Peripheral immune stimulation such as that provided by lipopolysaccharide (LPS) has been reported to increase brain levels of IL-1β mRNA, immunoreactivity, and bioactivity. Stressors produce many of the same neural and endocrine responses as those that follow LPS, but the impact of stressors on brain interleukin-1β (IL-1β) has not been systematically explored. An ELISA designed to detect IL-1β was used to measure levels of IL-1β protein in rat brain. Brain IL-1β was explored after exposure to inescapable shock (IS; 100 1.6 mA tail shocks for 5 sec each) and LPS (1 mg/kg) as a positive control. Rats were killed either immediately or 2, 7, 24, or 48 hr after IS. Brains were dissected into hypothalamus, hippocampus, cerebellum, posterior cortex, and nucleus tractus solitarius regions. LPS produced widespread increases in brain IL-1β, but IS did not.

Adrenal glucocorticoids are known to suppress IL-1β production in both the periphery and brain. Thus, it was possible that the stressor did provide stimulus input to the brain IL-1β system(s), but that the production of IL-1β protein was suppressed by the rapid and prolonged high levels of glucocorticoids produced by IS. To test this possibility rats were adrenalectomized or given sham surgery, with half of the adrenalectomized rats receiving corticosterone replacement to maintain basal corticosterone levels. IS produced large increases in brain IL-1β protein in the adrenalectomized subjects 2 hr after stress, whether basal corticosterone levels had been maintained. Thus elimination of the stress-induced rise in corticosterone unmasked a robust and widespread increase in brain IL-1β.

**Key words:** interleukin-1β; stress; brain; glucocorticoids; protein; rat
and escape learning failure that follows exposure to inescapable tail shock (Maier and Watkins, 1995).

There has been little work directed at determining whether stressors actually alter brain IL-1. Immobilization has been reported to increase IL-1β mRNA (Minami et al., 1991) and bioactivity (Shintani et al., 1995b). However, the generality of these results is unknown, and factors that might determine these brain IL-1 changes have not been studied systematically. Here, we sought to determine whether a different stressor, inescapable tail shock, would increase brain protein levels of IL-1β.

MATERIALS AND METHODS

Subjects. Adult male pathogen-free Harlan Sprague Dawley (Indianapo-

lis, IN) rats (300–350 gm) were individually housed in metal cages at 25°C with a 12 hr light/dark cycle (lights on at 7:00 A.M.). Subjects were acclimated to the colony for 14 d before experimentation began. Standard rat chow and water were freely available. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Inescapable tail shock treatment. All rats were handled and weighed 2 d before each study began. Animals either remained undisturbed in their home cages as controls (HCC) or were exposed to inescapable tail shock (IS). The IS protocol involved placing the rats in a Plexiglas restraining tube (23.4 cm long and 7 cm in diameter) and exposing them to 100 1.6 mA inescapable shocks for 5 sec each, with an average intertrial interval of 60 sec. The shocks were applied through electrodes taped to the tail. The animals were stressed between 8:00 and 10:00 A.M. and, after stressor termination, were returned to their home cages. Cages of stressed rats were placed on the opposite side of the room (14 x 12 ft) from the HCC animals.

Adrenalectomy. Bilateral adrenalectomies (ADX) were aseptically performed under halothane anesthesia (Halocarbon Laboratories, River Edge, NJ). All removed tissue was examined immediately to ensure complete removal of the adrenal gland. Sham-operated animals received the identical procedure, except that the adrenal glands were gently manipulated with forceps but not removed. Steroid replacement began for ADX animals immediately after surgery. ADX animals received corticosterone (CORT) replacement in their drinking water, because this method has been shown to mimic the normal circadian pattern of CORT secretion (Jacobson et al., 1988). Corticosterone (Sigma, St. Louis, MO) was initially dissolved in ethyl alcohol (ETHO) and diluted to a final concentration of 25 μg/ml in 0.2% ETHO. CORT-water also contained 0.5% saline. Sham animals received drinking water containing 0.2% ETHO. Animals were allowed 6 weeks to recover before any experiments were performed.

LPS treatment. LPS (Escherichia coli, 0111:B4; Sigma) was injected intraperitoneally at doses ranging from 1 μg/kg to 1 mg/kg dissolved in sterile, endotoxin-free 0.9% saline vehicle. Control injections were equal volumes of saline vehicle, with all injections given between 7:30 and 8:00 A.M. Animals were returned to their home cage and killed 6–7 hr later. These time intervals were chosen because increases in both mRNA and bioactivity for IL-1 have been reported 2–6 hr after LPS (Fontana et al., 1984; Buttini and Boddeke, 1995; Goujon et al., 1995).

Perfusion. To verify whether the IL-1β measured was from blood or tissue in the CNS, animals were perfused with 0.9% saline before they were killed. In the same experiment, perfused rats were compared with nonperfused rats, and levels of IL-1β protein were measured in the hippocampus, hypothalamus, and posterior cortex.

Tissue collection. Animals were anesthetized with a brief exposure to ether, and brains were quickly removed after decapitation. All dissec-

tions were performed on a frosted glass plate placed on top of crushed ice. The brain structures were quickly frozen on dry ice. Brain samples, which included hypothalamus, hippocampus, cerebellum, posterior cortex, and nucleus tractus solitarius, were stored at −70°C until the time of sonication.

Tissue processing. Each tissue was added to 0.5–1.0 ml of ice-cold culture medium containing 5% fetal calf serum and a cocktail enzyme inhibitor (in mM: 100 amino-n-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl fluoride). Total protein was mechanically dissociated from tissue using an ultrasonic cell disruptor (Heat Systems, Inc., Farmingdale, NY). Sonication consisted of 10 sec of cell disruption at the setting 10. Sonicated samples were centrifuged at 10,000 rpm at 4°C for 10 min. Supernatants were removed and stored at 4°C until an ELISA was performed. Bradford protein assays were also performed to determine total protein concentrations in brain sonication samples.

ELISA. Sheep anti-rat IL-1β immunoaffinity-purified polyvalent polyclonal antibo-
dy (Ab) was provided by Dr. Steven Poole (National Institute for Bio-

gical Standards and Control, Hertfordshire, England). The microtiter plates (96 flat-bottom wells, Immulon Maxisorp, Nunc, Roskilde, Den-
mark) were coated with this Ab overnight at 4°C. After washing the plates in assay buffer (0.01 M phosphate, 0.05 M NaCl, and 0.1% Tween 20, pH 7.2), 100 μl of rat IL-1β standards or samples was added to each well and incubated at room temperature for 4 hr. After washing the plates, 100 μl of biotinylated, immunoaffinity-purified polyclonal sheep anti-rat IL-1β Ab (1:2000) with 1% normal goat serum was added to each well and incubated at room temperature for 1 hr. The color was developed by use of avidin–HRP (Vector Laboratories, Burlingame, CA) and the chromogen ortho-

phenylene diamine (Sigma). Plates were read at 490 nm, and results are expressed as picograms of IL-1/100 μg of total protein. The detection limit of this assay was 10 pg/ml. A commercially available rat IL-1β ELISA kit (R & D Systems, Minneapolis, MN) was also used to verify that similar results can be obtained using a different Ab to rat IL-1β. This ELISA kit uses a goat anti-rat IL-1β polyclonal Ab that can recognize both recombinant as well as natural rat IL-1β. No significant cross-reactivity was ob-

served with the R & D Ab to recombinant human (r-human) IL-1α, IL-1β, IL-1ra, IL-1α, IL-2, and IL-4. Interference by tumor necrosis factor-α, and r-mouse IL-1α and IL-1ra. This R & D kit had a detection limit of 5 pg/ml.

Verification of rat IL-1β Ab specificity. The rat IL-1β Ab was tested for specificity to rat IL-1β by ECL Western blot analysis, ELISA on Seph-

adex column separation of brain sonicates, and immunoprecipitation. Western blot analysis consisted of loading ~150 μg of total protein onto a 15% polyacrylamide gel (20 cm x 12 cm, 0.75 mm thick) running for 90 min at 120 V. The proteins were then transferred onto a nitrocellulose membrane at 25 V for 90 min and blocked in 5% nonfat milk for 1 hr. The nitrocellulose blot was incubated in the sheep anti-rat IL-1β immunoaffinity-purified polyclonal Ab overnight at 4°C. After washing in Tris-buffered saline, the secondary Ab (rabbit anti-sheep IgG with HRP; Western Blotting Systems, Seattle, WA) was added and incubated at room temperature for 1 hr. Enhanced chemilu-

minescence reagents (Amersham, Buckinghamshire, England) were added, and the nitrocellulose blot was exposed to x-ray film. Dark bands were detected on the film, showing where the secondary Ab was bound to the sheep anti-rat IL-1β Ab. A molecular weight standard ladder (Life Technologies, Gaithersburg, MD) ranging from 43 to 3 kDa was also used to verify the molecular weights of the protein bands that were detected by the rat IL-1β Ab. A detectable band occurred at ~17 kDa, the approximate molecular weight of IL-1β. However, a second band was also present at 33 kDa, the approximate molecular weight of the IL-1β prohormone.

Total protein from the brain sonication supernatant was also separated according to the molecular weight of each protein on a Sephadex G-50 column. The Sephadex G-50 was packed into a 10 ml serological pipette according to the molecular weight of each protein on a Sephadex G-50 column separation of brain sonicates, and immunoprecipitation. Western blot analysis consisted of loading ~150 μg of total protein onto a 15% polyacrylamide gel (20 cm x 12 cm, 0.75 mm thick) running for 90 min at 120 V. The proteins were then transferred onto a nitrocellulose membrane at 25 V for 90 min and blocked in 5% nonfat milk for 1 hr. The nitrocellulose blot was incubated in the sheep anti-rat IL-1β immunoaffinity-purified polyclonal Ab overnight at 4°C. After washing in Tris-buffered saline, the secondary Ab (rabbit anti-sheep IgG with HRP; Western Blotting Systems, Seattle, WA) was added and incubated at room temperature for 1 hr. Enhanced chemilu-

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Verification of adrenalectomy. Whole trunk blood was collected by decapitation after a brief exposure to ether when rats were killed. Whole blood was allowed to clot overnight at 4°C, and serum was separated by
Effects of inescapable tail shock on brain IL-1β protein

Rats were exposed to IS and killed either immediately (n = 6) or 2 hr (n = 6), 7 hr (n = 6), 24 hr (n = 6), or 48 hr (n = 6) later. The immediate and 24 and 48 hr subjects were all killed at 10:00 A.M. because the stress session was held constant at 8:00–10:00 A.M. Thus, these groups required only a single home cage control group (HCC) killed at 10:00 A.M. Separate controls were also killed at 12:00 and 5:00 P.M. to match the times for the 2 and 7 hr IS groups. Only hypothalamus (Fig. 3A) and hippocampus (Fig. 3B) were examined. As can be seen, IS did not alter levels of IL-1β protein in either region [F(1,9) = 0.755; p > 0.05 for the hypothalamus; F(1,91) = 1.762; p > 0.05 for the hippocampus]. Interestingly, IL-1β protein levels varied with time of day [F(4,40) = 4.45; p < 0.005 for the hypothalamus; F(4,36) = 8.492; p < 0.0001 for the hippocampus]. Figure 3C shows this variation for HCC subjects in the hippocampus. Levels of IL-1β were highest at 10:00 A.M. near the beginning of the sleep cycle and lowest at 5:00 P.M. near the end of the sleep cycle [F(2,15) = 7.445; p < 0.01].

Effects of adrenalectomy on IS-induced increase in brain IL-1β protein

Adrenal corticosteroids inhibit IL-1β gene transcription (Lee et al., 1988; Amano et al., 1992). IS produces a rapid and prolonged rise in corticosteroids (Fleschner et al., 1995), and so it is possible that the corticosteroid response to IS masks possible alterations in IL-1β protein levels. To assess this possibility, rats were adrenalectomized or given sham surgery. Half of the adrenalectomized subjects received basal CORT replacement, and half did not. Figure 4A and B, shows brain IL-1β protein 2 hr after IS or HCC treatment in hypothalamus and hippocampus, respectively. With respect to hypothalamus, IS again had no effect on adrenal-intact subjects. However, IS produced a robust increase in IL-1β in adrenalectomized subjects, whether basal CORT was replaced [F(2,24) = 7.794; p < 0.005]. The pattern in hippocampus was similar, with the exception that the IL-1β increase produced by IS was only prominent in the adrenalectomized and basal CORT-replaced subjects [F(2,24) = 13.796; p < 0.0001].

These data were obtained using Ab from Dr. S. Poole. The implications of these data were sufficiently important that the experiment was repeated, and ELISAs were performed using the
Furthermore, cerebellum, nucleus tractus solitarius, and posterior cortex were analyzed in addition to hypothalamus and hippocampus. Results from hypothalamus (Fig. 5A) and hippocampus (Fig. 5B) were similar to those obtained using Dr. Poole’s Ab. IS increased IL-1\textsubscript{b} protein in adrenalectomized but not sham subjects. Again this increase occurred in both CORT-replaced and nonreplaced subjects at 2 hr after IS. Large increases were also observed in cerebellum (Fig. 5C) and nucleus tractus solitarius (Fig. 5D). In contrast, IS had no effect in posterior cortex (Fig. 5E) under any of the conditions [\(F_{(2,38)} = 5.777; p < 0.01\) for hypothalamus; \(F_{(2,41)} = 6.277; p < 0.005\) for hippocampus; \(F_{(1,42)} = 3.78; p < 0.05\) for cerebellum; \(F_{(1,40)} = 9.359; p < 0.0005\) for nucleus tractus solitarius; and \(F_{(1,37)} = 0.673; p > 0.05\) for posterior cortex].

Verification of adrenalectomy and corticosterone replacement

Adrenalectomy was verified in the last IS experiment by measuring serum CORT after a brief ether exposure, which has been shown to rapidly activate the hypothalamo–pituitary–adrenal (HPA) response. Adrenalectomy eliminated the CORT response to ether [\(F_{(2,45)} = 37.001; p < 0.0001\) (data not shown)]. The CORT replacement procedure was verified by examining thymus weight relative to total body weight of each animal. Given that the thymus gland is very sensitive to levels of circulating CORT, adrenalectomy should cause thymic hypertrophy, because no corticosterone is present to influence lymphocyte maturation in the thymus. If the corticosterone replacement therapy in the adrenalectomized rats mimics natural basal CORT levels, a normalization of thymus weight would be expected. Adrenalectomy caused a significant increase in thymus weight, and this increase was normalized in the adrenalectomized group that received CORT replacement [\(F_{(2,45)} = 22.517; p < 0.0001\)] compared with sham-operated controls (data not shown).

**DISCUSSION**

The purposes of the present experiments were to determine whether IL-1\textsubscript{b} protein could be detected in rat brain under basal nonstimulated conditions, and whether a stressor would alter these levels. The ELISA was adopted as the measurement technique, because Ab to rat IL-1\textsubscript{b} polyclonal Ab from Dr. S. Poole was available, and ELISAs are quite sensitive. Specificity of measurement is an issue with virtually all techniques designed to measure protein levels and is particularly problematic for Ab-based procedures. It is straightforward to determine whether an Ab recognizes the protein of...
interest, but it is not easy to prove that the epitope that it recognizes is not also contained on other proteins. To test Ab specificity for rat IL-1β, several approaches were used. First, Western blot showed a strong band at the molecular weight of IL-1β and only one other faint band. This band was at the molecular weight of the prohormone for IL-1β. Thus it may be that the data obtained partially reflect increases in the prohormone. This must simply be acknowledged, and the reader should bear this in mind. Second, the ELISA detected as much IL-1β in a fractionated sample as in the whole sample, suggesting that the predominant molecule measured was indeed mature IL-1β. Third, the Ab precipitated rat IL-1β in an immunoprecipitation procedure. Fourth, the Poole Ab has been studied for cross-reactivity with a large number of other proteins (Garabedian et al., 1995), and none was found. Finally, similar results were obtained with two different Abs.

To further address this issue, we next sought to determine whether the ELISA measured increases in brain IL-1β protein under conditions in which IL-1β increases have been detected with other measurement procedures. The peripheral administration of large doses of LPS has been reported to increase brain IL-1β bioactivity (Fontana et al., 1984; Coceani et al., 1988; Quan et al., 1994), immunoreactivity (Van Dam et al., 1992; Hagan et al., 1993; Hillhouse and Mosley, 1993), and mRNA (Ban et al., 1992; Buttini and Boddeke, 1995; Laye et al., 1994). A very large dose of LPS (1 mg/kg) was chosen as a positive control in the first experiment, not to test whether brain IL-1β is involved in mediating the usual effects of LPS. Lower doses of LPS (1–100 μg) were also examined using the R&D ELISA kit, which uses a different Ab specific to rat IL-1β. LPS did produce large increases in IL-1β protein in the brain, as measured by this ELISA as well. As with other measurement techniques, this increase appeared to be regionally nonspecific. In addition, brain IL-1β mRNA has recently been reported to have a circadian rhythm (Taishi et al., 1997) that is roughly the inverse of the glucocorticoid rhythm. IL-1β protein was therefore measured here at different times of the day, and a circadian rhythm was also obtained in which IL-1β protein levels were highest near the beginning of the light period and lowest near the dark period. This circadian rhythm is entirely in keeping with the proposal that brain IL-1 plays a physiological role in sleep regulation (Opp and Krueger, 1991).

In sum, all of the results were consistent with the proposition

![Figure 5. A–E, Effects of adrenalectomy on levels of IL-1β protein in the hypothalamus (A), hippocampus (B), cerebellum (C), nucleus tractus solitarius region (D), and posterior cortex (E) at 2 hr after the end of the stress session. IL-1β protein results were obtained using the R & D ELISA kit. Sham HCC, n = 8; sham IS, n = 6; ADX HCC, n = 6; ADX IS, n = 8; ADX-cort HCC, n = 8; ADX-cort IS, n = 8.](image-url)
that the ELISA detected IL-1β protein and was sensitive to changes in IL-1β protein levels. The detection of IL-1β protein in the undisturbed animal is consistent with the careful work of Quan et al. (1996) showing IL-1β bioactivity in undisturbed animals. IL-1β specificity was verified in their bioassay system by demonstrating that the measured bioactivity was completely blocked by the IL-1 receptor antagonist. It must be noted that IL-1β mRNA has often been undetectable under basal conditions (Gatti and Bartfai, 1993), but IL-1β protein may be a more sensitive index of basal IL-1β in brain, because IL-1β mRNA may only be transcribed after IL-1β has been recently released or used. In addition, IL-1β mRNA may not be an abundant brain mRNA and therefore may be difficult to detect, relative to background, without amplification such as that provided by reverse transcription-PCR (Laye et al., 1994; Taishi et al., 1997). Furthermore, glucocorticoids specifically reduce IL-1β mRNA stability (Lee et al., 1988; Amano et al., 1992), thereby rendering IL-1β mRNA difficult to detect under a variety of conditions.

Inescapable shock had no detectable effect on IL-1β protein levels measured immediately and 2, 7, 24, or 48 hr after exposure to the stressor. However, glucocorticoids are known to potentially suppress IL-1β transcription and mRNA stability (Lee et al., 1988), and IS produces a rapid increase in glucocorticoid levels that persist for roughly 1 hr after the termination of the stressor (Fleshner et al., 1995). Thus, the stressor might have provided input that would normally increase brain IL-1β signal, but any potential increases might have been inhibited by the glucocorticoids that are simultaneously stimulated. Indeed, Goujon et al. (1995) reported that adrenalectomy enhanced brain IL-1β mRNA increases produced by peripheral administration of LPS. Although an increase in IL-1β protein was not detected in adrenal-intact animals after stress, an effect of stress on brain IL-1β may nevertheless still exist. Stress could easily produce a small increase in IL-1β that is not detectable by ELISA, and small amounts of IL-1β may have biological effects given its potency. In addition, stress could modulate pro-IL-1 production or increase interleukin-1β-converting enzyme activity, changes that may occur at a later time than assayed here or be unmeasurable by the ELISA used in these studies.

The effects of IS on brain levels of IL-1β protein were therefore examined in adrenalectomized subjects. Indeed, adrenalectomy unmasked a stress-induced increase in brain IL-1β protein 2 hr after the stressor in both hypothalamus and hippocampus. This increase was also observed in adrenalectomized animals that were given basal corticosterone replacement, supporting the argument that it was the stress levels of glucocorticoids, rather than basal steroid, that inhibited the stress-induced production of brain IL-1β protein in the previous study. Interestingly, the IL-1β increase was regionally specific, with no detectable increase in the posterior cortex. The effects of immobilization observed by Minami et al. (1991) were also regionally specific, with increases in mRNA for IL-1β present in the hypothalamus but not cerebellum or midbrain. The brain regions examined in the present experiments were chosen based on previous observations implicating them in stress responses and peripheral immune challenge. The hypothalamus is a critical site mediating responses to IL-1β such as HPA activation and fever induction (Sapolsky et al., 1987; Klir et al., 1994). The cerebellum and the posterior cortex were chosen as control regions, largely because they have not been considered as important brain sites in the development of stress responses.

Although the source of brain IL-1β was not addressed in the present studies, several experiments have examined this issue. Human fetal microglia, as well as rat microglia, have been shown to express mRNA for IL-1β after peripheral LPS (Lee et al., 1993; Buttini and Boddeke, 1995). Immunoreactivity has also been found for IL-1β in ramified microglia and endothelial cells lining the venules (Van Dam et al., 1995). Furthermore, in situ hybridization has detected IL-1β mRNA for IL-1β in murine brain microvessel endothelial cells (Fabry et al., 1993). Although controversial, a neural source of IL-1β has also been reported (Moelenar et al., 1993; Pestarino et al., 1997).

The present data suggest a parallel between inflammatory stimuli and stressors, a parallel that has been noted often (Dunn, 1995). Similarities between the neurochemical, endocrine, and behavioral sequelae of immune challenge and stressors have been described frequently (Maier and Watkins, 1998). The physiological and psychological effects of immune challenge can resemble those caused by stressors. Influenza viral infection has been shown to increase plasma corticosterone and brain catecholamine metabolism (Dunn et al., 1989), effects that are also observed in response to stress (Shintani et al., 1995b). Yirmiya (1995) has recently reported depressive-like behaviors in rats after an endotoxin challenge that include reduced social interaction, food consumption, and locomotor activity, all of which have been implicated as responses to stressors (Johnson et al., 1992). The present data add one more parallel, namely, the production of increases in brain IL-1β. As with other sequelae of immune stimulation and stress, the IL-1β changes showed a family resemblance, not an identity. The regional distribution of IL-1β changes were not the same in the two cases, just as patterns of neural activation are not identical after stress and immune challenge (Rivest et al., 1995). However, patterns of neural activation differ between stressors (Li et al., 1996), so identity is not to be expected.

Finally, an activation of IL-1 usage by the IS stressor used in the present studies is entirely in keeping with documented similarities between the effects of IS and the intracerebroventricular administration of IL-1β. Intracerebroventricular IL-1β produces reductions in social interaction (Propes and Johnson, 1997), reductions in food and water intake (Kent et al., 1994), suppressed immune peripheral immunity responses (Sundar et al., 1989), fever (Ovadia et al., 1989), and aspects of the acute phase response such as increases in acute phase proteins and decreases in negative reactants, such as carrier proteins (Morimoto et al., 1988; Morimoto et al., 1989). IS produces all of these (Laudenslager et al., 1988; Fleshner et al., 1992; Short and Maier, 1993), even prolonged fever, increases in circulating acute phase proteins, and decreases in circulating carrier proteins (Deak et al., 1997). It is important to note that all stressors do not produce these outcomes; they are specific to the stressor used. It may be that these outcomes are mediated by the induction of brain IL-1β, and that there will be selectivity in terms of which stressors do, and do not, alter brain IL-1β.

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