T-Lymphocyte Activation Increases Hypothalamic and Amygdaloid Expression of CRH mRNA and Emotional Reactivity to Novelty

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Stimulation of T-cells with staphylococcal enterotoxin B (SEB) significantly elevates interleukin-2 (IL-2) and contemporaneous activation of the hypothalamic–pituitary–adrenal (HPA) axis and *c-fos* in the paraventricular nucleus (PVN) of BALB/cByJ mice. Such neural signaling may promote cognitive and emotional adaptation before or during infectious illness. Because corticotropin-releasing hormone (CRH) is an anxiogenic neuropeptide that may mediate the stressor-like effects of immunological stimuli, we measured neuronal CRH mRNA alterations in mice challenged with SEB. Increased CRH mRNA levels were observed in the PVN and central nucleus of the amygdala (ceA) 4–6 hr after SEB administration. This was associated with plasma ACTH increases, which could be abrogated by the systemic administration of anti-CRH antiserum. Additional experiments did not support a role for IL-2 or prostaglandin synthesis in activating the HPA axis. Behavioral experiments testing for conditioned taste aversion did not confirm that SEB

challenge promotes malaise. However, consistent with the notion that central CRH alterations induced by SEB may affect emotionality (e.g., fear), SEB challenge augmented appetitive neophobia in a context-dependent manner, being marked in a novel and stressful environment. It is hypothesized that immunological stimuli generate a cascade of events that solicit integrative neural processes involved in emotional behavior. As such, these data support the contention that affective illness may be influenced by immunological processes and the production of cytokines and are consistent with other evidence demonstrating that autoimmune reactivity is associated with enhanced emotionality.

Key words: T-lymphocytes; corticotropin-releasing hormone; staphylococcal enterotoxin B; adrenocorticotrophic hormone; psychoneuroimmunology; emotion; neophobia; interleukin-2; cytokines; prostaglandins; stress; amygdala; hypothalamus

The nervous and immune systems share a mutually interactive relationship that promotes various forms of physiological and behavioral adaptations in the face of pathogenic challenges (Besedovsky and Del Rey, 1996). There is also evidence that neural and behavioral adjustments to infection and/or cytokine treatment may impose additional and potentially subversive demands on neurochemical resources supporting emotional and cognitive functions (Anisman and Zacharko, 1992; Dunn, 1993; Maier and Watkins, 1998). Therefore, immunologically provoked behavioral repertoires such as anorexia, lethargy, and sleep (Kent et al., 1992; Plata-Salaman, 1996; Maier and Watkins, 1998) may occur at some expense to psychological well being, as further suggested by neurochemical mapping studies identifying immune activation as a stressor that engages neural substrates of emotional reactivity (Anisman et al., 1993; Dunn, 1993; Haas and Schauenstein, 1997). In particular, amygdaloid and hypothalamic neurons synthesizing the anxiogenic neuropeptide, corticotropin-releasing hormone (CRH; Dunn and Berridge, 1990; Heilig et al., 1994), are highly responsive to immunological stimuli (Berkensbosch et al., 1987; Sapolsky et al., 1987; Laflamme et al., 1997),

raising the possibility that fundamental alterations in mood and cognition may occur independently of or before more obvious signs of infectious illness. In apparent support of this, immunological cytokines [e.g., interleukin-1 (IL-1)] and the bacterial endotoxin lipopolysaccharide (LPS) have been shown to reduce exploratory behavior in novel or precarious test environments (Spadaro and Dunn, 1990a; Dunn et al., 1991; Montkowski et al., 1997; Connor et al., 1998; Lacosta et al., 1998), in some cases dependent on central CRH receptors (Dunn et al., 1991) but in others influenced by depressive illness-like effects on locomotion (Montkowski et al., 1997). Additionally, mice progressing into developmentally regulated stages of autoimmune reactivity display increased emotionality (Schrott and Crnic, 1996, 1998). Finally, clinical trials designed to boost T-cell immunodeficiencies with cytokine immunotherapy (e.g., IL-2) have reported neuropsychiatric and neuropsychological side effects (Walker et al., 1996, 1997), and immune responses have been implicated in the etiology or exacerbation of affective disorders (Maes, 1995).

Circumvention of undesirable CNS effects by otherwise beneficial immunotherapeutic protocols, as well as immune disorders, requires greater preclinical knowledge of the neurobehavioral impact of immunological factors. Although efforts to achieve this goal have embraced models encompassing the full extent of the immunological repertoire, less attention has been given to the combined neural and behavioral impact of T-lymphocytes, which orchestrate most immune responses (London et al., 1998). A prominent model of T-cell activation *in vivo* uses the staphylococcal enterotoxins (SEs) produced by the Gram-positive bacteria *Staphylococcus aureus*, a major cause of food poisoning (John-

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son et al., 1991; Kotzin et al., 1993; Marrack et al., 1993). One of the best characterized is staphylococcal enterotoxin B (SEB), which *in vivo* elicits major production of interleukin-2 (IL-2), interferon- γ (IFN- γ), and tumor necrosis factor (TNF) by CD4⁺ T-cells, with peak concentrations of these cytokines occurring by 2 hr after challenge and returning to baseline levels several hours later (Bette et al., 1993; Gonzalo et al., 1993, 1994; Florquin et al., 1994; Litton et al., 1994). Challenge with SEB dose-dependently activates the hypothalamic–pituitary–adrenal (HPA) axis, with coincident immediate early gene induction in the paraventricular nucleus (PVN) of the hypothalamus (Shurin et al., 1997). Moreover, T-cell-derived IL-2 production follows closely the time course of plasma corticosterone elevations after challenge with not only SEB but also other SEs (Shurin et al., 1997).

Because CRH release from the PVN serves as the primary source of secretagogue action on pituitary corticotrophic cells (Owens and Nemeroff, 1991; Smith et al., 1998), it was hypothesized that central CRH mediated the effects of T-cell reactivity on HPA activation. The results of the present investigation revealed that increased adrenocorticotrophic hormone (ACTH) production after T-cell activation with SEB relied on CRH release, but not on circulating IL-2 or prostaglandin synthesis. Furthermore, SEB challenge enhanced central CRH mRNA levels, which was not associated with illness behavior but was associated with appetitive neophobia. The latter was unmasked by a contributory influence of psychological stress, raising important implications for potential sensitizing and/or synergistic effects of systemic, immunological processes on adaptive neural mechanisms engaged by stress.

MATERIALS AND METHODS

Animals

Male BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME) arrived at 5–6 weeks of age and were acclimated for 10–14 d before experimentation. Food and water were provided *ad libitum*, under conditions of group housing ($n = 4$ per cage) and 12 hr light/dark illumination (lights on at 7:00 A.M.). All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Drugs and reagents

Staphylococcal enterotoxin B (SEB) was purchased from Sigma (St Louis, MO), lipopolysaccharide (LPS; from *E. coli* 055:B5) from Difco (Detroit, MI), and recombinant human interleukin-1 β (IL-1 β) from R & D Systems (Minneapolis, MN). Indomethacin (Sigma) was dissolved in 4% sodium bicarbonate. Sheep anti-rat CRH for *in vivo* immunoneutralization of blood-borne CRH was a kind gift from Dr. Wylie Vale (Salk Institute, CA). Rat anti-mouse IL-2 was from Genzyme (Cambridge, MA). Plasmids containing exonic segments of mouse IL-2 and CRH genes were provided by Cynthia Watson (National Institute of Allergy and Infectious Diseases) and Dr. Audrey Seasholtz (University of Michigan, Ann Arbor), respectively.

Neuroendocrine and histochemical studies

Animals (~25 gm in weight) were injected intraperitoneally with either 50 μ g of SEB or pyrogen-free 0.9% saline (Baxter Healthcare, Deerfield, IL). This dose of SEB was found to be optimal, as determined by dose–response studies published previously (Shurin et al., 1997). In some experiments, additional groups of animals were injected intraperitoneally with 50 ng of human IL-1 β . In experiments designed to immunoneutralize CRH and IL-2, the animals were pretreated with 0.2 ml of undiluted sheep anti-rat CRH or 200 μ g of rat anti-mouse IL-2. To test the effects of indomethacin (INDO), we briefly restrained the animals (no more than 1 min) in mouse tail bleeding tubes and administered 10 mg/kg INDO via a tail vein. Then 2 hr later they were challenged with 50 μ g of SEB or 50 ng of IL-1 β . In most experiments, death routinely occurred 2 hr after injection, between 12:00 P.M. and 2:00 P.M. The 2 hr time point was found in previous studies to be the peak time of the plasma ACTH and corticosterone response to SEB. However, for certain experiments the animals also were killed after 4, 6, and 24 hr to assess

splenic and neuronal CRH and IL-2 mRNA levels by *in situ* hybridization.

For the determination of hormonal and cytokine concentrations, the animals were decapitated, and trunk blood was collected into chilled EDTA-coated Vacutainer tubes (Beckton Dickinson, Rutherford, NJ).

Behavioral studies

Conditioned taste aversion

An experiment was conducted to test whether SEB challenge induces conditioned taste aversion (CTA), a form of associative learning that routinely occurs when animals experience illness after ingesting a novel drinking solution (Yamamoto et al., 1994). An additional experiment tested whether conditioned taste aversion could be induced by using LPS, as reported in the literature (Exton et al., 1995; Janz et al., 1996). Initially, group-housed mice were habituated every second day to placement in individual cages for 1 hr/d. Animals were given a total of five preexposures. This procedure was introduced to attenuate any effects of a new environment on consumption of a novel drinking solution. On the training day (2 d after the final preexposure to the drinking environment) the animals were separated into individual cages (as on habituation days) and presented with the conditioning stimulus (CS). The CS was Prosobee (Mead Johnson, Evansville, IN), a commercial infant formula liquid, and was presented in a 50 ml tube fit with a conventional metallic sipper tube. Preliminary studies had shown that animals voluntarily will consume this solution without the need for fluid deprivation. Animals were allowed to drink the solution for 1 hr, after which they were removed individually and injected intraperitoneally with either 50 μ g of SEB or an equal volume (0.2 ml) of nonpyrogenic saline. Our previous published results had shown this dose of SEB to be sufficient in activating the HPA axis and had shown that doubling the dose of SEB did not enhance this activation (Shurin et al., 1997). More to the point, in conducting this experiment we were interested in determining whether the HPA activation that occurs in response to 50 μ g of SEB is secondary to or associated with any illness effects.

Overall, there was a total of three main treatment groups, all of which received the CS solution at the same time: (1) conditioned animals were treated with SEB (or 5 μ g of LPS) immediately after drinking the CS solution; (2) nonconditioned animals received noncontingent pairing of the CS and SEB (or LPS), in that the animals were treated with SEB (or LPS) 24 hr before CS exposure—i.e., no further injections were provided to this group after they drank the CS on the training day; and (3) placebo animals were injected with saline either immediately after CS exposure on the training day (P1 group) or were injected with saline 24 hr earlier (P2 group) to simulate conditions experienced by the nonconditioned group. Animals were returned to their original cages and group housing until the test day (1 d after conditioning when the UCS was SEB and 3 d after when the UCS was LPS). On the test day the animals were reexposed to the CS solution for 1 hr as on the training day. After the test session the animals were returned to their group housing and tested again on 2–3 more consecutive days. Measures of consumption (expressed in grams) were determined by weighing the drinking tubes before and after each drinking session.

Disruption of fluid ingestion

Two experiments were conducted to test whether an injection of 50 μ g of SEB disrupts ongoing consumption of a familiar drinking solution. Thus, two groups of mice were presented with Prosobee for 1 hr/d for 2–4 d, and on the test day they were injected with either saline or 50 μ g of SEB 2 hr before another drinking session. Consumption over a 1 hr period was measured as in the taste aversion experiments. As with the above experiments the animals were habituated to separation and placement into a new environment.

Taste neophobia

Three experiments assessed the level of consumption of a novel solution in control and SEB-challenged animals. Exposure to novel stimuli, in particular food and contextual surroundings, elicits a form of avoidance behavior that suggests the presence of neophobia or fear of novelty. This behavior is the basis of many common tests of anxiety, especially those assessing anxiogenic and/or anxiolytic treatments (Stout and Weiss, 1994).

Experiment 1. Mice were challenged with saline or 50 μ g of SEB ($n = 8$ per group) 2 hr before being placed individually into a cage other than their home cage, where they were allowed to consume for 1 hr an

unfamiliar drinking solution. The novel cages were in a separate room from that in which the colony was housed. The novel drinking solution was Prosobee (see above). The environment into which animals were placed was made of opaque polypropylene and was fit with an empty (save for the bottle containing the drinking solution) standard stainless steel cage top. The physical dimensions of the cage were the same as the home cage (12 inches in length, 7 inches in depth, and 5 inches in width). The floor was covered with fresh wood shavings.

Experiment 2. Animals were challenged as in Experiment 1 and subsequently were exposed to either a novel or familiar cage containing an unfamiliar drinking solution. Novelty, under these circumstances, was defined as the first such experience for an animal (i.e., removal from the group and relocation to the new cage for 1 hr). Alternatively, familiarity was promoted by previous daily exposure (Habituation) to this process of removal into an individual cage. During habituation the animals remained in the cage for 1 hr, after which they were returned to the home cage in the colony room. For this experiment there was a total of four groups ($n = 8$ per group): (I) SEB/N, (II) SEB/H, (III) SAL/N, and (IV) SAL/H. In all groups the mice had never been exposed before to the distinct drinking solution, or any other solution, except for drinking water in their home cage. Thus, for all groups the Prosobee formula solution was a novel taste stimulus, whereas the drinking environment and relocation to this environment were familiar only to groups II and IV. This latter process of familiarization to 1 hr of individual housing (Groups II and IV) occurred over a period of 10 d, with animals being placed into individual housing every other day. Animals in groups I and III were handled on the same days. Handling for these animals consisted of being picked up briefly by the tail and put back down into a holding cage for 1 min. The holding cage had the same characteristics as the home cage. Once all animals from the same home cage had been handled in this way, they were returned immediately to the home cage.

Experiment 3. In contrast to the previous experiment, Experiment 3 manipulated the familiarity of the distinct drinking solution by preexposing one set of animals ($n = 16$) to Prosobee in their home cage while another set of animals ($n = 16$) simultaneously was presented with a bottle of water. These preexposures occurred on two consecutive days, and each lasted 1 hr. On the third day, one-half of the animals in each set were injected either with saline or SEB and 2 hr later were exposed to a new drinking environment, as in Experiment 1.

Effect of novelty on plasma ACTH

Additional experiments were conducted to determine whether the placement of group-housed animals into an individual housing condition served as a novelty stressor. For these experiments, plasma ACTH levels were assessed 15 and 30 min after individual placement into a novel cage. Control animals were picked up briefly by the tail but returned immediately to the home cage. These experiments were conducted to determine the neuroendocrine impact of relocation to a novel environment.

In situ hybridization (ISH)

Prehybridization

Brains and spleens were removed quickly, frozen in TissueTek OCT compound by immersion in 2-methyl butane at -30°C , and stored at -70°C until Cryostat-cut coronal sections (10 to 12 μm thick) were obtained from the optic chiasm to the mamillary bodies. Tissue sections were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.2, for 5 min, followed by a 3 min rinse with 0.1 M PBS and dehydration through a graded series of ethanols (50, 70, and 95%, for 1 min each). To reduce nonspecific binding of the riboprobe, we neutralized positive charges by a 10 min incubation in 0.25% acetic anhydride containing 0.1 M triethanolamine, pH 8.0. Sections then were rinsed with $0.2\times$ SSC, dehydrated through a graded series of ethanols, and dried.

Preparation of radiolabeled RNA probes

Detection of CRH and IL-2 mRNA was accomplished with antisense ^{35}S -labeled riboprobes. The CRH mRNA probe was generated from a linearized pGem4Z plasmid containing a 578 bp DNA fragment from exon II of the mouse CRH gene [(Keegan et al., 1994), kindly provided by Dr. A. Seasholtz, University of Michigan]. The IL-2 mRNA probe was generated from a pGEM-1 plasmid containing a 600 bp fragment of mouse IL-2 cDNA (kindly provided by Cynthia Watson, National Institute of Allergy and Infectious Diseases). Plasmids were linearized by digestion with *HindIII* (New England Biolabs, Beverly, MA), and antisense RNA probe transcription was conducted from the SP6 promoter

with the Promega Riboprobe Transcription System (Promega Biotech, Madison, WI) as follows. Each 20 μl reaction contained 1 μg of linearized plasmid DNA, transcription-optimized buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl_2 , 2 mM spermidine, 10 mM NaCl), 20 U SP6 RNA polymerase, 2.5 mM each of ATP, CTP, and UTP, 100 μM CTP, 100 mM dithiothreitol (DTT), 20 U RNase inhibitor, and 62.5 μCi ^{35}S -UTP (specific activity $\sim 2 \times 10^7$ cpm/pmol; DuPont NEN, Boston, MA). The reaction was allowed to proceed for 1 hr at 37°C , whereafter the DNA template was removed by a 15 min incubation at 37°C with 1 U RQ1 DNase (Promega), followed by centrifugation over a G-50 Sephadex column (Boehringer Mannheim, Indianapolis, IN). One microliter of the eluant was counted on a β -counter to ensure a count of $210\text{--}700 \times 10^5$ disintegrations per minute (DPM). For the control sense probe, plasmid DNA was linearized with *EcoRI*, and transcription was performed as above, using T7 RNA polymerase.

Hybridization

Antisense or sense radiolabeled probes were diluted in hybridization buffer to yield a specific activity of 2×10^6 DPM per 100 μl . The hybridization buffer consisted of 0.6 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, pH 8, 0.1 mg/ml sheared salmon sperm DNA (Stratagene, La Jolla, CA), 0.05 mg/ml yeast transfer RNA (Stratagene), 10 mg/ml total yeast RNA (Ambion, Austin, TX), $1\times$ Denhardt's solution, 10% dextran sulfate, 50% redistilled formamide (Kodak, Rochester, NY), 0.1% SDS, 0.1% nathiosulfate, and 0.1 M DTT (Stratagene). Approximately 25 μl /tissue of the hybridization mixture (5×10^5 DPM) was applied to each slide (50 μl per slide). Then the slides were coverslipped and incubated for 16 hr in a 50% formamide atmosphere at 55°C .

After hybridization

After hybridization the slides were soaked in $2\times$ SSC to remove coverslips and rinsed for a further 5 min in fresh $2\times$ SSC. Then the slides were treated with 2 $\mu\text{g}/\text{ml}$ ribonuclease A (RNase A, Boehringer Mannheim) in RNase buffer (40 mM Tris, pH 8.0, 0.5 M NaCl, and 1 mM EDTA) for 30 min at 37°C . This was followed by an additional 30 min incubation at 37°C in RNase-free RNase buffer and then a 5 min rinse at 37°C in $2\times$ SSC. All sections were subjected to a series of washes as follows: 45 min at 50°C in $2\times$ SSC, 45 min at 55°C in $0.2\times$ SSC, and 45 min at 60°C in $0.1\times$ SSC. The tissue sections were dehydrated through a series of 2 min washes in 50, 70, 80, and 90% ethanol/0.3 M sodium acetate, with a final 5 min in 100% ethanol. Subsequently, the sections were air-dried and subjected to film autoradiography.

Film autoradiography was conducted by exposing slides for 4 d to Kodak BioMax film in light-tight x-ray cassettes. Calibration of exposure intensity was achieved by exposing the film to ^{14}C plastic strips (American Radiolabeled Chemicals, St. Louis, MO) of variable specific activity (46.4–1580 DPM). After film development the slides were dipped at 45°C in emulsion (type NTB2, Kodak number 165-4433) and incubated at 4°C in light-tight slide racks for 10 d. They subsequently were developed in Kodak Dektol solution (2 min at 15°C) and fixed in Kodak fixer (5 min at 15°C). The slides were counterstained with nuclear red (Vector Laboratories, Burlingame, CA) and stored in a safe location until they were ready for examination by optical microscopy under dark- and light-field conditions.

Semiquantitation of in situ hybridization autoradiographs

Semiquantitation of autoradiographic images was achieved by using SigmaScan image analysis software (SPSS Science, Chicago, IL). For the brain a calibration curve was established on the basis of the gray level intensity of autoradiographs obtained from different radioactive concentrations of ^{14}C standards (American Radiolabeled Chemicals), as described by others (Miller, 1991; Brady et al., 1994). Units of measurement were DPM, and these were used to quantitate the activity from four to six sections per animal of the left and right PVN and central nucleus of the amygdala (ceA). A nonhybridized region outside of the PVN and ceA was quantitated also, and this was subtracted from the value obtained for the region of interest to give a final net value in DPM of antisense RNA probe hybridization. A similar semiquantitation procedure that used the ^{14}C calibration curve was applied for the spleen, in that all areas in the white pulp of the spleen that showed hybridization for the antisense IL-2 RNA probe were quantitated and adjusted for background obtained in nonhybridized regions. In all experiments the sense RNA probes showed no hybridization when applied to the spleen and brain. Selective RNase

Figure 1. IL-2 transcription is increased in the spleens of mice 2, 4, and 6 hr after SEB challenge. Mice were challenged intraperitoneally with 50 μ g of SEB at 0, 2, 4, or 6 hr before death and assessment of IL-2 mRNA by *in situ* hybridization of spleen tissue sections. Unchallenged mice (*A*) showed no detectable levels of IL-2 mRNA in the lymphocyte-rich white pulp (WP) regions of the spleen. This was not the case 2 hr (*B*) and 4 hr (*C*) after SEB challenge. Hybridization for IL-2 mRNA was dramatic at these times and confined principally to the WP regions (arrows) rather than to the macrophage-enriched red pulp (RP). The level of hybridization decreased appreciably by 6 hr (*E*). This is more clearly evident by comparing the images in *D* and *F*, which offer higher magnification views of silver grain density within the dotted circles in *C* (4 hr) and *E* (6 hr), respectively. Scale bars: *A*–*C*, *E*, 200 μ m; *D*, *F*, 20 μ m.

treatment of tissue sections before hybridization with antisense RNA probes failed to result in any hybridization.

Assays for ACTH and IL-2

Blood was collected into EDTA-coated tubes and centrifuged at 2500 rpm for 15 min at 4°C. Plasma was collected and stored at –70°C in separate tubes designated for ACTH and IL-2 measurements. Hormonal measurements were achieved by commercial radioimmunoassay kits pur-

chased from ICN Biochemicals (Irvine, CA). Cytokine measurements were performed with ELISA reagents purchased from Endogen (Cambridge, MA).

Statistical analysis

Where it was deemed necessary, data were analyzed by ANOVA or Bonferroni *t* test. Significant main effects obtained from ANOVA were analyzed further by a *post hoc* Tukey test, whereas interaction effects

Table 1. Plasma IL-2 and ACTH concentrations 2, 4, or 6 hrs after SEB treatment

Time (hr)	Plasma IL-2 (ng/ml)	Plasma ACTH (pg/ml)
0	0.78 ± 0.07	40.4 ± 4.1
2	43.9 ± 9.3	158.5 ± 12.1
4	19.8 ± 4.0	189.6 ± 45.0
6	7.58 ± 0.6	72.8 ± 15.9

All animals were killed around the same time of the day to avoid circadian influences. All values for 2, 4, and 6 hr time points were significantly different ($p < 0.05$) from 0 hr, which in the case of ACTH represented collapsed values for animals injected with saline 0, 2, 4, or 6 hrs before death.

$n = 6$ –8 for each time point, except $n = 24$ for 0 hr, ACTH column.

were explored by contrast analysis of group means. Treatment effects or group differences were considered to be significant at $p < 0.05$.

RESULTS

Stimulation of splenic IL-2 transcription and associated CRH mRNA expression in the brains of SEB-challenged mice

Animals were challenged with saline or SEB at 0, 2, 4, or 6 hr before death at the same time of the day. For all of the reported measures, saline-injected animals did not differ from animals killed immediately at 0 hr. Therefore, only the data for the 0 hr group are presented in relation to results obtained from SEB-injected animals. To confirm T-cell activation, we assayed spleens and plasma for IL-2 mRNA and protein levels. As is evident in Figure 1, after SEB challenge there was a dramatic induction of IL-2 mRNA in the spleen. The level of IL-2 mRNA was high for several hours (4 hr time point) but was found to decline by 6 hr (Fig. 1). No IL-2 mRNA was detected in the spleens of animals killed at 0 hr. As reported previously (Shurin et al., 1997), plasma IL-2 and ACTH concentrations displayed the same kinetics of response, with IL-2 achieving nanogram concentrations by 2 hr and declining by 6 hr (Table 1).

Determination of CRH mRNA levels was conducted on the brains and spleens of animals in the 0, 2, 4, and 6 hr groups. There was no detectable hybridization for CRH mRNA in the mouse spleens at any time point (data not shown). However, significant hybridization was observed in brains, in particular the PVN of the hypothalamus and ceA (Figs. 2, 3). Figure 4 shows the calculated mean DPM for the PVN and ceA. A one-way ANOVA of DPM obtained from the ceA revealed a significant effect of time ($F_{(3, 22)} = 4.23$; $p < 0.025$). Additional *post hoc* analysis with the Tukey test confirmed that the mean DPM for the 4 and 6 hr groups was each significantly higher ($p = 0.05$ and $p = 0.036$, respectively) than the 0 hr group. The 0 and 2 hr groups did not differ from each other. Similarly, one-way ANOVA of the PVN data revealed a significant effect of time ($F_{(3, 22)} = 4.8$; $p < 0.025$), which was attributable to significantly higher DPM in the 6 hr group relative to the 0 hr group (Tukey test, $p = 0.019$). For the PVN the level of CRH mRNA for the 2 and 4 hr groups did not achieve the criterion for statistical significance relative to the 0 hr group.

Immunoneutralization of circulating CRH blocks the ACTH response to SEB

The increased transcription of CRH in the PVN suggested that the high concentration of plasma ACTH observed after SEB challenge may be mediated by CRH secretion into the median eminence. To test this, we treated mice intraperitoneally with 0.2 ml of undiluted sheep anti-rat CRH antiserum and 15 min later challenged them intraperitoneally with 50 μ g of SEB. Animals

were killed 2 hr after the SEB challenge. Analysis of plasma ACTH values revealed a significant interaction effect between toxin challenge and serum administration [$F_{(1, 23)} = 15.15$; $p < 0.001$]. As shown in Figure 5A, this was attributable to the greatly attenuated ACTH response in SEB-challenged animals who were administered the anti-CRH antiserum.

Indomethacin treatment or immunoneutralization of IL-2 failed to block the ACTH response to SEB

Prostaglandin synthesis has been implicated in the HPA-activating effects of the proinflammatory cytokine IL-1, and a similar mechanism may be involved in SEB-mediated HPA axis stimulation. Figure 5B presents the plasma ACTH concentrations in saline, SEB, and rhIL-1 β -challenged animals who were administered indomethacin or vehicle. The rhIL-1 β group was included as a positive control of the ability of indomethacin to block the ACTH response to this cytokine. A two-way (Drug $\times$ Challenge) ANOVA revealed only a significant effect of Challenge ($F_{(1, 34)} = 6.11$; $p < 0.01$), but no Drug or Drug $\times$ Challenge effects. Because the IL-1 group was included as a positive control and the literature allowed us to expect an effect of indomethacin treatment, we conducted orthogonal contrasts comparing the two rhIL-1 β groups. This revealed a significant difference ($F = 4.16$; $p < 0.05$) and confirmed what is clearly evident in Figure 5B as a marked reduction of ACTH levels in indomethacin-treated rhIL-1 β animals.

Excessive exposure to IL-2 can stimulate the HPA axis (Hannisch et al., 1994). Therefore, we tested whether the immunoneutralization of circulating IL-2 would alter the increased ACTH response to SEB challenge by pretreating animals with rat anti-mouse IL-2 monoclonal antibody or nonimmune rat IgG. The results showed that anti-mouse monoclonal antibody treatment reduced plasma IL-2 levels to near-baseline concentrations as measured by ELISA [SEB + rat anti-IL-2, 1.34 ± 0.4 ng/ml; SEB + rat IgG, 10.73 ± 1.5 ng/ml]. However, this apparently effective immunoneutralization of circulating IL-2 did not alter the increase in plasma ACTH observed in response to SEB challenge [SEB + rat IgG ($n = 5$), 77.1 ± 17.9 pg/ml; SEB + rat anti-IL-2 ($n = 7$), 73.8 ± 13.0 pg/ml; saline + rat IgG ($n = 4$), 28.25 ± 7.4 pg/ml; saline + rat anti-IL-2 ($n = 4$), 26.7 ± 4.9].

T-cell activation with SEB augments taste neophobia in the absence of illness effects

Behavioral experiments were initiated to determine whether T-cell activation with SEB produced a generalized illness that could be associated with the neural effects of SEB challenge. The results of a conditioned taste aversion experiment failed to show that pairing a novel taste solution (conditioning stimulus) with SEB challenge results in the development of a learned aversion to the CS that is linked to T-cell activation (data not shown). Additional experiments showed that SEB challenge did not disrupt ongoing consumption of a familiar drinking solution or produce overnight changes in body weight (data not shown, but consider Fig. 6). In over a dozen experiments visual inspection of SEB-challenged animals in their home cages at the time they were to be removed for death or behavioral assessment did not reveal piloerection or reduced activity.

Because of the apparent lack of illness-like effects caused by challenge with SEB, appetitive behavior was assessed under conditions designed to probe neophobic behavior. As shown in Figure 6A, animals challenged with SEB and 2 hr later exposed to a novel drinking solution displayed reduced consumption of a

Figure 2. SEB increases CRH mRNA levels 6 hr after challenge in the hypothalamus (PVN) and amygdala (ceA). This figure shows representative autoradiographic film images (*insets*) and dark-field silver grain formation of the ceA and PVN from subsequently emulsion-dipped slides. *A*, ceA/control. *B*, ceA/SEB. *C*, PVN/control. *D*, PVN/SEB. Scale bars: *A–D*, Dark field, 100 μ m; *insets*, 2 mm.

novel drinking solution. A repeated measures ANOVA (Challenge $\times$ Test) revealed significant effects of Challenge ($F_{(1,11)} = 9.4$; $p < 0.025$) and Test ($F_{(2,22)} = 4.3$; $p < 0.025$). The absence of an interaction effect was consistent with the persistent difference in consumption between SEB- and saline-injected animals across the three test periods.

A replication of this experiment tested whether the novelty of the environment in which consumatory behavior was elicited influenced animals who were challenged with SEB. Figure 6*B* shows the results of this experiment. A three-way ANOVA (Challenge $\times$ Habituation $\times$ Test) with repeated measures across the Test variable revealed significant effects of Challenge ($F_{(1,28)} = 5.8$; $p < 0.025$), Habituation ($F_{(1,28)} = 8.0$; $p < 0.01$), and Test ($F_{(2,56)} = 107.7$; $p < 0.0001$) without any further main or interaction effects. The main effects of challenge and habituation were evidently a function of the reduced consumption by SEB-challenged animals who had received no previous habituation to being placed in the drinking environment (Fig. 6*B*). However, if the animals were familiarized with the drinking environment, SEB challenge did not influence consumption of the novel drinking solution differently from either of the saline-injected groups.

A final experiment tested whether the novelty of the drinking solution influenced the consumatory behavior of SEB-challenged animals. A two-way ANOVA (Taste Novelty $\times$ Challenge) conducted on the data in Figure 6*C* showed a significant effect of

Taste Novelty ($F_{(1,28)} = 29.2$; $p < 0.0001$) and interaction between Taste Novelty and Challenge ($F_{(1,28)} = 15.1$; $p < 0.001$). The latter was likely a function of the greater consumption by SEB-challenged animals who were familiar with the drinking solution, as well as the markedly reduced consumption by the SEB-treated group unfamiliar with the drinking solution, which likely accounted for the significant main effect of Taste Novelty. A planned Bonferroni *t* test comparing animals challenged with SEB and saline and subsequently exposed to novel drinking solution revealed a significant difference in consumption ($t_{(14)} = 4.078$; $p = 0.001$).

To test whether relocation to a novel drinking environment activated the HPA axis, we individually exposed different groups of naïve animals for 15 or 30 min to a novel environment identical to that in which appetitive behavior was tested in the foregoing experiments. Control animals were left in their home cages and killed at 15 and 30 min. A two-way ANOVA (Novel Cage $\times$ Sampling Time) conducted on the plasma ACTH values revealed significant main effects of being placed in a novel cage ($F_{(1,28)} = 15.3$; $p < 0.001$) as well as sampling time ($F_{(1,28)} = 12.0$; $p < 0.01$). A borderline interaction also was observed ($F_{(1,28)} = 3.4$; $p = 0.076$). The effect of being placed into a novel cage was clearly evident in the doubling of plasma ACTH concentrations by 30 min after removal from the home cage [Home Cage 15 min ($n = 8$), 54.3 ± 7.3 pg/ml; Home Cage 30 min ($n = 8$), 69.0 ± 9.4

Figure 3. Increased CRH mRNA hybridization in the central nucleus of the amygdala 6 hr after SEB challenge. The ceA region in emulsion-dipped and nuclear-counterstained brain tissue sections were captured digitally under light-field illumination. *A*, Control. *B*, SEB. Clear evidence of more intense and numerous silver grain formation over cytoplasmic domains is evident in the SEB-challenged animal (compare the areas indicated by arrows). Scale bars: *A*, *B*, 25 μ m.

pg/ml; Novel Cage 15 min ($n = 8$), 73.3 ± 9.7 pg/ml; Novel Cage 30 min ($n = 8$), 121.5 ± 9.8 pg/ml]. This provided neuroendocrine confirmation of the anxiogenic properties of individual relocation from the home cage and into a novel environment in which the animals were alone and exposed to a novel drinking solution.

DISCUSSION

Neurocircuitry subserving emotional reactivity can be activated by processive—e.g., psychological—stimuli as well as by systemic events acting via interoceptive mechanisms (Herman and Cullinan, 1997). The latter includes signals derived from the immune system, and the results of the present series of experiments confirm that T-lymphocytes can mediate neural and behavioral effects similar to other immune cells, such as macrophages (Derijk et al., 1991). This was demonstrated by using staphylococcal enterotoxin B (SEB), a protein antigen that readily binds MHC class II molecules on antigen-presenting cells and then cross-links with specific motifs on the variable (V) region of the β -chain of the T-cell receptor (Johnson et al., 1991). The $V\beta 8^+/CD4^+$ T-cell population is highly reactive to SEB, producing within 1–2

Figure 4. Mean disintegrations per minute (DPM $\pm$ SE) in the PVN and ceA of CRH mRNA-hybridized brains from control and SEB-challenged animals. Animals were killed at the same time of day 0, 2, 4, and 6 hr after challenge with 50 μ g of SEB ($n = 6$ –8 per group). Film autoradiographs were captured digitally and quantified as described in Materials and Methods. *Significantly different from 0 hr at $p < 0.05$.

hr highly measurable levels of cytokines and, in particular, IL-2 (Bette et al., 1993; Shurin et al., 1997). Such robust activation is impressive relative to the failure of most T-dependent protein antigens to induce cytokine production that achieves measurable quantities in plasma (Kelso et al., 1991; Troutt et al., 1992).

Consistent with this, the present results revealed prominent levels of splenic IL-2 mRNA and plasma IL-2 that persisted for up to 6 hr after the initial challenge with SEB. This frank activation of T-cells was associated with increased CRH mRNA in the PVN and ceA. This was observed 4–6 hr after SEB challenge, whereas pituitary–adrenal and behavioral changes (see below) occur well before this (at 2 hr). It has been documented that cytoplasmic CRH mRNA levels are stable and increase slowly, whereas CRH heteronuclear RNA is transcribed readily within minutes of stressor exposure, presumably in anticipation of the need for compensatory increases in mRNA because of augmented translational events leading to CRH secretion (Imaki et al., 1995; Kovacs and Sawchenko, 1996). Although *in situ* hybridization data that use heteronuclear CRH riboprobes would clarify the present set of results, it is likely that the increased CRH mRNA after SEB challenge is evidence of compensation. In support of this, the increased plasma ACTH response at 2 hr after SEB challenge was abolished by systemic immunoneutralization of CRH, which suggests that the release of CRH from neurosecretory cells in the PVN may be the principal driving mechanism for the increased ACTH output. As for the amygdala, we can only speculate as to whether CRH release occurred at 2 hr after SEB challenge, an event suggested by the likely compensatory increase in CRH mRNA at 4 hr and 6 hr.

It could be argued that the immunoneutralizable CRH arose from SEB-activated immune cells, because CRH mRNA has been detected in rat and mouse lymphoid tissue (Aird et al., 1993; Muglia et al., 1994; Brouxhon et al., 1998). However, there is also evidence of a failure to detect lymphoid cell CRH mRNA (Stenzel-Poore et al., 1992; Bamberger et al., 1998), supported in the present study with the failure of control and activated spleens

Figure 5. Effect of systemic CRH immunoneutralization and indomethacin treatment on plasma ACTH concentrations after SEB challenge. *A*, Animals were treated with either sheep anti-rat CRH or normal sheep serum and 15 min later were challenged intraperitoneally with 50 μ g of SEB or saline. It is evident that there was complete abrogation by anti-CRH treatment of the ACTH response to SEB ($n = 6$ –9 per group). *B*, Animals were treated intravenously with vehicle or indomethacin (10 mg/kg) and challenged with saline, 50 μ g of SEB, or 50 ng of hIL-1 β . Measurement of plasma ACTH 2 hr later revealed no alteration because of INDO treatment in the elevated ACTH concentrations observed in SEB-challenged animals. A statistically significant reduction in plasma ACTH was observed in the IL-1 group pretreated with INDO (see Results).

Figure 6. SEB challenge enhances the neophobic avoidance of a novel taste stimulus. Several experiments measured consumatory behavior under various conditions of environmental and food novelty (see Materials and Methods). Data represent the mean consumption $\pm$ SE ($n = 6$ per group, Experiment 1; $n = 8$ in Experiments 2 and 3; see Results for full statistical details). *A*, Effect of SEB challenge on consumption of a novel taste stimulus in a novel environment 2 hr after challenge, *Test 1*. Animals were reexposed to the drinking situation (same environment and drinking stimulus) on the next day (*Test 2*, i.e., day 2) and on day 6 after challenge (*Test 3*). Consumption levels for SEB-challenged animals were reduced significantly. *B*, Different groups of animals were preexposed (*H*, i.e., habituated) or remained naïve (*N*, i.e., novel environment) to the drinking test environment. Consumption 2 hr after SEB challenge was reduced significantly only if the environment was novel (*Test 1*, day 1). Consumption remained low on subsequent test days (*D4* and *D9*) but displayed recovery. *C*, Effect of varying the novelty of the drinking stimulus. One group of animals was preexposed to the drinking stimulus in the home cage (*Familiar Taste* group) while another group received water (*Novel Taste* group). Consumption of the distinct drinking stimulus in a novel environment was measured 2 hr after SEB or saline challenge. There was no effect on consumption by SEB challenge if the drinking stimulus was familiar.

(i.e., showing extensive IL-2 mRNA) to hybridize with the CRH riboprobe. Consequently, it is unlikely that neutralizable CRH in the present experiments was of immune origin.

Activation of T-cells with SEB increased CRH mRNA in the ceA. Amygdaloid function is fundamental to emotional responses (Davis, 1992), and CRH production in the ceA promotes

anxiogenic functions (Rassnick et al., 1993; Rodriguez et al., 1997). Increased CRH mRNA and activity in the ceA have been observed in response to psychological stressors and challenge with endotoxin (Laflamme et al., 1997; Hsu et al., 1998; Merali et al., 1998), although in the latter the indirect effects of illness were not ruled out. In the present study the neural effects of SEB were

shown not to be associated with various indices of illness, because animals lacked piloerection, loss of body weight, and reduced feeding. Moreover, although LPS effectively produces a conditioned taste aversion in BALB/cByJ mice (our unpublished observations), SEB challenge did not have the same conditioning properties. At higher doses of SEB than that used in the present experiments, illness effects might be possible, although this has been found to be inconsistent (Wood and Todd, 1995). However, the purpose of these experiments was to determine whether immunological challenge with SEB at a dose that reliably activates the CNS is associated with illness-like behavioral alterations. Given the lack of behavioral signs supporting this, it is unlikely that neural activation in response to SEB challenge involves cognitive reactivity to somatic sensations of malaise.

Additional experiments explored behavior that might reflect emotional reactivity. The amount of food or water consumed in a novel environment can be modulated by anxiolytic and anxiogenic agents (Stout and Weiss, 1994), and seemingly normal appetitive behavior can raise CRH activity in the ceA (Merali et al., 1998), implying that this form of behavior may have considerable emotional valency. Therefore, mice challenged with SEB were tested for consumption of a novel food substance presented in a similarly novel environment. The results showed significant suppression of consumatory behavior, which was governed by contextual novelty. However, although this was paramount, it was not sufficient to alter consumption of a familiar drinking solution in SEB-challenged mice (which incidentally confirmed that animals were not feeling ill). Ultimately, the necessary conditions for reduced consumption in the SEB-challenged animal were for *both* environmental context and gustatory stimulus to be novel. Indeed, the novelty of the environment was found to be stressful, as determined by plasma ACTH measures. Interestingly, the amygdala is required for HPA axis reactivity to psychological stressors (Van de Kar et al., 1991). Therefore, immunological challenge may sensitize animals to exhibit augmented neophobic behavior under conditions that most likely access neural substrates of emotionality, such as the amygdala.

Additional experiments are required to determine whether behavior in other paradigms that test anxiety and/or emotionality are altered by SEB challenge. Nonetheless, the present set of results argues strongly for an emotional influence of T-cell activation with SEB. As such, they are also consistent with other evidence showing the influence of autoimmune processes on emotional behavior in mice (Schrott and Crnic, 1996), although the mediatory role of T-cells in the latter is not known. In any case the behavioral effects of SEB may be a result of underlying changes in CRH gene transcription in the central nucleus of the amygdala. For example, despite evidence that centrally administered immune products, such as IL-1, influence novelty-induced behavior via a CRH-independent mechanism (Spadaro and Dunn, 1990), there is also evidence that systemically administered IL-1 can modify similar behavior via a mechanism that does involve CRH (Dunn et al., 1991). Although this is not to suggest that IL-1 mediates the behavioral effects of SEB, there does appear to be support for the hypothesis that peripheral immune processes may exert behavioral actions via recruitment of CRH synthesis and release. Such a direct link between behavior and CRH mRNA induction needs to be verified in the case of SEB.

The determination of central factors involved in activating CRH mRNA and neophobic behavior still remains. The presence of IL-1 in the hypothalamus has been suggested to influence the HPA axis; furthermore, central IL-1 receptors may influence the

effects of stress on emotional learning (Maier and Watkins, 1995). However, in preliminary studies we did not observe induction of IL-1 β mRNA in the hypothalamus after SEB challenge (our unpublished observations). Additional studies seeking to measure other cytokines and their receptors obviously are needed.

Previously, it was shown that treatment with cyclosporine A, which suppresses T-cell function by inhibiting IL-2 gene transcription, prevented the neuroendocrine effects of SEB challenge (Shurin et al., 1997). Interestingly, in the present study, immunoneutralization of IL-2 did not alter the ACTH response to SEB challenge. This is consistent with other evidence in which acute, but not chronic, administration of IL-2 fails to stimulate the HPA axis in rats and mice (Hanisch et al., 1994; Zalcman et al., 1994). Furthermore, the lack of IL-2 involvement in HPA activation is supported by similar noninvolvement in anxiogenic behavior in the elevated plus maze (Connor et al., 1998). However, IL-2 can stimulate the production of CRH by amygdaloid tissue (Raber et al., 1995), and limbic regions express IL-2 receptors (Hanisch and Quirion, 1995). Furthermore, IL-2 exerts anhedonic effects (Anisman et al., 1996; Hebb et al., 1998), which may or may not be operational in the present set of results. Although SEB challenge did not affect the palatability of the taste stimulus when it was familiar or consumed in a familiar context, it remains to be determined whether immunological challenge alters the manner in which novelty influences the hedonic properties of an otherwise palatable diet. As noted above, evidence that CRH release in the ceA is provoked by a normal eating bout (Merali et al., 1998) implies some form of arousal, but the significance of this is unknown. In short, although immunoneutralization of IL-2 does not affect SEB-induced HPA axis activation, future studies will determine whether neutralizing IL-2 or other cytokines alters the observed changes in behavior and CRH mRNA.

The generation of arachidonic acid metabolites, such as prostaglandin E, does not appear to be involved in the ACTH response to SEB, because indomethacin treatment did not affect ACTH output in challenged mice. However, consistent with the literature (Buller et al., 1998), pituitary–adrenal activation because of IL-1 β was attenuated significantly by indomethacin. This raises an interesting question as to whether HPA reactivity to SEB involves IL-1. Previously, we found that IL-1 production in response to SEB did not achieve readily detectable levels in plasma but could be measured in lymphoid tissue, such as the spleen, in which antigen-presenting cells are most likely the source of IL-1 (Shurin et al., 1997). Therefore, its role in mediating the neural and behavioral effects of SEB challenge via at least humoral mechanisms remains uncertain and requires further investigation.

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