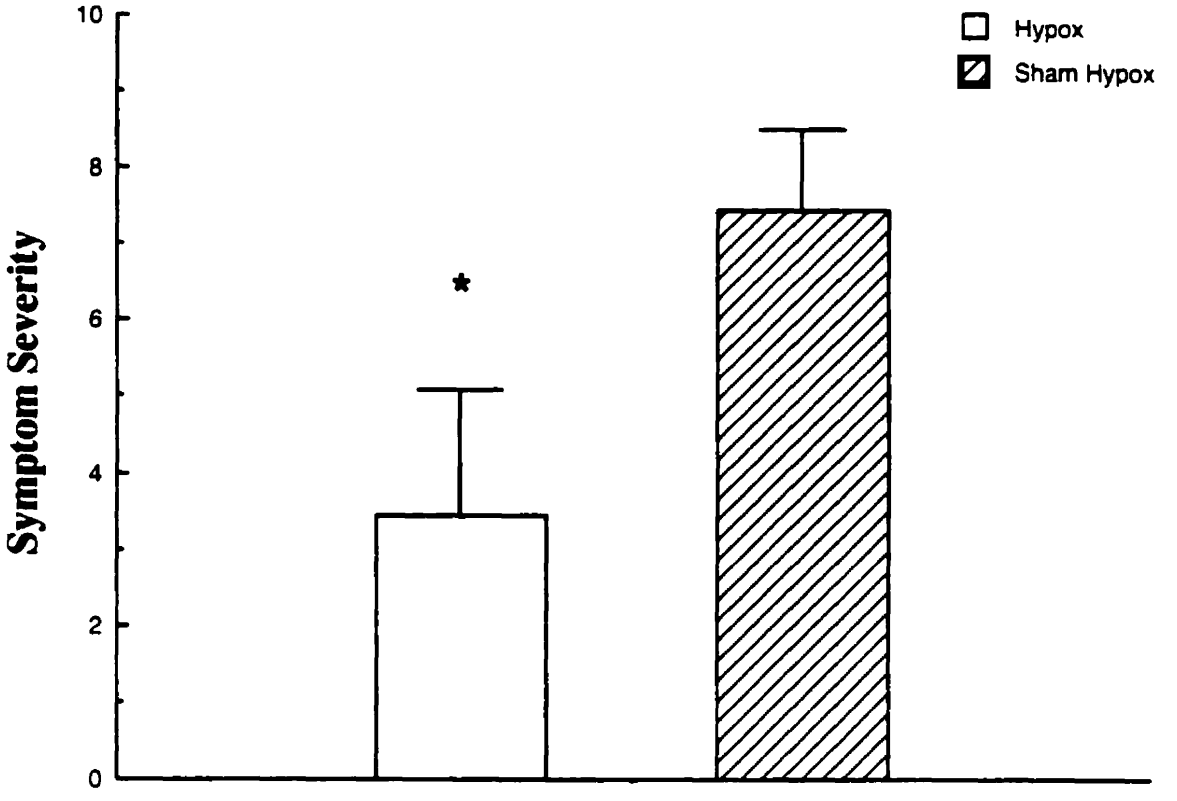

Figure 20. Pain-related symptom severity of hypophysectomized (Hypox) and sham hypophysectomized (Sham Hypox) rats measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base (n = 7-9). Error bars indicate SEM. Hypox rats show significantly less symptom severity than Sham Hypox rats (* $p < 0.05$).

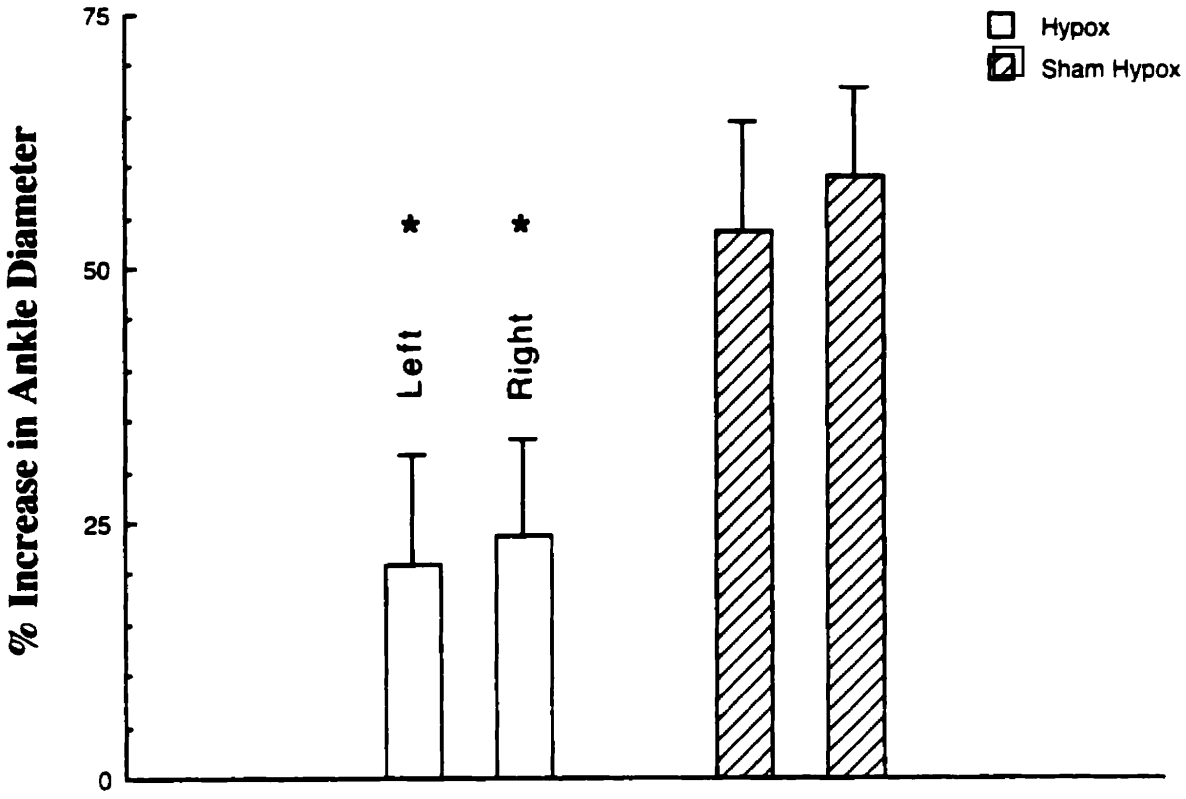

Figure 21. Increase in ankle diameter of hypophysectomized (Hypox) and sham hypophysectomized (Sham Hypox) rats measured 21 days after intradermal injection of complete Freund's adjuvant in the tail base (n = 7-9). Error bars indicate SEM. Hypox rats show significantly less paw swelling in both hind paws than Sham Hypox rats (* $p < 0.05$).

hypophysectomy on symptom severity ($p < 0.05$).

Formalin Test

A t test on the mean pain score for the entire 60-minute observation period after formalin injection shows that Hypox rats exhibit significantly less formalin-induced pain behaviour than Sham Hypox rats, with means ± SEM of 0.69 ± 0.13 versus 1.10 ± 0.06 (t (15) = 3.06; $p < 0.01$). The mean pain responses across time are illustrated in Figure 22. A repeated measures ANOVA on the 12 5-minute periods following formalin injection found a significant interaction of Group (Hypox or Sham Hypox) x Time (F (11, 165) = 2.04; $p <$ 0.05), since Hypox rats show less pain behaviour from 15 to 40 minutes postinjection ($2.85 < t(15) < 3.38; p < 0.05$). There is no significant difference between groups in the first phase of the formalin test, 5 minutes after injection, nor in the late second phase from 45 to 60 minutes ($0.47 < t(15) < 1.78; p > 0.05$). To correct for the lack of homogeneity of variance (F (2, 15) = 4.16; overall 60-minute mean pain scores), t tests were performed on the data points from 15 to 40 minutes and the mean pain score for the entire 60-minute period without pooling group variances. All comparisons remained significant ($2.71 < t$ (15) < 3.18; $p <$ 0.05).

Examination of the individual responses of Hypox rats shown in Figure 23 shows that the effect of hypophysectomy is to increase or prolong the interphase depression in pain responding relative to the Sham Hypox mean response. All but one of the Hypox rats shows less pain behaviour than the Sham Hypox mean during the interphase depression of the Sham Hypox rats 6-20 minutes after formalin injection. Three of the 8 Hypox rats show an onset of the characteristic second phase only when the Sham Hypox rats' mean pain scores are

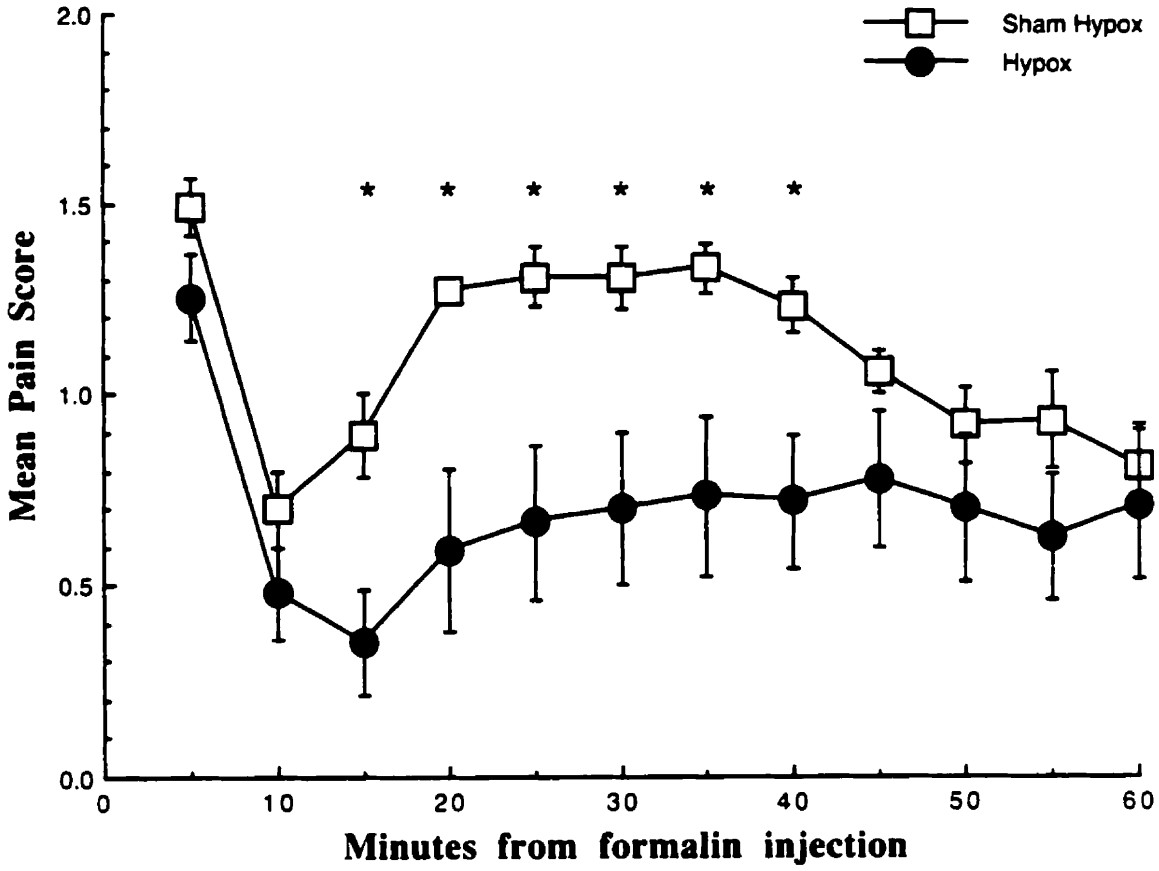

Figure 22. Mean pain scores of hypophysectomized (Hypox) and sham hypophysectomized (Sham Hypox) rats following intraplantar injection of formalin (n = 8-9). Error bars indicate SEM. Hypox rats show significantly lower mean pain scores than Sham Hypox rats from 15 to 40 minutes after formalin injection (* $p < 0.05$).

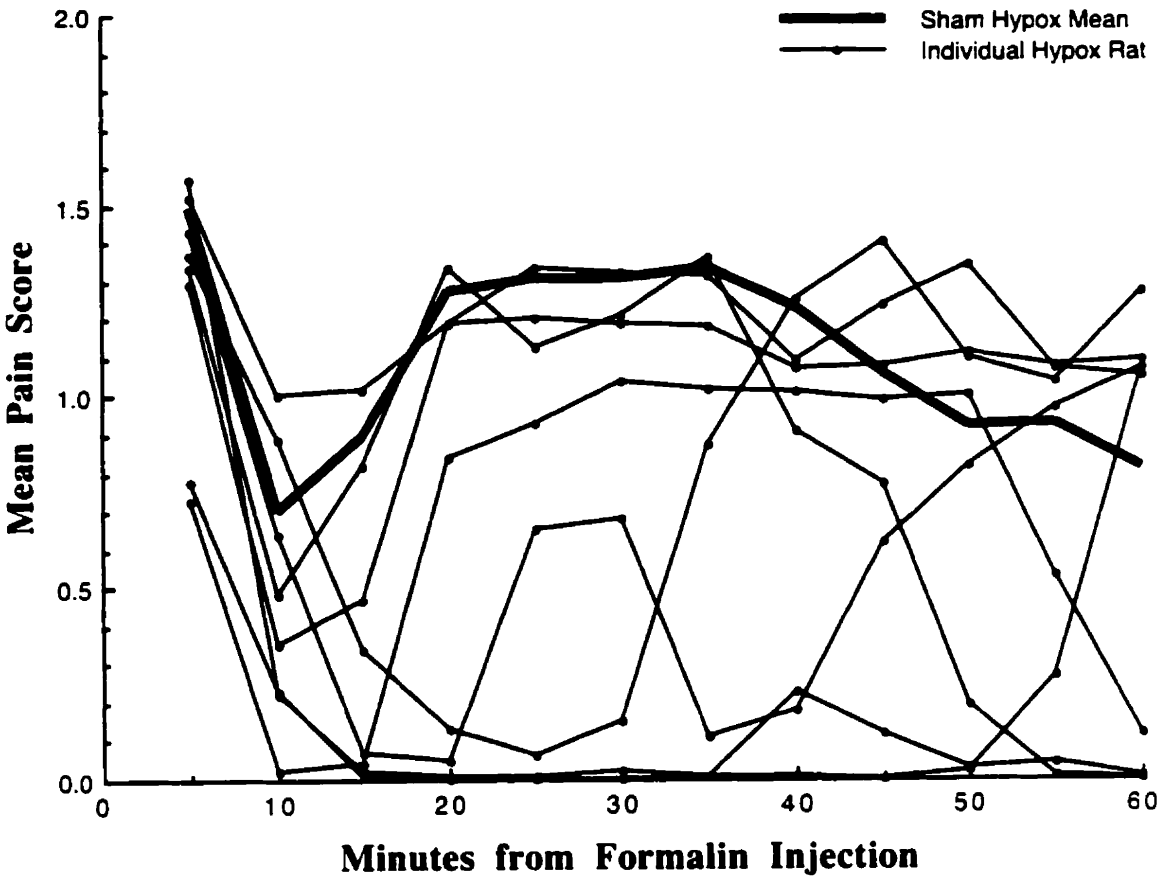

Figure 23. Formalin pain responses of individual hypophysectomized (Hypox) rats. Compared to the Sham Hypox mean response ($n = 9$), individual Hypox rats show increased pain suppression during the interphase depression of the Sham Hypox group from 5 to 20 minutes after formalin injection. Half of the 8 Hypox rats show extremely prolonged interphase depressions.

decreasing at the end of the second phase, and one Hypox rat does not show a second phase during the entire 60-minute observation period. As illustrated in Figure 24, Sham Hypox rats do not show similar exaggerations of the interphase depression.

Bee Venom Test

A *t* test on the mean pain scores for the 45-minute observation period shown in Figure 25 shows that there is no significant difference between Hypox and Sham Hypox groups ($t(16) = 1.39$; $p > 0.05$).

Discussion

This study shows that hypophysectomy significantly inhibits the development of inflammation associated with adjuvant-induced polyarthritis in the adult rat, confirming the previous findings in the young rat (Neidhart and Flückiger 1992). The results also show for the first time that hypophysectomy inhibits the development of pain-related behaviour associated with adjuvant-induced polyarthritis.

Hypophysectomy-induced analgesia is not simply due to the inhibition of all pain associated with inflammation. In the formalin test, hypophysectomy does not affect the pain behaviour late in the second phase of the formalin pain response, which is associated with significant inflammation (Rosland et al. 1990; Lariviere and Melzack 1996; Yashpal and Coderre 1998). Moreover, hypophysectomy is ineffective in the bee venom test despite the development of hind paw swelling several times greater than that seen in the formalin test (Lariviere and Melzack 1996). In fact, different pain models associated with inflammation have different neural contributions (Lam and Ferrell 1991), and hence, a manipulation such

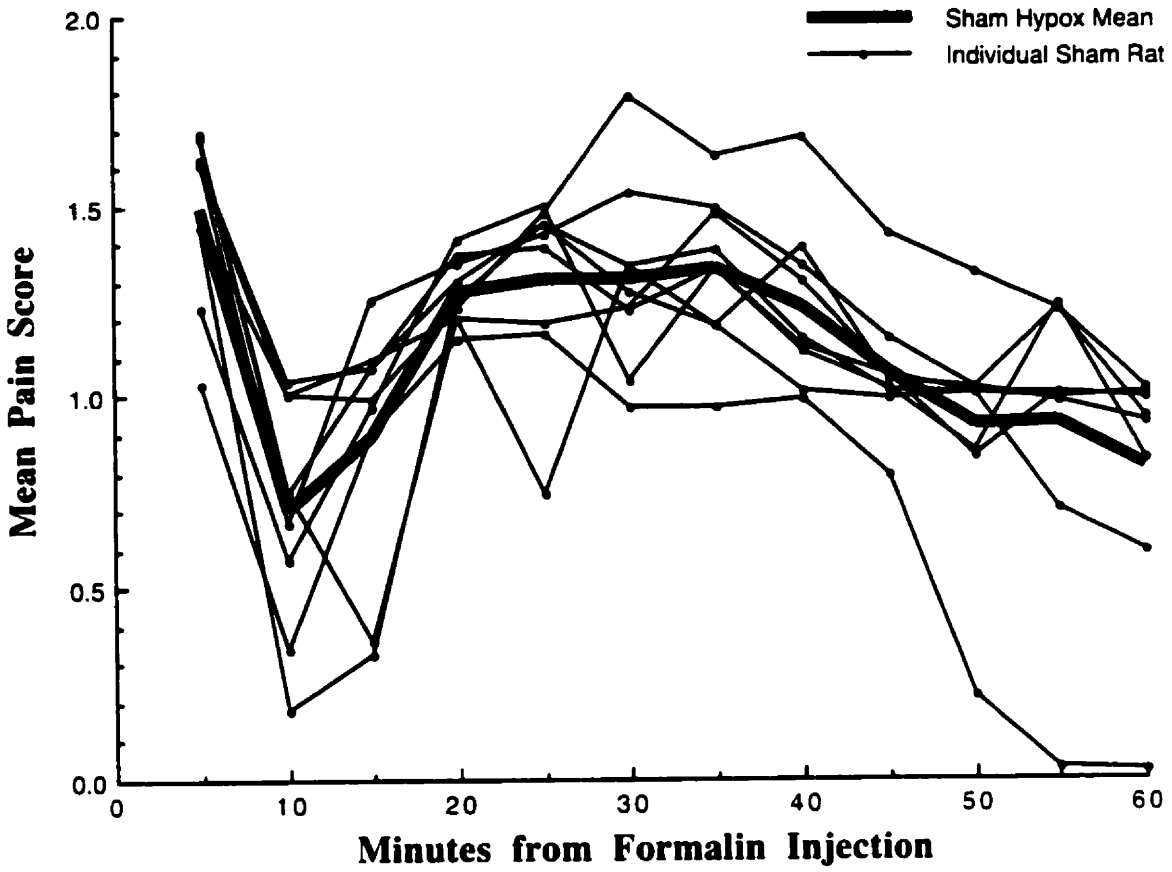

Figure 24. Formalin pain responses of individual sham hypophysectomized (Sham Hypox) rats. Compared to the Sham Hypox mean response (n = 9), individual Sham Hypox rats do not show exaggerations of the interphase depression.

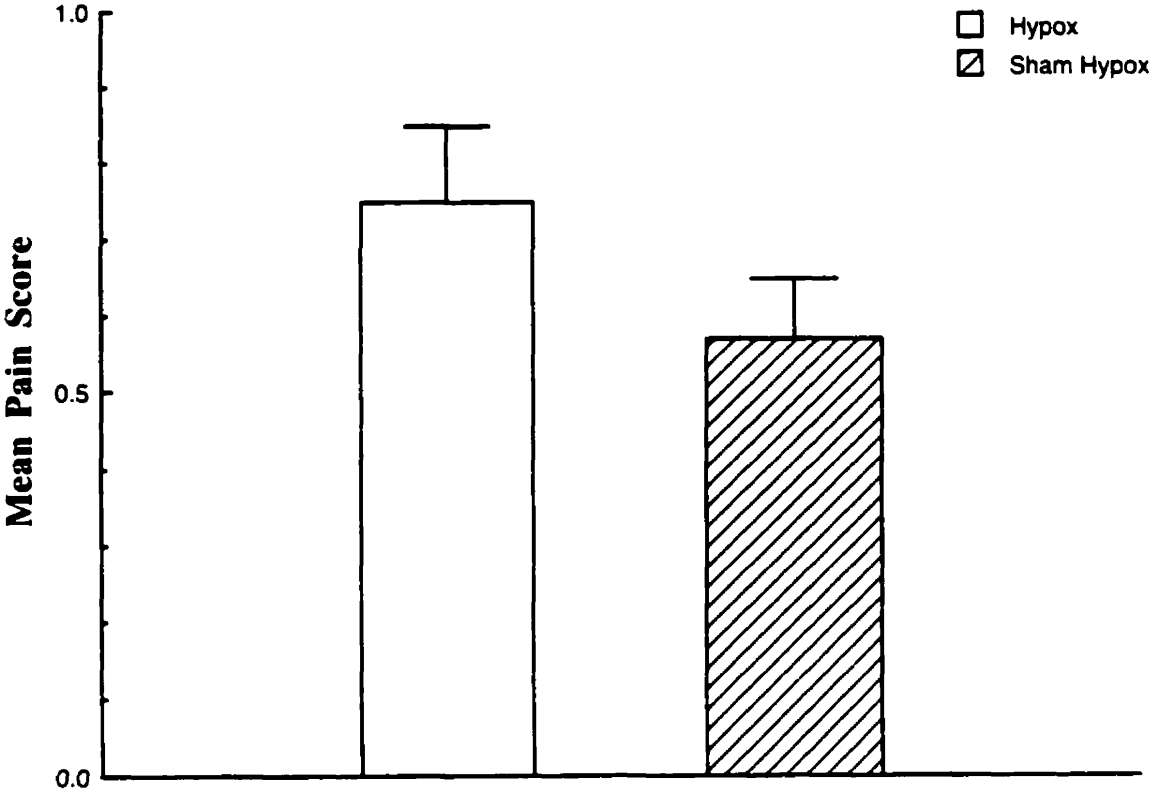

Figure 25. Mean pain scores of hypophysectomized (Hypox) and sham hypophysectomized (Sham Hypox) rats for the 45 minutes following intraplantar injection of bee venom ($n = 9$). Error bars indicate SEM. The mean pain scores of Hypox rats do not significantly differ from the mean pain scores of Sham Hypox rats ($p > 0.05$).

as hypophysectomy could affect the mechanisms of one model of pain and inflammation without affecting the mechanisms of another model as seen in the present study.

The results show that hypophysectomy-induced analgesia is specific to particular prolonged pain mechanisms. Hypophysectomy does not affect the pain responses of the first phase of the formalin test, which is considered to be a phasic pain like that evoked in the tail flick and hot plate tests since its duration is short and there are pharmacological similarities between the first phase and phasic pain tests (Melzack and Wall 1996). For instance, in the rat, systemic morphine is only half as potent against the pain responses of the first phase of the formalin pain response and the tail flick response to immersion in 48 °C water compared to the tonic pain of the second phase (Wheeler-Aceto & Cowan 1991a, 1991b). In fact, a low dose of 2 mg/kg of morphine strongly depresses pain responding in the second phase, but has little effect on the pain of the first phase (Dubuisson and Dennis 1977). The lack of effect of hypophysectomy on the phasic pain of the formalin test may explain the inconsistent effects of hypophysectomy in phasic pain tests. Hypophysectomy does, however, strongly affect the mechanisms responsible for the interphase depression in pain responding in the formalin test.

The interphase depression has traditionally been considered to be a passive response to decreased afferent input from peripheral tissue. More recently, it has been recognized as due to active pain suppression that can be inhibited and induced, and which originates from within the central nervous system.

The view of the interphase depression as a response to decreased afferent input is based on electrophysiological studies of responses of primary afferent neurons and of spinal cord dorsal horn cells to intraplantar formalin injection. Subsets of primary afferent fibres,

dorsal root ganglion cells and dorsal horn cells have a biphasic response to formalin injection. They vigorously increase their rate of firing immediately after formalin injection, become quiet within 10 minutes, and then show a prolonged increase of firing following the quiescent period (Dickenson and Sullivan 1987a, 1987b; Tjølsen et al. 1992; Porro and Cavazutti 1993; McCall et al. 1996; Puig and Sorkin 1996; Henry et al. 1999). Since the behavioural response to formalin injection shows a similar time course, the interphase depression has been attributed to the relative inactivity of peripheral afferent pathways (Tjølsen et al. 1992; Porro and Cavazutti 1993). That is, the interphase depression has been described as a passive response to a state of nervous system inactivity. However, electrophysiological recordings of the response to subcutaneous formalin injection of supraspinal structures, including the preoptic area, raphe nuclei, and the bulboreticular formation, are less similar to the behaviour (Tjølsen et al. 1992), questioning the attribution of the interphase depression to nervous system inactivity.

Cortical electroencephalographic (EEG) recordings in the rat show that the central nervous system is highly active during the interphase depression. In fact, cortical EEG recordings during the interphase period are like those during the first phase of the formalin response, showing a pattern of low amplitude, high frequency activity characteristic of vigilance (Ichinose et al. 1999). Moreover, the interphase cortical EEG activity is unlike the high amplitude, low frequency activity seen in the late second phase when pain behaviour is decreasing and when pain behaviour has ceased. In addition, a pilot study conducted in our lab found that although adult rats in the interphase depression appear inactive, ultrasonic recordings show that the rats make 50-60 ultrasonic calls per minute when auditory feedback of their calls is provided. The calls cease with the onset of the second phase of formalin pain

responding (Lariviere et al., unpublished data). Thus, the interphase depression is not simply a period of nervous system and behavioural inactivity.

The interphase depression is an active response that can be selectively inhibited. For instance, decerebration by complete transections made between the anterior tectum and the mid-hypothalamus abolishes the interphase depression in the rat without affecting the first and second phases compared to sham operated rats (Matthies and Franklin 1992). Thus, the decerebrate rat exhibits relatively intense pain behaviour continuously from immediately after formalin injection without any significant decrease in pain responding during the 60-minute observation period. In addition, partial decortication by aspiration increases pain responding during the interphase depression, although the effect was reported to be not statistically significant (Matthies and Franklin 1995). The interphase depression is also selectively inhibited by the administration of anxiolytics. The administration of the anxiolytics pentobarbital, diazepam, and ethanol dose-dependently increases the pain behaviour during the interphase depression without any effect on the first and second phases (Franklin and Abbott 1993).

Furthermore, a second period of active pain suppression can be induced by a second formalin injection. A second intraplantar formalin injection given 20 minutes after the first injection results in a significant increase in pain behaviour in the next five minutes followed by a decrease in pain behaviour that lasts 10-15 minutes, lowering the mean pain scores below those expected if only the first formalin injection were given (Henry et al. 1999).

The underlying mechanisms responsible for the interphase depression are unknown. The mechanisms appear to be supraspinal since decerebration just above and below the pons abolishes the interphase depression in formalin pain behaviour. The observation of cortical

activity during the interphase depression and the effect of partial decortication is also consistent with this conclusion.

In contrast, the mechanisms have been proposed to be spinal since, following a second formalin injection, a second period of decreased spinal dorsal horn cell firing is observed in anaesthetized, acutely spinalized rats (Henry et al. 1999). The authors argue that the inhibition seen in their electrophysiological recordings would also be observed as an interphase depression in pain behaviour of the acutely spinalized rats since they have observed a biphasic pattern of hindpaw flinching in rats spinalized 21 days earlier (Coderre et al. 1994). However, awake rats administered the formalin test 48 hours after spinalization at the same thoracic level as in the above study do not show the typical biphasic response. When tested 48 hours after surgery, the flinching and licking of the first phase is significantly reduced, and the second phase of flinching and licking (observed from 20 to 35 minutes postinjection) is completely abolished (Wheeler-Aceto and Cowan 1991b). Therefore, it can not be concluded from the available data that a second formalin injection would produce a second interphase depression in pain behavior in awake, acutely spinalized rats. Hence, it also can not be concluded that the mechanisms responsible for the behavioural interphase depression are spinal. The electrophysiological recordings of dorsal horn cell responses to a second formalin injection may simply reflect the response to decreased peripheral afferent fibre activity that is presumed to occur in rats that do not show an interphase depression following decerebration or the administration of anxiolytics. In conclusion, the available evidence strongly suggests that the pain suppression mechanisms responsible for the interphase depression in formalin pain responding are supraspinal.

The neuropharmacological basis of the mechanism is unknown. The present study

demonstrates that disruption of the HPA axis by hypophysectomy has profound effects on the interphase depression, and thus, the HPA axis likely plays a significant role in the interphase depression. In addition, twenty-five day old rat pups show the typical biphasic formalin pain response, whereas rat pups 15 days old or younger show a monophasic pain response after formalin injection (Teng and Abbott 1998). This demonstrates that the interphase depression develops between 15 and 25 days after birth in the rat, which is approximately the time at which rats are emerging from the early period of HPA-axis hyporesponsiveness (Aksentijevich et al. 1994). Furthermore, the study in Chapter 2 shows that there are sex differences in the interphase depression in the Fischer rat which are correlated with sex differences in HPA axis function, producing higher baseline plasma corticosterone in the female rat.

CRF neurotransmission may underlie the formalin interphase depression. Anxiolytics inhibit the interphase depression, and CRF administration is anxiogenic, producing behaviours that are indicative of anxiety such as decreased time spent on the open arms of the elevated plus maze and decreased amount of food eaten in the centre of an open field (Dunn and Berridge 1990). Moreover, it has been suggested that GABA is involved, particularly the $GABA_A$ receptor (Franklin and Abbott 1993), and CRF and GABA interact. For instance, intracerebroventricular administration of GABA decreases the concentration of immunoreactive CRF in the hypophysial portal circulation (Plotsky et al. 1987). Furthermore, hypophysectomy has profound effects on CRF production and release. Following hypophysectomy, CRF plasma levels are increased (Yokoe et al. 1988). In the paraventricular nucleus of the hypothalamus, CRF increases seven fold after hypophysectomy, and in the parietal cortex CRF mRNA is doubled (Moldow and Fischman

1982; Yokoe et al. 1988; Frim et al. 1990). Thus, CRF is a likely candidate as the underlying mechanism of hypophysectomy-induced analgesia in the formalin test. It is also likely that CRF underlies the inhibitory effect of hypophysectomy on the development of adjuvant-induced polyarthritis, since hypophysectomy inhibits the development of inflammation in the rat hind paw immersed in 58 °C water, and this effect is blocked by systemic administration of the CRF receptor antagonist, α-helical CRF (Wei et al. 1990).

In conclusion, this study shows that hypophysectomy preferentially affects prolonged pain mechanisms over phasic pain mechanisms. Specifically, hypophysectomy prolongs the formalin interphase depression, the underlying mechanisms of which are supraspinal. Since the interphase depression is inversely correlated with adjuvant-induced polyarthritis susceptibility in the Fischer rat, and hypophysectomy inhibits the development of adjuvant-induced polyarthritis, a role for the supraspinal mechanisms in the development of polyarthritis is strongly supported. Hypophysectomy-induced hyperalgesia, or no effect on pain at all, is expected 4 or more weeks after surgery (see Figure 2). This hyperalgesia would increase the severity of the pain behaviour following hypophysectomy if the development of adjuvant-induced pain behaviour were not specifically related to the interphase depression mechanisms. Instead, a decrease in adjuvant-induced pain behaviour is observed, further supporting a specific role of the formalin interphase depression mechanisms in the development of adjuvant-induced polyarthritis. The next chapter examines the effect of CRF in the formalin test since CRF may underlie the effects of hypophysectomy on polyarthritis development and the formalin interphase depression.

Chapter 4

Study of Corticotropin-Releasing Factor-Induced Analgesia

To determine whether corticotropin releasing factor (CRF) has effects on pain mechanisms which are affected by hypophysectomy and associated with adjuvant-induced polyarthritis susceptibility, this study examines the effect of CRF in the formalin test. To determine the site of action, CRF is administered by 3 modes of administration: centrally by intracerebroventricular injection, systemically by intravenous injection, and locally by intraplantar injection.

Methods

Subjects, Formalin Testing, and CRF

Adult male Sprague-Dawley rats weighing 275-405 g at the time of testing were used. Male Long-Evans rats of the same weight range were also used in the study of subcutaneous administration since the responses of Sprague-Dawley rats in pilot studies were too variable (SEM up to 0.35) to have confidence in non-significant results. The animals were given free access to standard rat chow and tap water, and were maintained on a 12 h light/dark cycle with the lights on from 7:00AM.

Rats were handled for 5 minutes and habituated to the observation box in the testing room for 30 minutes on the two days prior to the day of testing and immediately prior to testing. The rats were then injected with 50 µl of 2.5% formalin under the plantar surface of the hind paw, and the pain behaviour was scored for 60 minutes using the same method and 3-point scale used in Chapters 2 and 3. All pain testing was performed during the light phase.

Rat/human CRF (Sigma) was dissolved in 0.9% saline and kept frozen at -70 °C in

aliquots until immediately prior to testing. Control rats received an equivalent volume of sterile, non-pyrogenic 0.9% saline. Vials of CRF and saline were coded to ensure that the experimenter was blind to the drug treatment during testing.

Intracerebroventricular administration

The rats were handled by the experimenter for 5 minutes on two occasions prior to surgery. Rats were anaesthetised with acepromazine (0.5 mg/kg), ketamine (50 mg/kg), and xylazine (5 mg/kg). Atropine (0.5 mg/kg) and 24% Tribrisson (0.5 ml/kg) were also given to inhibit mucous secretion and prevent infection. With the use of a stereotaxic apparatus, a guide cannula (23G) was implanted with the tip 0.7 mm dorsal to the wall of the right lateral cerebral ventricle (mm from Bregma: -0.9 AP, -1.7 ML, -3.0 VD). Testing was performed 5 to 7 days after surgery.

On the day of testing, an inner cannula (30G) was inserted into the guide cannula to extend 1.0 mm beyond the tip of the guide cannula and enter the ventricle. Using a Hamilton microinjection pump, 10 µl containing saline or 300, 500, 700, or 900 ng of CRF was injected at 5 µl/min (n = 6, 7, 7, 6, 6). The inner cannula was then left in place for 1 min to allow for diffusion of the drug away from the cannula. The inner cannula was then removed and an insect pin obdurator was placed in the guide cannula. Five minutes after CRF injection, an intraplantar formalin injection was administered in the right hind paw, and the pain behaviour was recorded.

At least 24 hours after pain testing, the rats were sacrificed with a lethal dose of chloral hydrate and perfused with 0.9% saline followed by 10% formal saline. The brains were removed and stored in formal saline for several days, after which histological analysis

was performed on 30-μm coronal sections stained with formal thionine. Guide cannulas made clear tracts of damage through the brain to within 1 mm of the ventricle, and inner cannulas made faint tracts that lead to the lateral ventricle. Rats whose cannula did not lead to the ventricle were excluded from the analysis.

Intravenous administration

Three hours prior to formalin testing, 5 or 10 μg/kg CRF in 100 μl/kg was injected into the penile vein of rats anaesthetized with methoxyflurane (Metofane) in a closed bell chamber, and maintained with a nose-cone (n = 8, 9). Control rats received an injection of 100 μl/kg saline (n = 7). The rats were then returned to their home cage for 2.5 hours until habituated to the observation box for 30 minutes before receiving the intraplantar formalin injection. Formalin testing was done 3 hours after CRF injection since CRF has maximal effects 3 hours after systemic administration (Hargreaves et al. 1989). The 3-hour interval also allows for the anaesthetic effects to wear off, although most of the rats were awake within five minutes of the intravenous injection.

Intraplantar administration

After habituation, 1.0 μg CRF in 50 μl of saline or 50 μl of saline was injected subcutaneously under the plantar surface of the hindpaw 10 minutes prior to intraplantar injection of formalin (n = 6). To examine the effect of degree of inflammation induced in the pain test, the effect of intraplantar CRF injection was examined in the bee venom test, which produces hind paw swelling several times that seen in the formalin test (Lariviere and Melzack 1996). Ten minutes after CRF injection, 0.1 mg of bee venom in 50 μl of saline

was injected, and the pain behaviour was scored for 60 minutes (n = 6).

In addition, the effect of preestablished inflammation of the hind paw on CRF-induced analgesia was examined in the formalin test. Four days prior to formalin testing, 50 µl of complete Freund's adjuvant (10 mg Mycobacterium butyricum/ml paraffin oil) was injected subcutaneously under the plantar surface of the hind paw. A local injection of adjuvant produces obvious marked swelling within 12 hours of the injection and infiltration of immune cells that is maximal 4 days after injection (Cabot et al. 1997). As above, CRF or saline was injected subcutaneously under the plantar surface of the hindpaw 10 minutes prior to formalin injection (n = 6).

Data Analysis

The interphase depression in formalin pain responding is expected to be affected since hypophysectomy affected the interphase depression. Furthermore, the underlying mechanisms are distinct from the underlying mechanisms of the first and second phases of pain responding. Thus, in the formalin test, mean pain scores were calculated for the first phase, the interphase depression, and the second phase. Based on the responses of control rats (see Figures 3 and 22), these periods were determined to be from 1-5 minutes, 6-20 minutes, and 21-60 minutes. In the bee venom test, a mean pain score was calculated for each 5-minute period from bee venom injection.

Results

Intracerebroventricular administration

The formalin responses following intracerebroventricular CRF are shown in

Figure 26. A two-way repeated measures ANOVA shows a significant interaction of Group (4 doses of CRF or saline) x Time (F (8, 54) = 2.416; $p < 0.05$). There is no significant difference between groups in mean pain scores during the first or second phases of formalin pain responding (F (4, 27) = 2.71, 0.51; $p > 0.05$), although there is a tendency for the 700 ng and 900 ng groups to be significantly different from the saline group during the first phase (t (10) = 1.96, 2.07; $p = 0.08, 0.07$). During the interphase depression, the 700 ng CRF-injected group has significantly lower mean pain scores compared to the saline-injected group (t (10) = 3.11; $p < 0.05$). As Figure 27 illustrates, the effect of CRF is to consistently prolong the interphase depression, delaying the onset of the second phase.

Intravenous administration

The formalin responses following intravenous administration of CRF are shown in Figure 28. A two-way repeated measures ANOVA showed that there is no significant interaction of Group (2 doses of CRF or saline) x Time (F (4, 42) = 1.12; $p > 0.05$), and a significant effect of Group (F (2, 21) = 4.76; $p < 0.05$) since the CRF-injected groups show less pain behaviour during the first and second phases and during the interphase depression. There is a significant effect of time (F (2, 42) = 30.4; $p < 0.001$) due to the nature of the formalin test. However, when the group that received 10 μg/kg is excluded from the analysis, the main effect of Group (5 μg/kg or saline) is no longer significant (F (1, 13) = 3.42; $p = 0.09$), and only the decrease in pain responding during the interphase depression is significant (t (13) = 2.49; $p < 0.05$; first, second phases: t (13) = 0.72, 1.19).

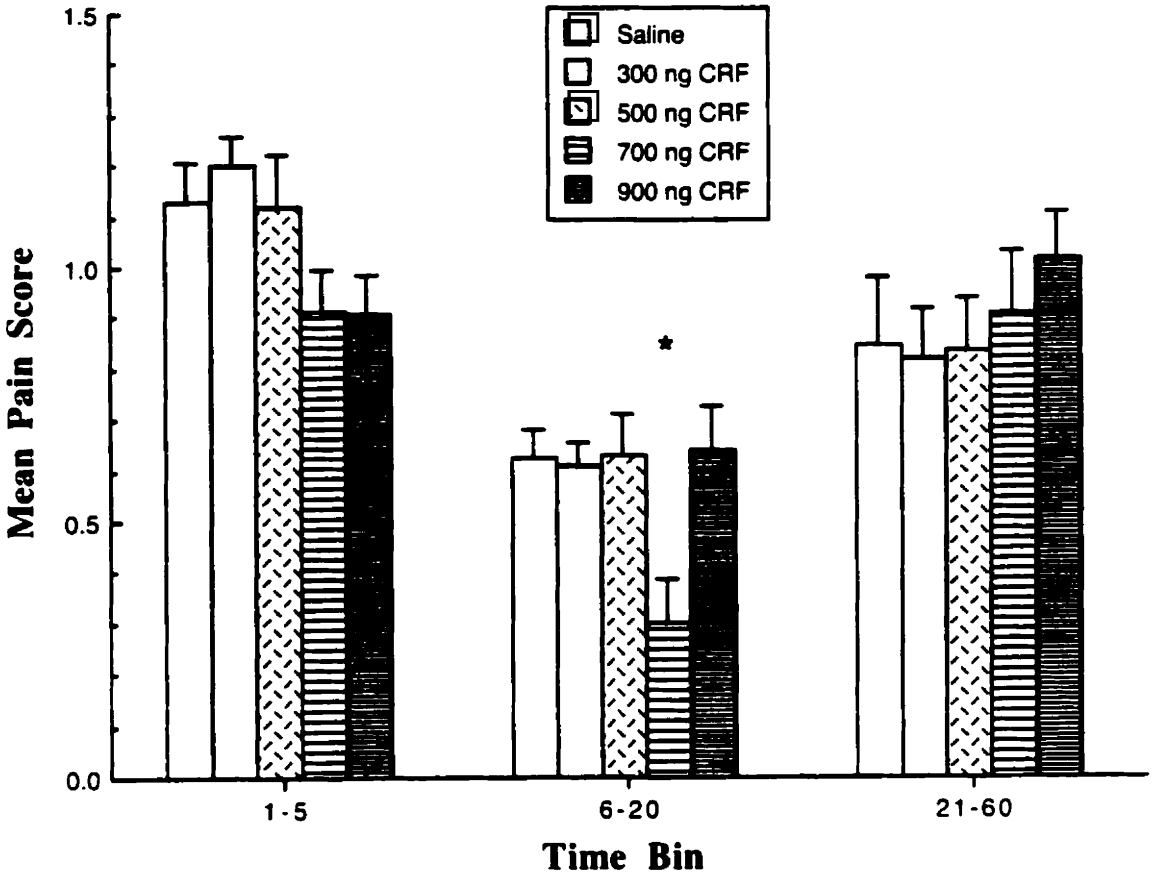

Figure 26. Formalin pain responses of rats five minutes after intracerebroventricular injection of CRF or saline (n = 6-7). Error bars indicate SEM. There is a significant interaction of Time x Group ($p < 0.05$). 700 ng-injected rats have significantly lower mean pain scores than saline-injected rats during the interphase depression, 6-20 minutes from formalin injection (* $p < 0.05$).

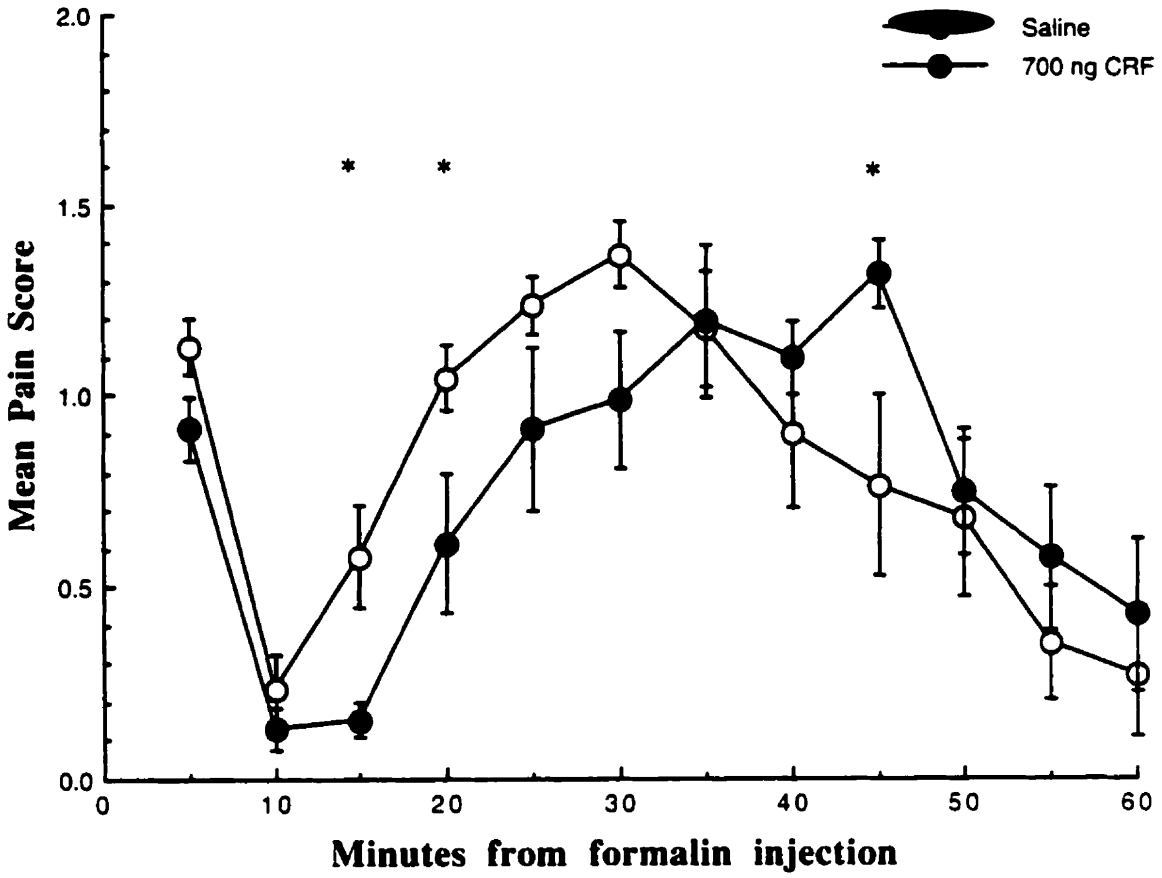

Figure 27. Formalin pain responses of rats five minutes after intracerebroventricular injection of 700 ng of CRF or saline ($n = 6$). Error bars indicate SEM. Compared to saline-injected rats, CRF-injected rats consistently show a prolonged interphase depression, delaying the onset of the second phase of the formalin pain response (* $p \leq 0.05$).

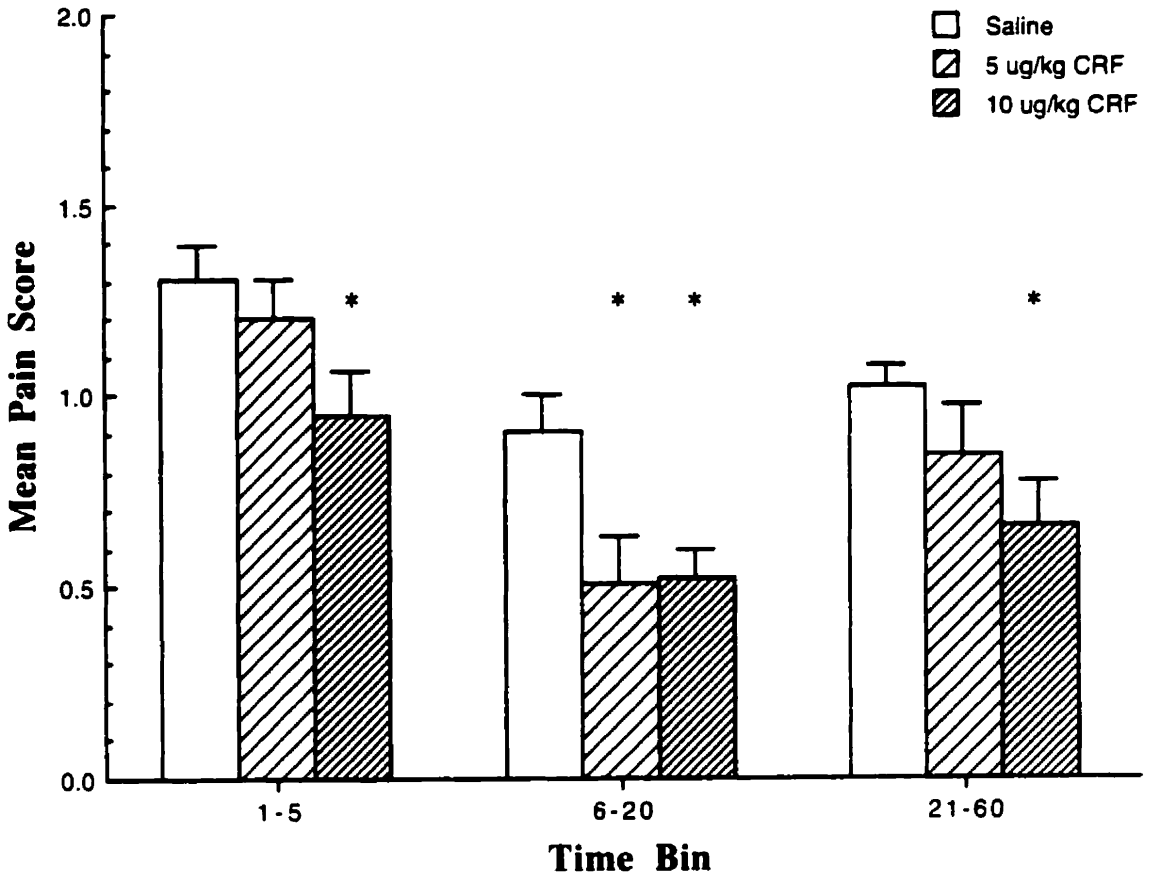

Figure 28. Formalin pain responses of rats 3 hours after intravenous injection of CRF or saline (n = 7-9). Error bars indicate SEM. There is a significant main effect of intravenous CRF injection ($p < 0.05$). However, the low dose of CRF significantly decreases mean pain scores only during the interphase depression, from 6 to 20 minutes (* $p < 0.05$, compared to saline injection).

Intraplantar administration

The responses to intraplantar injection of CRF in the formalin and bee venom tests are shown in Figure 29. Two-way repeated measures ANOVAs demonstrate that, in both pain tests, there is no significant interaction of Group (1.0 μg CRF or saline) x Time (formalin, bee venom: $F(11, 110) = 1.04, 1.10; p > 0.05$), and no main effect of intraplantar injection of CRF in the non-inflamed hind paw ($F(1, 10) = 0.001, 1.29; p > 0.05$). There is an effect of time in each pain test due to the nature of the pain tests ($F(11, 110) = 14.2, 46.5; p < 0.001$).

Local injection of adjuvant 4 days prior to testing significantly affected the response to formalin injection. As shown in Figure 30, there is a significant interaction of Adjuvant Injection (injected or not) x Time (first phase, interphase depression, and second phase) ($F(2, 40) = 10.3; p < 0.001$). Local adjuvant injection 4 days prior increases formalin pain responding during the interphase depression compared to non-adjuvant-injected rats ($t(22) = 2.41; p < 0.05$), and has no significant effect on the first or second phases ($t(22) = 0.11, 1.56; p > 0.05$). There is no significant interaction with, or main effect of, CRF administration ($F(11, 220) = 0.96, 1.26; F(1, 20) = 0.40$).

Although there is no effect on the overall mean for the second phase, a repeated-measures ANOVA of the 12 5-minute periods following formalin injection shows that adjuvant injection significantly decreases pain responding from 35 to 45 minutes after formalin injection compared to non-adjuvant-injected rats ($t(22) = 2.90, 2.89, 2.15; p < 0.05$). In addition, there is a tendency for CRF to produce analgesia compared to saline-injected rats 50 minutes after formalin injection ($t(10) = 2.04; p = 0.07$).

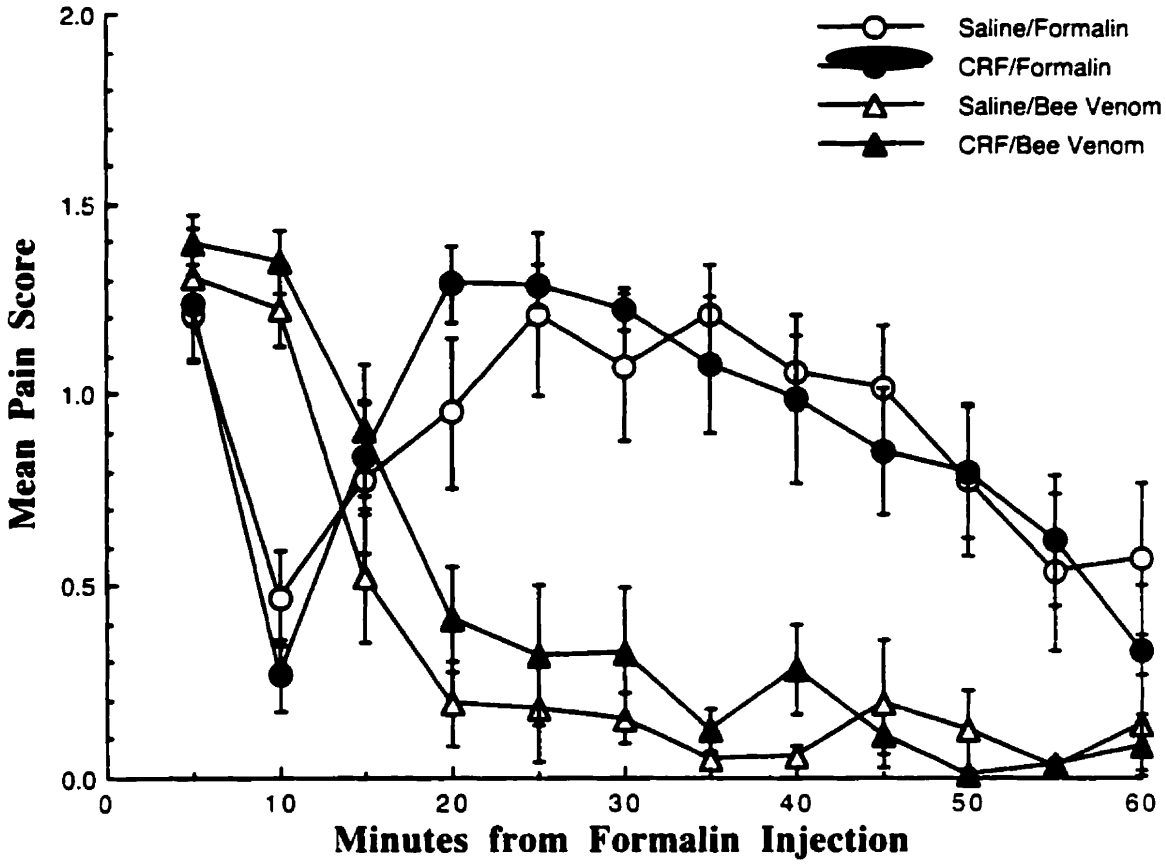

Figure 29. Formalin-induced and bee venom-induced pain responses of rats 10 minutes after intraplantar injection of CRF or saline (n = 6). Error bars indicate SEM. There is no significant interaction with, or effect of, intraplantar CRF injection ($p > 0.05$). There is a significant effect of Time from formalin or bee venom injection ($p < 0.001$).

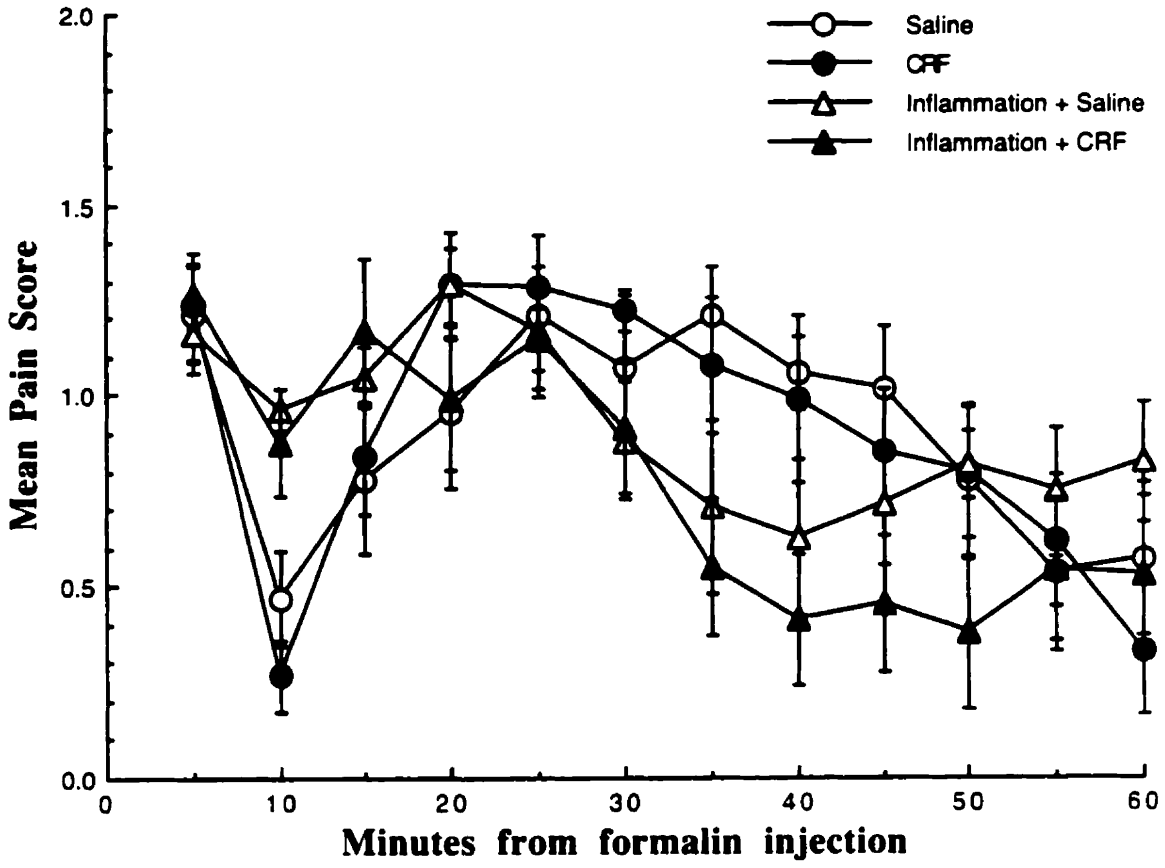

Figure 30. The effect of preestablished inflammation on formalin pain responses of rats 10 minutes after intraplantar injection of CRF or saline (n = 6). Error bars indicate SEM. Inflammation was induced by intraplantar injection of complete Freund's adjuvant 4 days prior to formalin testing. Adjuvant injection has no effect on the first phase of the formalin pain response ($p > 0.05$), increases pain responses during the interphase depression (6-20 min), and decreases pain responses from 35 to 45 from formalin injection ($p < 0.05$). There is a tendency for CRF to produce analgesia in the inflamed hind paw in the late second phase compared to saline injection in the inflamed hind paw ($p = 0.07$, 50 min from formalin injection).

Discussion

This study shows that intracerebroventricular administration of CRF produces analgesia in the formalin test during the interphase depression in pain responding, confirming the only other demonstration of intracerebroventricular CRF-induced analgesia in the rat in two reports that use the same parameters (Bianchi et al. 1991; Bianchi and Panerai 1995). The observation of only one effective dose is somewhat expected. In the rat hot plate and tail flick tests, analgesia is produced by 500 ng of CRF, but not by 300 ng or less or by 1.0 μg or more (Britton et al. 1985; Sherman and Kalin 1986; Wei et al. 1986; Sherman and Kalin 1987, 1988; Ayesta and Nikolarakis 1989; Bianchi et al. 1991; Bianchi and Panerai 1995). In addition, in the mouse, an increase from 50 ng to 100 ng causes a shift from a non-significant effect to a significant effect on pain (Kita et al. 1993). In the rabbit, an increase from 0.25 to 0.5 μg brings the effect on pain to significance, and a increase from 1.0 to 2.0 μg returns the effect to non-significance (Williams et al. 1986). Thus, a narrow effective dose range in the order of nanograms is also expected in the rat formalin test. The present results show that the dose-response curve for intracerebroventricular CRF in the formalin test is between 500 ng and 900 ng. Furthermore, the results show that intracerebroventricular CRF specifically affects the formalin interphase depression.

Intravenous administration of CRF also produces analgesia during the interphase depression. Although administration of a high dose of CRF produces analgesia throughout the formalin pain response, the response to a lower dose of CRF demonstrates the specificity of CRF's effects. With a low dose of intravenous CRF the interphase depression is significantly affected and the first and second phases are not significantly affected. Previous studies that compared the efficacy of CRF between phasic and tonic pain tests found CRF-

induced analgesia to be specific for prolonged, tonic pain mechanisms (Hargreaves et al. 1987; Song and Takemori 1990, 1991; Schäfer et al. 1994; Cabot et al. 1997; Lautenbacher et al. 1999). Thus, the present results confirm that although CRF can affect phasic pain mechanisms, there is a specificity for prolonged pain mechanisms. In the formalin test there is a specificity of intravenous and intracerebroventricular CRF-induced analgesia for the mechanisms underlying the interphase depression.

Intraplantar administration of CRF has no effect in the formalin and bee venom tests without preestablished inflammation. With preestablished inflammation evoked by adjuvant injection four days prior to formalin testing, intraplantar CRF decreases the pain behaviour during the second phase of pain responding, although not significantly. It is suspected that the detection of significant effects in the second phase was made more difficult by the decrease in second phase pain responding evoked by adjuvant injection. Nonetheless, an effect only in inflamed tissue is expected since previous studies found an analgesic effect of intraplantar injection of the same dose or less in the inflamed paw and no effect in the non-inflamed paw (Schäfer et al. 1994; Cabot et al. 1997). The lack of effect in the formalin test in the non-inflamed hind paw is not simply due to insufficient inflammation. Although the 2.5% concentration of formalin used does not evoke enough inflammation (compared to the injection of 5% formalin) to detect the effect of some anti-inflammatory agents (Yashpal and Coderre 1998), the bee venom test evokes marked swelling several times the swelling evoked by 2.5% formalin injection (Lariviere and Melzack 1996). Thus, it is more likely that preestablished inflammation with significant immune cell infiltration is necessary. In fact, the only known mechanism within peripheral tissue for CRF-induced analgesia is the CRF-receptor-mediated release of β-endorphin from immune cells that have infiltrated the

inflamed tissue (Schäfer et al. 1997).

Intraplantar CRF has no effect on the formalin pain mechanisms related to adjuvant-induced polyarthritis susceptibility, those responsible for the interphase depression, even though local adjuvant injection increases the interphase pain responding, which should have facilitated observation of analgesic effects. Thus, a peripheral site of action of CRF on the interphase depression mechanisms is ruled out.

Although intravenous CRF affects the pain mechanisms associated with adjuvant-induced polyarthritis susceptibility, the site of action of CRF can not be concluded from the effects of this mode of administration. Due to the lipophilicity of CRF, some intravenous-injected CRF is expected to cross the blood-brain barrier where it could act within the central nervous system (Martins et al. 1996). Therefore, intravenous CRF could affect mechanisms both within and outside of the central nervous system.

The effects of intracerebroventricular administration of CRF strongly suggest that the site of action on the interphase depression mechanisms is within the central nervous system. Although some intraventricular-injected CRF is expected to be pumped out across the blood-brain barrier where it could affect mechanisms outside of the central nervous system, the dose-response curve of intracerebroventricular administration indicates that the analgesic effects are due to actions within the central nervous system. Following intracerebroventricular injection of 700 ng of CRF, half of the injected amount is expected to be pumped out into the circulating blood within 11 minutes, providing the equivalent of approximately 1 μg/kg CRF by intravenous injection. Although this is lower than the intravenous doses examined in the present study, it is possible that such a dose could have the same effect as 5 μg/kg of intravenous CRF shown in Figure 28. However,

intracerebroventricular injection of 900 ng of CRF has no effect on the interphase depression. Thus, an effect of CRF on the interphase depression via mechanisms outside of the central nervous system is excluded since a dose 200 ng greater of intracerebroventricular CRF would bring the circulating CRF levels closer to the doses used in the intravenous CRF administration experiment, both of which have significant effects on the interphase depression. Therefore, the site of action of CRF on the interphase depression mechanisms is within the central nervous system.

The pattern of results following the three modes of administration of CRF is congruent with the evidence reviewed in the previous chapter that places the mechanisms responsible for the interphase depression within the supraspinal central nervous system. Intraplantar injection of CRF has no effect on the interphase depression since the injected CRF does not reach the brain. Intravenous injection of CRF has effects on the interphase depression because CRF can cross the blood-brain barrier to act within the central nervous system. Moreover, intracerebroventricular injection of CRF affects the supraspinal mechanisms simply by diffusing to the site of action. In conclusion, the present study suggests strongly that the site of action of CRF on the pain mechanisms responsible for the interphase depression and associated with adjuvant-induced polyarthritis is within the brain.

Based on the electrophysiological effects of intracranial injection of CRF and on the distribution of CRF and CRF-receptor immunoreactivity, CRF may potentially modulate pain processing at loci in all major subdivisions of the brain (Lariviere and Melzack 2000). The effect of hypophysectomy on CRF in the paraventricular nucleus of the hypothalamus and the prefrontal cortex suggests that these areas are involved in the interphase depression. However, direct action on the paraventricular nucleus can be ruled out since bilateral lesions

of the nucleus do not have an effect on formalin-induced pain (Lariviere et al. 1995). The effects of partial decortication and the observation of cortical EEG activity during the interphase depression further suggests that cortical structures are involved. Moreover, since anxiolytics can abolish the interphase depression, likely candidates include structures involved in anxiety including amygdaloid nuclei, the hippocampus, and the cingulate cortex.

A pilot study conducted in our laboratory examined the effect of ipsilateral microinjection of 0.1 μg CRF into the central nucleus of the amygdala in the rat formalin test. Compared to CRF injection in neighbouring structures ($n = 6$), injection into the central nucleus of the amygdala ($n = 6$) 5 minutes prior to formalin injection decreases overall 60-minute mean pain scores, although not significantly (0.90 ± 0.11 versus 1.12 ± 0.03; $t(10) = 1.89$; $p = 0.09$). The decrease in mean pain scores from 20 to 30 minutes shown in Figure 31 is due to the prolongation of the interphase depression in half of the amygdala-targeted rats, whose onset of second phase pain behaviour was at 25, 35, and 45 minutes, and whose injection sites were found to be in the medial portion of the central nucleus. Examination of the individual responses of the control group and the three more laterally amygdala-targeted rats shows that the onset of the second phase in these rats is consistently at 20 minutes after formalin injection. Re-analysis after subdivision of the amygdala-targeted group found a significant analgesic effect of CRF injection in the medial central nucleus compared to in neighbouring structures and to CRF injection in the lateral central nucleus (overall 60-minute mean pain scores: $F(2, 9) = 13.1$; $p < 0.01$). Thus, although the number of subjects per group is small in this pilot study, it is highly likely that CRF acts in the medial portion of the central nucleus of the amygdala to affect the interphase depression.

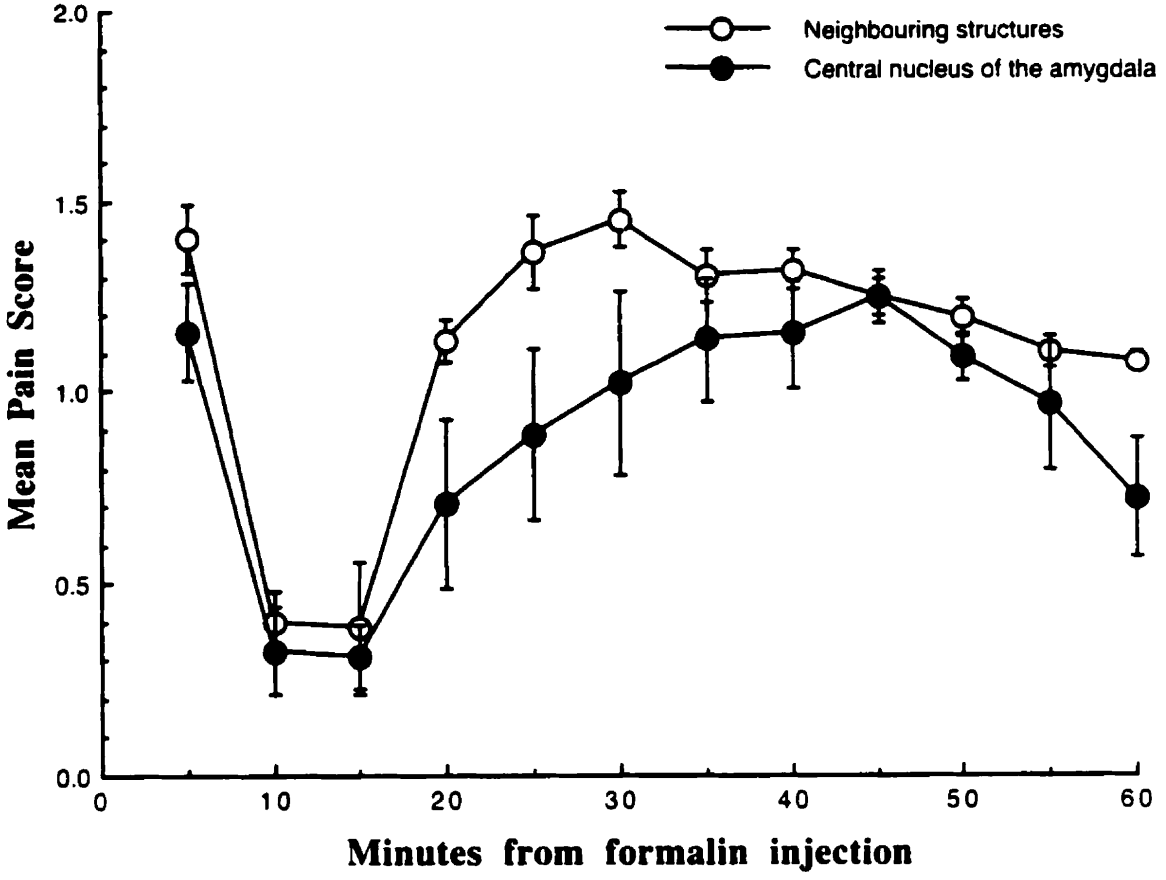

Figure 31. Formalin pain responses following microinjection of CRF into the central nucleus of the amygdala and neighbouring structures (n = 6). Error bars indicate SEM. There is a tendency for microinjection of CRF in the central nucleus of the amygdala to significantly decrease the overall 60-min mean pain scores compared to injection in neighbouring structures (p = 0.09). This tendency is due to the significant analgesic effect of microinjection of CRF into the medial aspect of the nucleus compared to in the lateral aspect and in neighbouring structures (n = 3, 3, 6; $p < 0.01$; data not shown).

Chapter 5

Conclusion

The experimental evidence presented in the preceding chapters shows that specific pain mechanisms contribute to the development of adjuvant-induced polyarthritis among groups of rats that differ in HPA-axis function. Paradoxically, sensitivity to the pain associated with inflammation in the late second phase of the formalin response is not correlated with adjuvant-induced polyarthritis susceptibility, ruling out a nonspecific 'inflammatory pain' sensitivity as responsible for the differential susceptibility. In contrast, the Lewis rat, which is more sensitive to the pain evoked in the tail flick test than the Fischer rat, is also more susceptible to adjuvant-induced polyarthritis. In addition, the degree of pain suppression during the formalin interphase depression is inversely correlated with susceptibility in the Fischer rat. That is, the female Fischer rat, whose pain suppression mechanisms underlying the formalin interphase depression are not as effective as those of the male Fischer rat, is more susceptible to adjuvant-induced polyarthritis.

Experimental manipulation of the HPA axis in Chapter 3 further confirms the role of the interphase depression mechanisms in adjuvant-induced polyarthritis susceptibility. The results show that hypophysectomy inhibits the development of adjuvant-induced inflammation and pain behaviour. In addition, hypophysectomy prolongs the formalin interphase depression compared to sham hypophysectomy. However, hypophysectomy does not affect the phasic pain of the first phase of the formalin test, or the pain associated with inflammation in the second phase of the formalin test or in the bee venom test. A specific role of the interphase depression mechanisms in the development of adjuvant-induced polyarthritis is further suggested by the expectation of hypophysectomy-induced hyperalgesia at the time adjuvant-induced pain behaviour was measured. This hyperalgesia would

increase the severity of the pain behaviour following hypophysectomy if the development of adjuvant-induced polyarthritis pain behaviour were not specifically related to the interphase depression mechanisms, but instead, a decrease in adjuvant-induced pain behaviour is observed. The development of adjuvant-induced polyarthritis inflammation is significantly inhibited by 2 or 3 days of intracerebroventricular morphine every 2 hours (Levine et al. 1985b, 1986). Therefore, it is postulated that although adjuvant injection was administered 12 days after hypophysectomy in the present study, and hypophysectomy-induced analgesia was observed only up to 14 days after surgery in the present study, as little as 2 days of analgesia is sufficient to inhibit the development of adjuvant-induced polyarthritis.

Furthermore, the formalin interphase depression mechanisms are affected by intraplantar injection of complete Freund's adjuvant, which produces the peripheral manifestations of adjuvant-induced polyarthritis and almost abolishes the formalin interphase depression without affecting the pain behaviour of the first phase of the formalin pain response. This convincingly demonstrates that the interphase depression mechanisms are integrally involved in the susceptibility to adjuvant-induced polyarthritis.

In summary, the formalin interphase depression mechanisms are affected by the peripheral manifestations of adjuvant-induced polyarthritis, they are correlated with the susceptibility to the chronic pain model, and they are specifically affected by a procedure that inhibits its development. Together, these results strongly support a role of the interphase depression mechanisms in the development of adjuvant-induced polyarthritis. Hence, rats with relatively ineffective interphase depression mechanisms are predisposed to develop adjuvant-induced polyarthritis. Conversely, rats whose interphase depression mechanisms are more effective, resulting in less pain behaviour during the interphase depression, are

more resistant to adjuvant-induced polyarthritis.

The experimental evidence presented in Chapter 4 demonstrates that the formalin interphase depression is also specifically affected by intracerebroventricular administration of the appropriate dose of CRF. In addition, a low dose of intravenous CRF preferentially affects the interphase depression due to the effect of CRF within the central nervous system. Moreover, preliminary findings suggest that the medial central nucleus of the amygdala is a central site of action of CRF on the interphase depression. Injection of CRF into inflamed peripheral tissue decreases the pain associated with inflammation in the late second phase of the formalin test in the inflamed hind paw, but does not affect the pain during the interphase depression. Hence, intraplantar CRF may decrease the pain of adjuvant-induced polyarthritis after inflammation has developed. It is likely since elevated levels of CRF are seen in inflamed tissue, correlating significantly with the degree of immune cell infiltration (Crofford et al. 1993). However, central CRF affects the pain suppression mechanisms involved in the development of polyarthritis, and therefore, likely contributes to adjuvant-induced polyarthritis susceptibility.

Indeed, central CRF production and release is correlated with adjuvant-induced polyarthritis susceptibility. Levels of genetic precursors (mRNA) of CRF in the paraventricular nucleus of the hypothalamus decrease with the progression of adjuvant-induced polyarthritis (Lightman and Harbuz 1993). Eleven days after adjuvant injection, when inflammation begins to develop, CRF mRNA is significantly less than on the day of injection. When inflammation and pain behaviours are maximal approximately 3 weeks post-injection, CRF mRNA in the hypothalamus are minimal at less than 60 % of initial pre-injection levels. These markers of CRF production are also inversely correlated with

polyarthritis susceptibility between the Lewis and Fischer rats. In response to a variety of inflammatory and non-inflammatory stressors, the highly susceptible Lewis rat has a blunted CRF mRNA response in the paraventricular nucleus of the hypothalamus compared to the less susceptible Fischer rat (Sternberg et al. 1992b; Aksentijevich et al. 1994).

In addition, the central effects of CRF differ among groups with differential polyarthritis susceptibility. Intracerebroventricular administration of CRF increases the spontaneous, tonic electrophysiological activity of locus coeruleus neurons in adult male Sprague-Dawley rats, but has no effect in immature males (Borsody and Weiss 1996). Immature male Sprague-Dawley rats are also highly susceptible to adjuvant-induced polyarthritis, with a 100% induction rate, whereas adult males of this strain have a susceptibility that is between that of Lewis and Fischer rats (Neidhart and Flückiger 1992; Lariviere and Melzack 1997).

The production and effects of central CRF are associated with adjuvant-induced polyarthritis susceptibility, and central CRF directly enhances the supraspinal pain suppression mechanisms associated with resistance to polyarthritis development. Therefore, the evidence shows that the central effects of CRF modulate the development of this chronic pain model and underlie the effects of hypophysectomy. Moreover, the difference in susceptibility between male and female Fischer rats may also be due to the central effects of CRF on the formalin interphase depression mechanisms.

Although the Lewis rat shows a blunted central CRF response to various stressors compared to the Fischer rat, it is unknown why the Lewis rat does not show more pain behaviour during the interphase depression than the Fischer rat. The Lewis rat does, however, have greater sensitivity in the tail flick test, which may be related to HPA axis

function. Despite a preference for the modulation of the interphase mechanisms, hypophysectomy can affect the pain evoked in phasic pain tests, albeit not consistently (see Table 1 and Figure 2). Moreover, CRF can also affect pain mechanisms not related to the interphase depression. A high dose of intravenous CRF affects the first phase of phasic pain in the formalin test and in several other phasic pain tests (see Table IV CRF). In addition, intracerebroventricular CRF also shows a tendency to inhibit the pain of the first phase of the formalin test. Therefore, the difference in tail flick test sensitivity between the Lewis and Fischer rat may be related to their differences in HPA axis function, although it is unknown why the Lewis rat does not show more pain behaviour during the formalin interphase depression compared to the Fischer rat. It is possible that pain suppression mechanisms not examined in the formalin test are involved and related to their differential adjuvant-induced polyarthritis susceptibility. For instance, the Lewis rat exhibits less morphine analgesia than the Fischer rat (Vaccarino and Couret 1995), which may be related to HPA axis function since the HPA axis and endogenous opioids interact.

In addition to acting directly on the polyarthritis-related pain mechanisms, central CRF may also inhibit the development of adjuvant-induced polyarthritis indirectly by modulating neurogenic inflammation mechanisms (Wei et al. 1990). Central mechanisms activated by intracerebroventricular morphine inhibit the development of adjuvant-induced inflammation in the hind paw (Levine et al. 1986). Thus, the central effects of CRF may similarly activate mechanisms that have an inhibitory effect on the development of adjuvant-induced polyarthritis in the peripheral tissue. The contribution of peripheral afferent fibres to neurogenic inflammation may be particularly important in the Lewis rat. It has been proposed that the tail flick test is more spinally and peripherally mediated compared to other

pain tests including tonic pain tests (Carstens 1996). Thus, although it is merely speculation, the high sensitivity of the Lewis rat in the tail flick test may indicate a greater activation of peripheral afferents which could contribute to the development of inflammation in the peripheral tissue via axon reflex mechanisms or via spinal loops to sympathetic efferent fibres.

Nonetheless, the evidence indicates that CRF acts within the brain to enhance pain mechanisms that underlie the formalin interphase depression, and which are associated with resistance to adjuvant-induced polyarthritis. In conclusion, the evidence strongly suggests that the HPA axis contributes to the development of chronic pain in the rat via effects within the central nervous system.

Other components of the HPA axis also have effects on central pain mechanisms. Although the effects of ACTH on pain are equivocal and not well understood (Chrubasik et al. 1993), ACTH has analgesic effects following microinjection into the posterior arcuate nucleus, and hyperalgesic effects following intracerebroventricular administration (Bertolini et al. 1979; Takeshige et al. 1991). Furthermore, ACTH interacts with central opiate receptors, which may be responsible for the antagonism of stress-induced analgesia and morphine-induced analgesia by ACTH (Terenius 1976; Gispen et al. 1976). Corticosterone also has effects on central pain mechanisms. Microelectrophoretic application of corticosterone has an excitatory effect on raphe neurons (Avanzino et al. 1984), which may increase the descending inhibition originating from these neurons, and may underlie an opioid form of stress-induced analgesia that is abolished by adrenalectomy and replaced by corticosterone administration (MacLennan et al. 1982). In addition, chronic --but not acute-- intrathecal administration of corticosteroids produces analgesia in the second phase of the

formalin pain response of the rat (Abram et al. 1994). Therefore, the direct effects of these components of the HPA axis on central pain mechanisms may also contribute to the development of chronic pain . The evidence presented shows that elucidation of the central effects of the HPA axis will lead to a greater understanding of the role of systems activated by stress in the development of chronic pain associated with HPA axis abnormalities such as rheumatoid arthritis, fibromyalgia, and chronic pain in patients with post-traumatic stress disorder (Clauw and Chrousos 1997).

The recognition that the HPA axis affects central pain mechanisms and the susceptibility to chronic pain indicates that chronic pain is not merely affected by the effects of stress on peripheral tissue. The central effects of systems activated by stress must also be considered, especially in the development of chronic pain. The specificity seen in the interactions between CRF and the chronic pain model of adjuvant-induced polyarthritis demonstrate the complexity of the interactions, and provide some understanding of the equivocal results seen in studies of the effects of stress on arthritis in animals and humans (Koehler 1985). Finer consideration of the mechanisms affected by particular events that vary widely but are grouped as "stressors" may be fruitful. Indeed, the subclassification of stressors as major versus minor, or as evoking fear versus anxiety, shows that stressful events can have opposite effects on pain (Huyser and Parker 1998; Rhudy and Meagher 2000). Investigation of the effects of various stressors on central pain mechanisms will provide a better understanding of their role in the susceptibility to particular chronic pain syndromes.

Appendix 1. Corticosterone assay protocol.

All assays were performed by the laboratory of Dr. Shree Mulay at the Royal Victoria Hospital and paid for by an NSERC grant to Dr. Ronald Melzack. Dr. Mulay provided this assay protocol.

Reagents for Assay:

Assay Buffer:

17.4 g	sodium phosphate diabasic (MW 142)
10.8 g	sodium phosphate monobasic (MW 138)
2.0 g	sodium azide (MW 65)
18.0 g	sodium chloride (MW 58)
2.0 g	gelatin

Dissolve the above chemicals by first dissolving the gelatin in about 1 litre warm double-distilled water, then add all the other salts, leaving sodium azide to the last, cool to room temperature than add the sodium azide and make up the final volume to 2 litres in a volumetric flask.

Dextran-coated Charcoal:

625 mg	Norit A charcoal
62.5 mg	Dextran T-70
100 ml	Assay buffer

Corticosterone Standards:

Purchased at 1μg/ml in ethanol from ICN Biochemicals (Catalogue # 245-198)

Prepare sequential dilutions ranging from 0.025-10 ng in 0.5 ml assay buffer.

Anti-corticosterone antibody:

Stock antisera purchased from ICN Biochemicals (# 1472). Prepare a working stock solution at the time of assay (1:1200 dilution).

^{3}H-Corticosterone tracer:

10 μci/ml purchased from ICN Biochemicals (Catalogue # 198)

Prepare a working stock solution by diluting 85 μl in 10 ml assay buffer. Check that the counts are approximately, 10 000-cpm/0.1 ml of tracer.

Corticosterone in rat serum/plasma:

Sample preparation:

(Warm all reagents to room temperature before starting the assay.)

1. Dilute rat plasma 1:500 in duplicate in 10 x 75 mm glass, not plastic, tubes
2. Pipette total, NSB, blank and standards (0.6 ml and 0.5 ml assay buffer and 0.5 ml of each standard, respectively).
3. Pipette diluted plasma samples to tubes (0.5 ml)
4. Incubate all tubes for ten minutes in a boiling water bath (98 °C) to denature the corticosterone binding globulin. Cool to room temperature.
5. Add tracer and antibody (working stock solutions) to the tubes as shown belown and

incubate tubes overnight at 4 °C.

(Incubate at 98 °C) (Incubate at 4 °C)

Tubes	Buffer	Tracer	Antibody	Charcoal
Total (2)	0.8 ml	0.1 ml	–	–
NSB	0.6	0.1 ml	–	0.2 ml
Reference	0.5	0.1 ml	0.1 ml	0.2 ml
Standard G	0.025 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Standard F	0.05 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Standard E	0.1 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Standard D	0.25 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Standard C	0.5 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Standard B	1.0 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Standard A	2.0 ng/0.5 ml	0.1 ml	0.1 ml	0.2 ml
Unknown plasma samples	0.5 ml diluted sample	0.1 ml	0.1 ml	0.2 ml

DO NOT ADD CHARCOAL TO FIRST TWO TUBES

6. Following overnight incubation of tubes, add 0.2 ml charcoal solution, which is placed on the magnetic stirrer. Vortex each tube. Incubate for 10 minutes at 4 °C.

7. Centrifuge tubes at 2 500 rpm at 4 °C.

8. Decant supernatant in a counting mini-vial and add 3.5 ml opti-phase scintillation cocktail.

9. Count in a LKB 2016 beta counter and calculate the results using an LKB multicalc program. Multiply with the dilution factor to express as ng/ml plasma.

Assay characteristics:

The specificity of the antisera as provided by the supplier is given on the next page. The intra- and inter-assay coefficients of variance for this assay were 5.6 % and 7.4 %, respectively.

Comparison with previously used methods:

This method was compared with one in which plasma/serum was first extracted with methylene chloride, then the aliquots were dried and reconstituted in assay buffer, and then assayed as described in steps 5-9. The results were highly comparable (95-102%).

Conversion of ng/ml to nmol/L:

Multiply all values in ng/ml with 2.886 to convert them to nmol/L

Antisera specificity as provided by ICN Biomedicals, Inc.:

Catalogue Number:	07-120016
Antisera for:	Corticosterone
Antigen used for immunization:	Corticosterone-3-Carboxymethyoxime:BSA
Sensitivity of the standard curve:	10-25 pg
Titer (Final):	1: 8 400 (Using 10 000 cpm CpB-1, 2, 6, 7 3H)
Purification prior to assay:	Rat: Heat denaturation only; Human: Extraction with ethyl acetate: hexane (3:2) followed by chromatography (system III).
Specimen requirement for assay:	Rat: 10 µL serum/plasma Human: 0.5 ml serum/plasma
Steroid RIA reference:	RSL unpublished data. Assay procedure provided on request.

CHARACTERIZATION DATA

STEROIDS	% CROSS REACTIVE
Corticosterone	100.00
Desoxycorticosterone	6.10
Progesterone	0.29
Cortisol	0.19
Aldosterone	0.08
20a-Dihydroprogesterone	0.08
Testosterone	0.08
11-Desoxycortisol	0.03
Androstenedione	0.01
Cholesterol	< 0.01
Dehydroepiandrosterone	< 0.01
Dehydroepiandrosterone-sulfate	< 0.01
Dihydrotestosterone	< 0.01
Estradiol-17B	< 0.01
Estradiol-17a	< 0.01
Estrone	< 0.01
Estriol	< 0.01
Pregnenolone	< 0.01
17a-Hydroxypregnenolone	< 0.01
17a-Hydroxyprogesterone	< 0.01

Appendix 2. Data comparing measurement of the development of adjuvant-induced polyarthritis with the scale of pain-related behaviour used in Chapters 2 and 3(shown in Table 6) with the measurement of paw swelling. The following abstract (Lariviere and Melzack 1997) was presented as a poster at the 1997 Meeting of the Canadian Pain Society.

GENETIC INFLUENCES IN THE SUSCEPTIBILITY TO ADJUVANT-INDUCED POLYARTHRITIS IN THE RAT

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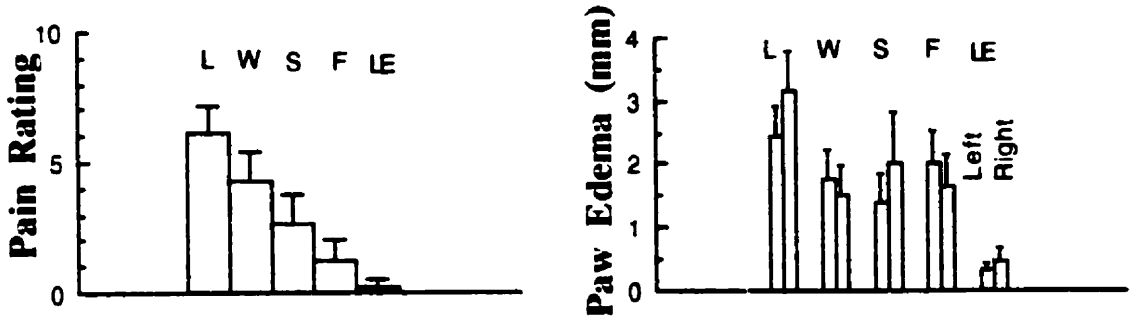
INTRODUCTION Genetic differences in the susceptibility to adjuvant-induced arthritis (AIA) have been demonstrated: Lewis (L) rats are highly susceptible and Fisher (F) rats are hardly susceptible. Moreover, the most commonly used strain of rat in studies that employ the model is Sprague-Dawley (S). In our experience, S and Long-Evans (LE) rats from Charles River Montreal showed very low susceptibility. Therefore, a systematic study was performed to ascertain the relative susceptibilities of several strains of rat.

METHODS Six female and six male rats of five strains (L, Wistar (W), S, F, and LE) were anaesthetized prior to intradermal injection of complete Freund's adjuvant (1.0 mg Mycobacterium butyricum/300 g rat). On the 21st day following injection, pain behaviour was scored using a ten-point rating scale. The mediolateral dimension of the ankle of both hind paws were measured with calipers prior to adjuvant injection and on the 21st day.

RESULTS



There was a significant effect ($p < 0.05$) of strain on the mean pain rating and on edema. Significant pairwise comparisons: L vs LE for pain rating and edema; W vs LE for pain rating only.

DISCUSSION This difference in susceptibility needs to be considered when choosing a strain of rat and when interpreting the presence or absence of effects of a manipulation. These data highlight the genetic contribution in this animal model of chronic pain and support the investigation of genetic predispositions to chronic pain disorders in animals and humans. [In addition, the data also show that the use of the scale of pain and disability is a valid method of measurement of the development of adjuvant-induced polyarthritis.]

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