Differences in the susceptibility to chronic inflammatory disease between inbred rodent strains have long been appreciated and show the influence of genetic factors in chronic inflammation. One very important inbred strain comparison for the study of the role of the hypothalamic-pituitary-adrenal (HPA) axis in the susceptibility and development of chronic inflammation is between Lewis rats and Fischer 344 rats. Female Lewis rats are highly susceptible to adjuvant-induced arthritis (AAIA; a model of rheumatoid arthritis) and other prolonged inflammation models and, in response to stressors, have a blunted activation of the HPA axis and release of corticosterone compared with the more inflammation-resistant female Fischer rats (Sternberg et al. 1989a,b; Wilder 1993). Corticosterone has anti-inflammatory effects, and thus the strain differences in susceptibility have been ascribed to levels of circulating corticosterone (Sternberg et al. 1992; Sternberg 1995). Later studies suggest that mechanisms other than circulating corticosterone must be involved in the strain differences (Chover-Gonzalez et al. 1998, 1999, 2000; Griffin and Whitacre 1991; Harbuz et al. 1994; Jessop et al. 2001).

The responses of the nociceptive system to acute inflammatory stimuli may also differ between the inbred strains and may be partly responsible for the differences in the responses of the endocrine systems of the inbred strains to inflammatory stimuli. That is, differential baseline inflammatory nociceptive sensitivity may evoke differential endocrine responses (including corticosterone), which in turn, modulate the development of chronic inflammation. Nonendocrine, neural pain mechanisms shown to be involved in the development of AAIA, may also differ between the strains, directly or indirectly affecting the development of inflammation through afferent and efferent fibers terminating in the peripheral tissue (Colpaert et al. 1983; Cruwys et al. 1995; Donaldson et al. 1995; Levine et al. 1985a,b, 1986; Wheeler-Aceto and Cowan 1991).

Similarly, the inherent ability of the inbred rats to modulate inflammatory nociception may also depend on inherent differences in baseline nociceptive sensitivity. Recently shown, inherited sensitivity to analgesic compounds can be negatively correlated with inherited baseline sensitivity in the assay used to assess the analgesia (Wilson et al. 2003). That is, strains of inbred mice that are more sensitive to thermal or inflammatory nociception are less able to modulate the nociception. Thus baseline differences in thermal and inflammatory nociception may underlie reported differences in nitrous oxide antinociception, morphine antinociception, and tolerance to morphine antinociception observed between Lewis and Fischer rats (Fender et al. 2000; Vaccarino and Couret 1995). In neither of these studies was the baseline sensitivity examined or reported despite expected differences, and in no reported study has the acute inflammatory nociceptive response been compared between the two strains.
METHODS

The experimental protocol is summarized in Table 1 and is described in detail below.

Subjects

Pregnant Fischer 344 and Lewis rats, respectively, arrived from the supplier (Charles River, St. Constant, Quebec, Canada) 4–6 days before giving birth to male and female pups that were tested as adults ($n = 9–10$ /group). The animals had free access to rat chow and water and were maintained on a 12-h light/dark cycle, with the lights on from 7:00 A.M. All procedures were approved by the McGill University Animal Care Committee and adhere to the guidelines of the Committee for Research and Ethical Issues of IASP (Zimmermann 1983).

Early postnatal treatments

On the second day of life, pups were equally distributed among the available dams of the same strain (7–11 pups/dam; total of 7 Fischer and 9 Lewis dams) and were immediately exposed to one of three treatments each day for 21 days: maternal separation for 15 or 180 min (MS15, MS180) or no daily handling by the experimenter (Control). Maternal separation consisted of removing the dam from the home cage after which the pups were placed as a group into a similar clean cage with bedding. The dam was returned to her home cage for the period of separation. The pups were taken to another cage until 1 wk before testing, when they were housed alone for the remainder of the experiment.

Great care was taken to not stress the rats as adults when handling for any reason, introducing the hand into the rat’s cage, and allowing the rat to approach and explore the hand before handling, for instance. The rat was never “chased” around the cage. This was done to minimize the effects of stress on the measures (including stress-induced analgesia) caused by the experimenter, because it has been shown that the experimenter is an important variable in nociception assays in which the animal is restrained during the test (Chesler et al. 2002a,b). In addition, W.R.L.’s handling and habituation may produce less nociceptive modulation by procedural stress because he reports greater behavioral pain responding in the formalin and bee venom inflammatory nociception assays than others (Lariviere et al. 2002, 2004). In addition, handling of every animal was done extremely methodically, using a timer to standardize handling time across groups. To reduce possible effects of being housed alone, rats were also handled once per week for 3 min when not in an experiment. Throughout the experiment, the animals seemed extremely well habituated, approaching the experimenter when the cage was opened and even during exploration after arthritis induction.

Estrous cycle determination

To control for the effect of the estrous cycle, female rats were tested in diestrous. At least 2 h before testing, a vaginal smear was performed, and the presence of mainly leukocytes indicated that the rat was 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 plastic 1-ml syringe was pressed against the anogenital region for 10 s. Pairing of males with females on test days was done as much as possible.

Tail flick test

At 3 mo of age, rats were handled for 3 min and habituated to the testing room for ~1 h on two occasions before the day of testing and again on the day of testing. The rat was removed from its home cage and gently restrained in a towel, and its tail was immersed in 54°C water, a temperature used to reduce the effects of stress on the measures (d’Amore et al. 1992). The latency to flick the tail was recorded three times, each time separated by 10 s, and the average of the three measures was calculated. The responses were also analyzed as a repeated measure. All rats responded within 5 s, before the predetermined cut-off of 10 s used to prevent tissue damage had the rats not responded. All tail flick testing was performed between 9:00 A.M. and 1:00 P.M. Notes were also taken of whether the rat made a vocalization audible to the experimenter without amplification or modification at each trial after insertion of the tail in the water.

Formalin test

At least 7 days later, the formalin test was administered. Tail flick testing 1 week before is not expected to affect formalin pain responses because others have reported no effect of repeated (formalin) testing at 1-wk intervals (Matthies and Franklin 1992, 1995; Rosland et al. 1990). The rats were habituated to the 30 $\times$ 30 $\times$ 30-cm transparent Plexiglas observation box for 30 min on two occasions before the day of testing and immediately before testing. The rat was removed from the observation box and restrained in a towel, and 50 $\mu$l of 1.5% formaldehyde (10% NaPO$_4$ buffered neutral formalin, VWR, diluted in sterile saline) was injected under the plantar surface of the left hind paw. The rats were placed in the observation box, and the pain behavior was scored for 60 min. Below the floor of the box, a mirror at a 45° angle facilitated viewing of the injected paw. The behavior 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; or 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 last score entered once every half-second. A mean pain score (a weighted sum of the durations of each behavior) was calculated for each 5-min 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 9:00 A.M. and 2:00 P.M.

Airpuff startle

To assess HPA axis responsiveness, the plasma corticosterone response to airpuff startle was measured ≥14 days after formalin testing. While in their home cage, the rats were habituated to a room (not the pain testing room) for 30 min on two occasions before 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 45 s, the distal 2 mm of the tail was excised with a scalpel. The tail was milked, and 0.3 ml of blood was collected. The procedure was repeated 30 and 120 min after airpuff without further excision of the tail. All blood samples were collected between 9:00 A.M. and 12:30 P.M.

Airpuff startle (Engelmann et al. 1996) was administered immediately after the first sampling of blood. The unrestrained rat was placed in an empty 48 × 25 × 20-cm shoebox cage without a lid. Three sets of airpuffs were directed toward the side of the head of the rat from ~15 cm. Each set consisted of three 5-s air blasts from a pressurized air can (Kensington Dust Blaster), and each air blast was separated by a 10-s interval. A 1-min interval separated each set of three airpuffs. The rat was then returned to its home cage.

Blood samples were collected directly into a microcentrifuge tube containing 5 µl of heparin (1,000 IU/ml). The tube was centrifuged at 2,000 rpm for 15 min at 4°C. The plasma was drawn off, immediately frozen on dry ice, and stored at −70°C until the assay was performed. Corticosterone assays were performed by standard radioimmunoassay (ICN Biomedicals) on five plasma samples per group. The intra-assay and interassay CVs were 5.6 and 7.4%, respectively.

AIA

At least 7 days after airpuff startle, complete Freund’s adjuvant (1.0 mg Mycobacterium butyricum/300 g; 10 mg/ml paraffin oil; Difco) was injected subcutaneously at the base of the tail of rats anesthetized with 2.5 mg/kg acepromazine and 75 mg/kg ketamine. Polyarthritis was induced and examined instead of monoarthritis after intraplantar injection of adjuvant because the former is considered to have a better fit of the model.

### Statistical analyses

For the tail flick test, the formalin test, and airpuff startle, ANOVAs with repeated measures (where appropriate) were performed using the factors of Strain (Lewis, Fischer), sex (male, female), maternal separation (MS180, MS15, Control), and time (where appropriate). A significance level of $P < 0.05$ was used, with Bonferroni corrections made for the number of post hoc comparisons made. Parametric analyses were used for the formalin pain behaviors because the metric used has been shown to have interval properties in the formalin test when the composite mean pain score (a weighted sum of durations) is calculated as described above (Watson et al. 1997). Because the scale of measurement of symptom severity is an ordinal scale, we used ordinal logistic regression. Logistic regression applies maximum likelihood estimation after transforming the dependent into a logit variable (the natural log of the odds of the dependent occurring or not). In this way, logistic regression estimates the probability of a certain event occurring, with calculation of changes in the log odds of the dependent value. For percent increase in ankle diameter, ANOVA was used after logarithmic transformation of the data, which produced a better fit of the model.

### RESULTS

#### Tail flick test

Analyzed as the mean of three trials (Fig. 1), Fischer females had significantly lower mean latencies than Fischer males (2-way ANOVA: $F_{1,54} = 6.76; P = 0.01$). Regardless of sex, Lewis rats had lower mean latencies than Fischer rats (post hoc ANOVA: $F_{1,54} = 104.0$ and 40.8 for males and females, respectively; $P < 0.001$ for each sex). In Lewis rats, although there was a significant sex × maternal separation interaction ($F_{2,54} = 7.54; P = 0.001$), none of the pairwise comparisons of maternal separation groups were significant ($P > 0.05/10$ post hoc tests). Thus overall there was no effect of maternal separation. When analyzed with trial as a repeated-measures factor (Fig. 2), latencies increased with trial only in Lewis rats ($F_{2,108} = 7.95; P < 0.001$). In Fischer rats, all but one group showed stable or slightly decreasing latencies.

A striking difference in vocalizations emitted during the tail flick test occurred between Lewis and Fischer rats. Of the 60 Lewis rats, 87% vocalized audibly in at least one of the three trials, 50% in all three trials (22 of these 30 were females), and 26 males and 25 females (of 30) vocalized in the third trial (vs. 14 males and 23 females in the 1st trial). Lewis rats from all maternal separation groups vocalized. Only 1 (2%) of the 60 Fischer rats vocalized audibly in the tail flick test.
Formalin test

There was no effect of maternal separation ($F_{2,108} = 1.65; P = 0.20$) on formalin pain responses (Fig. 3). Surprisingly, Lewis rats displayed less pain behavior than Fischer rats during the “inflammatory,” second phase of the formalin response at 50, 55, and 60 min after formalin injection and in the interphase period at 10 and 15 min in females (post hoc 1-way ANOVA for each sex: $F_{1,58} = 12.3; P = 0.001$). Sex differences were observed only in Fischer rats in the first phase of the formalin response at 5 min and during the interphase depression at 10 and 15 min after formalin injection (post hoc 1-way ANOVA: $F_{1,58} > 9.01; P < 0.002; \alpha = 0.05/24$ post hoc tests). In males only, Lewis rats showed significantly more pain behavior than Fischer rats at 5 min after injection, although the effect size was very small (post hoc 1-way ANOVA: $F_{1,58} > 12.0; P = 0.001$). The results at 5, 10, and 60 min were confirmed with post hoc nonparametric statistics (Mann-Whitney $U$ tests); however, Watson et al. (1997) have shown that the composite mean pain score (a weighted sum of durations) used here has interval properties, permitting parametric analyses.

Airpuff startle

Baseline corticosterone levels in males were comparable, or slightly less than, baseline levels reported with sampling from indwelling jugular catheters of Sprague-Dawley rats, 50 ng/ml (Engelmann et al. 1996). Corticosterone levels returned to values similar to baseline 120 min after the tail excision, suggesting that the sampling procedure had little effect on corticosterone levels. For baseline corticosterone levels, there was no effect of maternal separation or strain (Fig. 4). Females had significantly higher baseline corticosterone levels than males (2-way ANOVA: $F_{1,24} = 27.4$ and 9.77 for Lewis and Fischer rats, respectively; $P = 0.005, P < 0.001$), as reported previously (Chisari et al. 1995).

For corticosterone responses to airpuff startle, there was no effect of maternal separation. There was a significant interaction of strain $\times$ sex ($F_{1,48} = 5.76; P = 0.02$): in males, Lewis rats had lower areas under the curve (AUCs) than Fischer rats (post hoc 1-way ANOVA: $F_{1,24} > 5.90; P = 0.02$); in Lewis rats only, females had greater AUCs than males (ANOVA: $F_{1,24} > 37.1; P < 0.001$).

AIA

On the 21st day after adjuvant injection (Fig. 5), Lewis rats had higher symptom severity than Fischer rats (ordinal logistic regression; $P < 0.001$; odds ratio = 7.64), and there was no effect of sex or maternal separation (except MS15 vs. Control in male Fischer rats, $P = 0.038$, odds ratio = 12.8, nonsignificant with 10 post hoc ordinal logistic regressions; and a significant difference between male and female Fischer MS15
rats, $P = 0.002$, odds ratio = 3.76). The same pattern of results was obtained when the 11-point scale was converted to a 4-point scale, with 0 = 0; 1 = 1–3; 2 = 4–6; 3 = 7–10 (effect of strain: $P < 0.001$, odds ratio = 8.62), suggesting that the results are not idiosyncratic to the 11-point scale.

On symptom severity from days 15 to 20 (Fig. 6), Lewis rats had higher symptom severity than Fischer rats ($P = 0.002$; odds ratio = 5.87), and within Fischer rats, females had higher symptom severity than males ($P < 0.001$; odds ratio > 6.05). There was no effect of maternal separation ($P > 0.05$), and an effect of sex in Fischer rats ($F_{1,54} = 14.6$ and 12.1 for left and right paws, respectively; $P = 0.001$, $P = 0.001$), and an effect of strain in males ($F_{1,54} = 17.4$ and 14.0 for left and right paws, respectively; $P < 0.001$, $P = 0.001$). A total of four post hoc GLM analyses were performed ($\alpha = 0.05/4$). These results are similar to the pattern of results observed from days 15 to 20 for symptom severity.

**DISCUSSION**

Maternal separation has been shown to affect HPA axis responsiveness and other measures in adulthood in Long-Evans, Sprague-Dawley, and Wistar strains of rat (Huot et al. 2000; King and Edwards 1999; Lehmann et al. 2000; Plotsky and Meaney 1993), but it is not appreciated that other rat strains may be resistant. In this study, early postnatal maternal separation of Lewis and Fischer rats had no statistically significant effect on the measures examined (with only 1 exception for 2 subgroups), strongly suggesting that the two strains are resistant and that sensitivity to maternal separation is highly dependent on genetic factors of rat strain, sex, and species. Sparse results from somewhat related models of maternal separation (for 24 h on postnatal day 9) or handling (for 3 min/day until postnatal day 21) showed that, whereas Wistar rats are susceptible to long-term effects of the former, Lewis and Fischer rats are not, and Fischer rats are not affected by the latter (Amkraut et al. 1971; Ellenbroek and Cools 2000).
Thermal sensitivity is not affected by maternal separation (for 2 h/day until weaning) in Fischer rats and is only slightly affected in female Lewis rats in the hot plate test but not in the tail flick test (examined only in the Lewis rat) (Stephan et al. 2002). In female Long-Evans rats, but not in males, maternal separation (for 15–20 min/day from postnatal days 1 to 14) elevates paw lick latencies in the hot plate test (Smythe et al. 1994). Finally, in the mouse, maternal separation (15 min/day from postnatal days 1 to 13) reduces sensitivity in the radiant heat tail flick test and the formalin test (D’Amato et al. 1999). Thus it is most likely caused by genetic factors (of strain and species) that the Fischer and Lewis rat strains are resistant to the effects of maternal separation on HPA axis responsiveness and on the pain and inflammation measures in this study.

Congruent with earlier findings from AIA and other prolonged inflammatory models (Holmdahl 1995; Karalis et al. 1995; Lariviere and Melzack 1997; Misiewicz et al. 1996; Sternberg et al. 1989a,b; Wilder 1993; Wilder et al. 1982), we found Lewis and female rats to be more susceptible than Fischer and male rats to AIA, respectively. This study found that lower basal plasma corticosterone and corticosterone responses to an acute stressor were not associated with increased AIA severity in the adult rat. Specifically, baseline corticosterone was not different between strains, and females showed higher levels despite their increased AIA symptoms and severity. With respect to corticosterone responses to airpuff startle, higher responses were observed in the more susceptible female Lewis rats compared with male Lewis rats, and there were no sex differences in Fischer rats, despite clear sex differences in AIA measures in that strain. Combined with the demonstration of stressor-specific strain differences between Lewis and Fischer rats (Spinedi et al. 1994), these data support that non-specific HPA axis responsiveness is not likely solely responsible for differences in AIA susceptibility and that other mechanisms must be involved.

The clearest pattern of association with increased AIA severity observed in this study is with sensitivity in the tail flick test. The more susceptible Lewis rats are more sensitive in the tail flick test, and sex differences in both responses are more robust in Fischer rats than in Lewis rats. Pain-related neural mechanisms have been shown to be involved in the development of AIA, including capsaicin-sensitive high-threshold and other peripheral afferent fibers, efferent fibers of the sympathetic nervous system, spinal, and even supraspinal mechanisms (Colpaert et al. 1983; Cruwys et al. 1995; Donaldson et al. 1995; Levine et al. 1985a,b,c, 1986). Pain sensitivity in the tail flick test is proposed to be mediated in large part by spinal mechanisms and peripheral afferent fibers (Carstens 1996);
thus it is possible that strain and sex differences in the afferents, efferents, and spinal loops contribute to the differences in both the sensitivity in the tail flick test and AIA symptom severity.

Paradoxically, the Lewis rats exhibited less pain behavior in the formalin inflammatory pain test, and there were no sex differences in either strain during the second phase of the formalin test, which is associated with significant inflammation (Lariviere and Melzack 1996; Taylor et al. 2000; Wheeler-Aceto and Cowan 1991; Yashpal andCoderre 1998). In what is the first reported direct comparison of acute inflammatory nociception between Lewis and Fischer strains, our data show that the differences in AIA severity of symptoms are not simply reflective of a nonspecific response to painful inflammatory stimuli. This study remained necessary because a related study found greater thermal hyperalgesia induced by intraplantar injection of complete Freund’s adjuvant in male Fischer rats compared with male Lewis rats (Zhang et al. 2003), but the observed hyperalgesia is confounded by a floor effect in the hyperalgesia measure combined with decreased baseline thermal sensitivity of the Fischer rats.

The formalin test results of this study are also important because the strain differences in baseline inflammatory nociception, and not differences in HPA axis responsiveness, may underlie reported differences in morphine analgesia and tolerance observed between Lewis and Fisher rats (Vaccarino and Couret 1995), because baseline sensitivity and analgesic potency have been shown to be negatively correlated in inbred strains of mice (Wilson et al. 2003). Similarly, baseline thermal nociceptive sensitivity may also underlie reported differences in nitrous oxide antinociception between these strains (Fender et al. 2000).

Interestingly, Lewis rats were consistently less sensitive on repeated trials of tail flick testing, usually vocalizing audibly (87% of the time), whereas Fischer rats’ sensitivity generally remained unchanged or increased with repeated testing and did not vocalize audibly. In the formalin test, Lewis rats responded significantly less in the second phase. This suggests that the strains may differ integrally in their handling of repeated or prolonged painful stimuli. Although decreased pain responding may seem desirable or advantageous, it is becoming apparent that there is a constant balance between excitatory mechanisms from the periphery and descending inhibitory mechanisms controlling the ability of further input to produce pain (Straub and Cutolo 2001), comprising a negative feedback mechanism of inflammatory responses as has been proposed to contribute to these strains’ differences (Zhang et al. 2003). For instance, electrical stimulation of the rat hind paw at intensities that excite C-fibers inhibits bradykinin-induced plasma extravasation in the knee joint, an effect that requires an intact neuraxis and an intact HPA axis, because it is inhibited by thoracic spinal cord transection, hypophysectomy, and adrenalectomy (Green et al. 1995). Thus it is possible that with repeated or continuous stimulation, Lewis rats produce less of an afferent response to the stimulus, which in turn may produce less activation of descending negative feedback mechanisms to inhibit inflammatory responses.

Lewis rats have also shown that they respond more passively to stressors such as a noise stress or swim stress, freezing instead of actively behaving as the Fischer rat does (Metz et al. 2001; Michaud et al. 2003). However, tail flick responding is a mostly spinal reflex, whereas freezing is a coordinated behavior, and Lewis rats do behave during the second phase of the formalin test; that is, they do not freeze. Future studies using measures of neural activity in peripheral afferent fibers and spinal cord neurons are needed to test this alternative hypothesis.

In conclusion, this study suggests that, although postnatal experience with stress is currently viewed to lead to long term detrimental effects, genetic influences must be taken into consideration as a potent mitigating factor. Furthermore, although there are clearly effects of chronic inflammation on the HPA axis and vice versa, circulating corticosterone may not be a major determining etiological factor. In combination with previous experimental studies, our results suggest that mechanisms underlying inherent responses to repeated or prolonged painful stimuli may contribute to the severity of AIA, a rheumatoid arthritis model, and that the identification and further study of the therapeutic value of manipulating these mechanisms are warranted. Finally, these data further characterize the pain phenotypes of this important pair of inbred rat strains for the study of chronic inflammatory pain, providing a stronger basis for the interpretation of previous and future studies of chronic inflammatory pain and analgesia mechanisms.
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