Neuronal, Endocrine, and Anorexic Responses to the T-Cell Superantigen Staphylococcal Enterotoxin A: Dependence on Tumor Necrosis Factor- α

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Staphylococcal enterotoxin A (SEA) is a microbial superantigen that activates T-lymphocytes and induces production of various cytokines, including tumor necrosis factor- α (TNF α). Previously, it was shown that SEA activates the hypothalamic–pituitary–adrenal axis and augments gustatory neophobic behaviors. In the present study, it was hypothesized that these effects involve neuronal activation in forebrain regions mediating fear and/or anxiety and are dependent on the production of TNF α . Male C57BL/6J mice were given intraperitoneal injections of 10 μ g of SEA and 5 μ g of lipopolysaccharide (LPS) or saline and perfused 2 h later for histochemical determination of brain c-Fos immunoreactivity (IR). The results showed increased c-Fos IR in the paraventricular nucleus, arcuate nucleus, central nucleus of the amygdala, bed nucleus of the stria terminalis, and lateral septum. Challenge of TNF $^{-/-}$ mice with SEA did not produce a significant increase in brain c-Fos IR, although c-Fos was increased after exposure to a psychogenic stressor (i.e., open field). In additional experiments, the elevated corticosterone response to SEA was abrogated in TNF $^{-/-}$ mice and was shown to be corticotropin-releasing hormone dependent. Finally, the augmented reduction in novel food intake after SEA challenge was attenuated in TNF $^{-/-}$ mice as well as in wild-type mice administered antibody to TNF α . In conclusion, challenge with SEA recruits brain regions mediating stress and anxiety responses, an effect that requires endogenous TNF α . Whether this is indicative of all T-cell superantigens remains to be determined, although it stands in contrast to other models of neuroimmunomodulation (e.g., LPS) that involve multiple cytokine influences.

Key words: anorexia; corticosterone; immunity; limbic; neuromodulation; tumor necrosis factor; staphylococcal enterotoxin A

Introduction

Superantigens (SAGs) are microbial products implicated in toxic-shock syndrome and autoimmune disease (Torres et al., 2001). The best characterized SAGs are the staphylococcal enterotoxins (SEs) (Proft and Fraser, 2003) and, in particular, staphylococcal enterotoxin A (SEA) and B (SEB). These SEs display affinity for distinct V β regions on human and murine T-lymphocyte receptors (Marrack et al., 1993; Torres et al., 2001; Petersson et al., 2004) and in the mouse, stimulate V β 3⁺ and V β 8⁺ T-cells, respectively, in a major histocompatibility complex class II restricted manner (Gonzalo et al., 1993). Moreover, challenge with SEA and SEB induces high plasma levels of cytokines, such as tumor necrosis factor- α (TNF α), interleukin-2 (IL-2), and interferon- γ (IFN γ) (Bette et al., 1993; Rosendahl et al., 1997).

Considerable evidence shows that administration of SEA or SEB exerts neurobiological effects. This includes activation of the hypothalamic–pituitary–adrenal (HPA) axis, sympathetic nervous system, anorexic behavior, altered nociception, and pyro-

genesis (Gonzalo et al., 1993; Shurin et al., 1997; Kusnecov et al., 1999; Goehler et al., 2001; Del Rey et al., 2002; Kawashima and Kusnecov, 2002; Kawashima et al., 2002; Pacheco-Lopez et al., 2004). Studies in rats also showed that SEB activates a number of limbic brain regions, including the lateral septum (LS), central amygdala (ceA), and paraventricular nucleus (PVN) (Goehler et al., 2001; Wang et al., 2004), which is consistent with murine studies in which SEB increased hypothalamic and amygdaloid corticotropin-releasing hormone (CRH) mRNA (Kusnecov et al., 1999). These neuroanatomical data imply the induction of anxiety/fear-like responses and anorexia (Davis, 1998; Sheehan et al., 2004) after superantigenic challenge. Indeed, although SEA and SEB induce anorexia, this appears to be independent of malaise and more likely is a result of augmented novelty-induced arousal (Kusnecov et al., 1999; Kawashima and Kusnecov, 2002; Rossi-George et al., 2004).

The mechanism by which SAGs impact the CNS likely involves endogenous cytokine production. In mice, IL-2 does not activate the HPA axis (Zalcman et al., 1994; Kusnecov et al., 1999), whereas the more likely candidate, IL-1 β , is not detected in plasma after SAG challenge (Kawashima and Kusnecov, 2002). Alternatively, SEA elevates circulating TNF α (Kawashima and Kusnecov, 2002), a cytokine capable of increasing plasma corticosterone, hypophagia, and brain c-fos (Bluthe et al., 1994; Ando and Dunn, 1999; Hayley et al., 2001, 2002). Therefore, it was

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hypothesized that the primary influence for the CNS effects of SAg challenge may reside with TNF α .

To test this hypothesis, the present study used TNF α knock-out animals generated on a C57BL/6J background. Consequently, SEA was used because it reliably activates T-cells and TNF α production in C57BL/6 mice (Rosendahl et al., 1997; Kawashima and Kusnecov, 2002). Assessment of neuronal activation in limbic forebrain regions involved immunohistochemical detection of the immediate-early gene *c-fos*. We report for the first time that SEA challenge increased *c-Fos* immunoreactivity (IR) in key brain regions implicated in stress and regulation of ingestive behavior. Moreover, the findings revealed that in contrast to lipopolysaccharide (LPS) (Perlstein et al., 1993; Ebisui et al., 1994; Turnbull and Rivier, 1998), neurobehavioral and endocrine changes pursuant to SEA challenge were fully dependent on the production of TNF α .

Materials and Methods

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5–6 weeks of age and housed four per cage. TNF α -deficient and IL-1 receptor 1 (IL-1RI)-deficient mice bearing a C57BL/6 background were also obtained from The Jackson Laboratory. The TNF $^{-/-}$ population was expanded by backcrossing with C57BL/6J mice, followed by maintenance of breeding between F1 heterozygous (TNF $^{+/-}$) males and females, with the knock-out and wild-type (TNF $^{+/+}$) members of the F2 generation selected for experimentation. Animals were weaned at postnatal day 21 and housed in same-sex groups until genotyping (within 1–2 weeks). Subsequent to genotyping, animals were further subdivided into groups matched for age and genotype. For all experiments involving C57BL/6J mice ordered from the vendor (i.e., not bred in our animal facility), ~2 weeks of acclimation was provided before testing. In some experiments, animals were subjected to a daily food deprivation schedule (see below). Otherwise, animals were housed four per cage under 12 h light/dark illumination (lights on at 5:00 A.M.) and *ad libitum* access to food and water. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Rutgers University.

Genotyping of TNF α -deficient mice

Shortly after weaning, 2–3 mm tail sections were excised and subjected to DNA extraction by overnight digestion at 54°C in 200 μ l of lysis buffer containing 100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 1 mg/ml proteinase K. The samples were then centrifuged at 1200 rpm for 5 min, and the DNA was precipitated from the supernatant with isopropanol, rinsed with 70% ethanol, and resuspended in 100 μ l of Tris-EDTA buffer, pH 8. Five microliters of DNA were devoted to a 50 μ l PCR using the TaqPCR Master Mix kit (Qiagen, Valencia, CA). The primer sequences and protocol for the TNF tm1Gk1 were obtained from The Jackson Laboratory, and the oligonucleotides were synthesized by the DNA synthesis facility at the University of Medicine and Dentistry of New Jersey. PCR-amplified DNA was separated by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide and read using the EDAS120 Kodak Gel Imaging System (Eastman Kodak, Rochester, NY). The genotype of the subjects was determined as follows: knock-out, single product of 146 bp; heterozygous, two products of 146 and 280 bp; wild-type, single product of 280 bp. Once the animals were genotyped, they were housed four per cage corresponding to their genotype and allowed to acclimate for at least 2 weeks.

Reagents

SEA, LPS (055:B5), and astressin were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine IL-1 β was purchased from R & D Systems (Minneapolis, MN). Hamster anti-murine TNF α IgG antibody was kindly provided by the Biological Resources Branch, National Cancer Institute (Bethesda, MD). Hamster IgG isotype control was purchased from Fisher Scientific (Pittsburgh, PA). Antibodies were administered intraperitoneally.

Immunological and psychogenic stressor exposure

Immunological stressors. In experiments examining the effects of immunological challenge on CNS activation, animals were challenged with 10 μ g of SEA, 5 μ g of LPS, or 100 ng of IL-1 β . After injection, wild-type and transgenic mice were (1) perfused for *c-Fos* immunohistochemistry (only for experiments involving SEA and LPS treatment), (2) killed by decapitation for measurement of corticosterone, or (3) tested for food intake. In most experiments, killing the mice or testing took place 2 h after injection, because this was previously shown to be the optimal time for increased pituitary–adrenal activation (Kawashima and Kusnecov, 2002). For experiments involving IL-1 β , ingestive behavior was assessed 1 h after injection, whereas in other experiments, corticosterone measures were taken 2 h after injection. In all experiments, vehicle controls consisted of animals that were given injections of pyrogen-free saline.

Exposure to an open field. To confirm whether TNF $^{-/-}$ mice were capable of responding to a psychogenic stressor with increased recruitment of neuronal activity in limbic brain regions, both wild-type and knock-out mice were exposed for 15 min to an open field as described previously (Kawashima and Kusnecov, 2002) and returned to their home cage for 45 min. Subsequently, mice were perfused, and the brains were assayed for *c-Fos* IR as described below.

c-Fos immunocytochemistry

The procedure for immunohistochemical detection of *c-Fos*-immunoreactive cells conformed to previously published methods (Pezzone et al., 1992). Animals were anesthetized with 50 mg/kg sodium pentobarbital (Nembutal) and perfused transcardially for 3–5 min with isotonic saline containing 2% sodium nitrite. This was followed by a 5–10 min perfusion with 4% paraformaldehyde containing 2.5% Acrolein (Polysciences, Warrington, PA). After an additional 5 min rinse with sodium nitrite, the brains were removed and stored at 4°C in 5 ml of 25% sucrose solution until ready to be sectioned for immunocytochemistry. Anterior-to-posterior coronal brain sections (25 μ m thickness) were collected using a freezing microtome. Sectioning commenced from the prefrontal cortical region anterior to the anterior commissure and terminated at the posterior end of the diencephalon. The sections were cryopreserved as free-floating sections in a –20°C freezer until ready for assay.

For the assay, tissue sections were incubated initially for 72 h at 4°C in 0.4% Triton X-100 in PBS, pH 7.2, containing a 1:15,000 final dilution of rabbit anti-human Fos antibody (Oncogene Science, Cambridge, MA). This antibody is directed at the N-terminal region of Fos. After rinsing in PBS, the tissues were incubated for 1 h at room temperature in PBS/0.4% Triton X-100 containing a 1:500 final dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). After an additional rinse, tissues were treated for 1 h at room temperature with an avidin–biotin–peroxidase complex solution from the Vector Elite ABC kit (Vector Laboratories), followed by sequential washes in PBS and 0.175 M sodium acetate (NaOAc). The enzyme–substrate reaction was subsequently generated by the addition of a 3,3'-diaminobenzidine (DAB) substrate solution consisting of 0.175 M NaOAc containing 25 mg/ml NiSO $_4$, 0.2 mg/ml DAB, and 0.28% H $_2$ O $_2$. The addition of NiSO $_4$ allowed for dark blue to black stains to become visible during the reaction process, which were allowed to proceed for 10–20 min. Termination of the reaction was achieved by rinsing the tissues in 0.175 M NaOAc and then in PBS, after which the tissues were mounted on Superfrost slides (Fisher Scientific), dehydrated in a graded series of alcohols, and clarified and coverslipped using HistoClear and HistoMount (VWR Scientific, Westchester, PA).

For quantitation of *c-Fos*-immunoreactive cells, stained slides were examined under a Nikon Eclipse E400 microscope equipped with a high-resolution CCD camera. Neuroanatomically distinct regions as defined by the mouse atlas of Franklin and Paxinos (1997) were digitally captured, and immunopositive cells were enumerated using the NIH Image software program. Up to four to six sections per animal for each region of interest were quantified bilaterally and then averaged. Demarcation of nuclei was conducted by two observers who were blind to group treatments. Confirmation of software accuracy was conducted by two observers hand-counting randomly selected regions of interest. Variation in number between human and software counts was not >1–2%.

Behavioral testing for food consumption

Consumption of a liquid diet. Animals were tested for consumption of Prosobee, a commercially available baby liquid formula (Mead Johnson, Evansville, IN). This solution is highly palatable and readily consumed by mice without the need for food and water deprivation (Kusnecov et al., 1999; Kawashima and Kusnecov, 2002; Kaneta and Kusnecov, 2005). This approach is consistent with other laboratories that have used short-term exposure to chocolate milk or sweetened condensed milk to examine ingestive responses to immunological stimuli (Dunn and Swiergel, 1998; Hayley et al., 2002). Preparation of full-strength Prosobee was according to instructions provided on the product label. Before giving animals Prosobee, the solution was diluted 1:2 using distilled water. For measurement of consumption, each animal was placed for 1 h in an opaque box with a preweighed bottle of Prosobee, after which the animals were returned to their home cages. The bottles were then weighed to determine the amount consumed. Exposure to the bottle of Prosobee occurred 1–2 h after injection. That is, testing for the effects of SEA commenced 2 h after injection, whereas 1 h was allowed to elapse before animals were tested after IL-1 β injection (see Fig. 6B).

Consumption of food pellets. This experiment tested the effect of SEA challenge on consumption of small 20 mg food pellets (Noyes pellets; Research Diets, New Brunswick, NJ) to which animals were previously oriented (familiar group, $n = 20$) or remained naive (novel group, $n = 20$). Wild-type C57BL/6 mice were housed four per cage after arriving in the laboratory and during acclimation received *ad libitum* access to food and water. After commencement of the experiment, cages were allocated randomly to the different food preexposure conditions, and food was restricted for 3 d to a daily 4 h period that occurred before the onset of darkness. There was no restriction of availability to water. Subjects in the familiar group received in their home cage exposure to a glass Petri dish containing 10 g of the Noyes 20 mg food pellets. No other food was provided. Animals that were not oriented (i.e., the novel group) to the Noyes pellets ate regular laboratory chow (a pile of 3.5–4.5 g LabDiet pellets) accessible through the metal cage top. On the test day, one-half of the animals in the familiar and novel groups was given injections of 10 μ g of SEA, and the other half was given injections of saline, after which they were returned to the home cage without any exposure to food (i.e., the deprivation period was still in effect). Two hours after injection, the animals were removed from their home cage and placed individually in a test apparatus containing at one end 10 g of Noyes food pellets in the glass Petri dish. The apparatus was a metal chamber fitted with a metal grid floor and measuring 38 $\times$ 25 $\times$ 18 cm (length by width by height). Multiple test chambers were available to allow for cohorts of SEA- and saline-injected animals to be tested at the same time. Each animal was placed in the apparatus initially at the end opposite the location of the Petri dish. Animals were allowed to investigate and consume the food pellets for 30 min, after which they were returned to the home cage. After removal of the animal, the contents of the Petri dish were weighed, and adjustments were made after weighing spilled food captured by a sheet of white paper ($\sim$ 19 $\times$ 25 cm) placed immediately below the area where the Petri dish was located. Because of the large number of animals, the experiment involved testing two cohorts containing equal numbers of animals (i.e., $n = 20$). Each cohort was tested on succeeding days, although equal representation of all injection and novelty/familiarity conditions was present in each cohort: SEA/novel pellets ($n = 5$); SEA/familiar pellets ($n = 5$); saline/novel pellets ($n = 5$); saline/familiar pellets ($n = 5$). There were no differences between the cohorts, and therefore the results were pooled (see Results and Fig. 7).

Testing of TNF $^{-/-}$ animals. The preceding experiment was repeated using TNF-deficient animals. However, no groups were included that received home-cage preexposure to the pellets. Therefore, animals were subjected to the same regimen of daily restricted food intake of regular laboratory chow. On the test day, they were given injections of SEA ($n = 8$) or saline ($n = 7$) as described above and tested 2 h later for consumption of novel pellets presented in the test apparatus.

Measurement of plasma corticosterone, TNF α , and IFN γ

Trunk blood was collected by rapid decapitation into heparin-treated vacutainer tubes (Becton Dickinson, Rutherford, NJ). The blood was

Table 1. PCR primers (all sequences are 5' to 3')

Gene amplified	Forward primer	Reverse primer
IFN γ	agctcatccgagtggtccac	gcttctcaggctggattcc
IL-2	gtcaacagcgaccactt	tgcttcgctgtagagcttg
GAPDH	aactccctcaagattgtcagcaa	ggctaagcagtggtgtgtgc

centrifuged immediately at 2000 rpm for 15 min, and the plasma was collected and stored at -70°C . Corticosterone was measured by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA), and TNF α and IFN γ were measured using a Quantikine ELISA kit from R & D Systems.

Reverse transcription and real-time PCR for splenic IFN- γ and IL-2 mRNA

Confirmation of T-cell activation in TNF-deficient mice challenged with SEA was conducted by real-time PCR. Relative quantitation of spleen mRNA for the T-cell cytokines IFN γ and IL-2 used a validated quantitative reverse transcription (RT)-PCR method as described previously by others at the Keck Center for Collaborative Neuroscience, Rutgers University (Pan et al., 2004). Wild-type ($n = 8$) and mutant ($n = 8$) mice were given injections of saline ($n = 4$ /strain) or 10 μ g of SEA ($n = 4$ /strain), and the spleens removed 2 h later. Total RNA was extracted from all 16 spleens in a single run using a Totally RNA extraction kit (Ambion, Austin, TX) followed by quantification using Ribogreen (Molecular Probes, Eugene, OR). Generation of cDNA from 1 μ g of RNA was accomplished with the Advantage RT-for-PCR kit (BD Biosciences Clontech, Palo Alto, CA), followed by real-time PCR using mRNA primers for IFN γ , IL-2, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR reactions were performed in a 10 μ l volume using 5 ng of cDNA, 25 nM of reverse and forward primers (see Table 1 for PCR primer sequences), and PCR master mix containing the fluorescence agent SYBR Green (Applied Biosystems, Warrington, UK). Real-time PCR was conducted using an Applied Biosystems 7900HT system, with threshold cycles for each sample being compared with a standard curve. The standard curve was generated using a fourfold dilution series of cDNA from spleen RNA obtained from an animal that was given an injection of SEA (undiluted control cDNA, 10 arbitrary units). This allowed for comparative or relative quantification of starting gene-of-interest cDNA in each of the samples. The same control cDNA was used to generate separate standard curves for each primer set. Analysis of the melting point for each sample revealed the presence of only a single amplified product. For each gene of interest, the sample data were expressed in arbitrary units based on the standard curve.

Data analysis

Most experiments conformed to a factorial design and were therefore analyzed by ANOVA using Statview, a statistical software package. The c-Fos data were expressed as the number of c-Fos-immunoreactive cells per brain region, whereas food intake was measured in grams. Finally, corticosterone was expressed as nanograms of plasma per milliliter. *Post hoc* comparisons were conducted using the Fisher's least significant difference test when the omnibus F achieved a 0.05 level of significance. In some cases, in which a priori predictions based on previous data were warranted, an unpaired t test was conducted.

Results

Brain c-Fos IR

Mice were challenged with either saline, 10 μ g of SEA, or 5 μ g of LPS and killed 2 h later for assessment of brain c-Fos IR. A group challenged with LPS was used as a positive comparison, because LPS has been shown to increase brain c-Fos expression in C57BL/6 mice (Laflamme et al., 1997; Matsuoka et al., 2003; Reyes et al., 2003). Hypothalamic nuclei were examined that are associated with ingestive behavior and stress responses. Of the five hypothalamic nuclei that were analyzed [PVN, arcuate nucleus, dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), lateral hypothalamus (LH)], the PVN ($F_{(2,12)} = 11.9$; $p < 0.001$) and arcuate nucleus ($F_{(2,12)} =$

Figure 1. Mean number ($\pm$ SE) of c-Fos-immunoreactive cells in hypothalamic nuclei 2 h after intraperitoneal injection with saline ($n = 5$), 10 μ g of SEA ($n = 6$), or 5 μ g of LPS ($n = 4$). Each bar represents the mean $\pm$ SE. Arc, Arcuate nucleus. * $p < 0.05$ relative to saline.

Figure 2. Mean number ($\pm$ SE) of c-Fos-immunoreactive cells in the LS, CeA, and BNST 2 h after intraperitoneal injection with saline ($n = 5$), 10 μ g of SEA ($n = 6$), or 5 μ g of LPS ($n = 4$). Each bar represents the mean $\pm$ SE. * $p < 0.05$ relative to saline.

6.15; $p < 0.05$) showed significantly increased numbers of c-Fos-immunoreactive cells after SEA and LPS challenge (Fig. 1). However, in neither SEA- nor LPS-treated animals did the VMH and LH neurons show increased c-Fos IR relative to saline controls (Fig. 1). Interestingly, although a one-way ANOVA revealed a main effect of toxin treatment on c-Fos IR in the DMH ($F_{(2,12)} = 13.7$; $p < 0.001$), *post hoc* analyses revealed this to be attributable to LPS treatment (Fig. 1).

Extrahypothalamic forebrain regions that mediate emotional responses such as fear and anxiety were also examined, with the focus directed on the lateral septum (LS), bed nucleus of the stria terminalis (BNST), and CeA. As shown in Figure 2, the results revealed that both SEA and LPS induced significant increases in the number of c-Fos-immunoreactive cells in all three regions (BNST: $F_{(2,12)} = 11.15$, $p < 0.005$; LS: $F_{(2,12)} = 16.1$, $p < 0.001$; CeA: $F_{(2,12)} = 4.73$, $p < 0.05$).

Administration of 1–10 μ g of SEA to C57BL/6J mice produces by 1 h after injection a significant elevation in plasma TNF α that persists for several hours (Table 2). This is consistent with our previous observations and occurs in the absence of detectable IL-1 β in plasma (Kawashima and Kusnecov, 2002), which typically rises coincidentally with TNF α in response to LPS challenge (Netea et al., 1996). Therefore, a second experiment was conducted in which TNF $^{-/-}$ mice were challenged with either 10 μ g of SEA or saline. The results showed that in TNF $^{-/-}$ mice, exposure to SEA did not increase the number of c-Fos-immunoreactive cells in brain regions that were shown to be activated in wild-type TNF $^{+/+}$. This is shown in Figures 3 and 4, with the latter showing the percentage of change from saline controls in the number of c-Fos-immunoreactive cells from SEA-challenged TNF $^{+/+}$ and TNF $^{-/-}$ mice. Corresponding photomicrographs of c-Fos expression in representative wild-type

Table 2. Time course of plasma TNF α elevations after SEA challenge

Dose ^a	Time after challenge (h)	Plasma TNF α (pg/ml $\pm$ SE)
1 μ g ($n = 3$ /time point)	1	44.2 $\pm$ 7.2
	2	66.8 $\pm$ 7.5
	4	17.3 $\pm$ 3.7
5 μ g ($n = 3$ /time point)	1	79.5 $\pm$ 19.7
	2	87.7 $\pm$ 12.6
	4	26.8 $\pm$ 8.8
10 μ g ($n = 3$ /time point)	1	58.6 $\pm$ 7.6
	2	80.8 $\pm$ 8.5
	4	20.9 $\pm$ 1.5

^aControl animals ($n = 3$ /time point) were given injections of saline. ELISA results revealed no detectable TNF.

Figure 3. Brain c-Fos IR in SEA-challenged (10 μ g/mouse) wild-type (TNF $^{+/+}$) and TNF α knock-out (TNF $^{-/-}$) mice. Representative images of the LS (A, D), CeA (B, E), and BNST (C, F) are shown. The images display the greatest numbers of c-Fos-immunoreactive cells in wild-type mice (A–C) compared with knock-out mice (D–F). Magnification, 10 $\times$. LV, Lateral ventricle; ac, anterior commissure.

and knock-out mice challenged with SEA or saline are presented in Figure 3 (see Fig. 5 for the PVN).

To confirm that TNF $^{-/-}$ mice were not deficient in c-Fos induction, an experiment was conducted in which knock-out and wild-type mice were exposed to a psychogenic stressor (i.e., an open field) and killed 1 h later. Increased c-Fos IR was widespread throughout the brain. Quantitative data for the PVN is presented in Figure 5, where it can be seen that both TNF $^{+/+}$ and TNF $^{-/-}$ mice displayed significantly increased numbers of c-Fos-immunoreactive cells after exposure to the open field ($F_{(1,14)} = 12.89$; $p < 0.05$).

Food intake

Previously, it was demonstrated that 5 μ g of SEA administered to male C57BL/6J mice reduced consumption of a novel liquid diet

Figure 4. Percentage increase in the number of c-Fos-immunoreactive cells in the brains of SEA-challenged wild-type ($TNF^{+/+}$; $n = 6$) and $TNF\alpha$ knock-out ($TNF^{-/-}$; $n = 5$) mice. For each individual animal that was given an injection of SEA, the quantitation for each brain region was expressed as a percentage above the group mean of the corresponding saline-injected control of the same strain. The mean number of c-Fos-positive cells in wild-type saline-injected ($n = 5$) (see Fig. 1) and saline-injected $TNF\alpha$ knock-out mice ($n = 5$) did not differ (mean $\pm$ SE for TNF knock-out saline: PVN, 60 ± 34 ; arcuate nucleus, 55 ± 12 ; LS, 58 ± 36 ; BNST, 10.7 ± 2.2 ; ceA, 8.9 ± 1.9). * $p < 0.05$ (unpaired Student's t test) relative to the mean percentage of change of the $TNF\alpha$ knock-out group. Arc, Arcuate nucleus.

Figure 5. Increased c-Fos IR in the PVN of wild-type and $TNF\alpha$ knock-out mice exposed to a psychogenic stressor (open field). Relative to wild-type mice challenged with SEA (**A**), similarly challenged $TNF\alpha$ knock-out mice failed to increase c-Fos IR in the PVN (**B**) but displayed a robust increase after exposure to the open field (OF; **C**). **D**, Quantitation of the number of c-Fos-immunoreactive cells in wild-type and $TNF\alpha$ knock-out mice revealed no differences in magnitude of responsiveness to open-field exposure (each bar represents mean $\pm$ SE for $n = 5$ animals). Magnification, **A–C**, $10\times$.

(Kawashima and Kusnecov, 2002). The foregoing c-Fos data suggest that $TNF\alpha$ production in response to SEA may be important to the effects of SEA on food intake.

Liquid food experiments

The anorexic (or hypophagic) effect of SEA was assessed by challenging wild-type $TNF^{+/+}$ and mutant $TNF^{-/-}$ mice with SEA or saline and testing 2 h later for food intake. The results revealed a main effect of SEA treatment ($F_{(1,20)} = 20.84$; $p < 0.001$) that interacted with mouse genotype ($F_{(1,20)} = 10.29$; $p < 0.001$). Figure 6A presents these results, where it can be seen that wild-type mice challenged with SEA showed a reduction in food intake, whereas SEA-challenged $TNF^{-/-}$ knock-out mice did not differ from corresponding saline-injected controls.

This lack of SEA effect in $TNF^{-/-}$ mice may have been a result of an inherent failure to respond to anorexigenic signals. How-

ever, as shown in Figure 6B, both knock-out and wild-type mice that were given injections of 100 ng of recombinant murine IL-1 β displayed a similar reduction in food intake ($F_{(1,18)} = 100.1$; $p < 0.0001$). There was no interaction between IL-1 β treatment and genotype ($F_{(1,18)} = 0.706$; $p = 0.4$). Hence, these data ruled out the possibility that $TNF^{-/-}$ mice do not respond to anorexigenic signals.

In an additional assessment of the role of $TNF\alpha$ in SEA-induced hypophagic behavior, wild-type C57BL/6J mice were intraperitoneally administered hamster anti-murine $TNF\alpha$ antibody before challenge with SEA. The results are presented in Figure 6C. Antibody treatment significantly attenuated the reduction in Prosobee consumption after SEA, as revealed by significant main effects ($F_{(1,15)} = 27.3$; $p < 0.001$) and interaction effects ($F_{(1,15)} = 9.0$; $p < 0.01$). These results complemented the consumption data obtained with $TNF^{-/-}$ mice and strongly suggest that the hypophagic influence of SEA on food intake is dependent on $TNF\alpha$.

Food pellet experiments

An additional experiment was conducted to determine whether SEA challenge would alter ingestion of solid food pellets, as opposed to a liquid diet. To this end, wild-type $TNF^{+/+}$ mice were familiarized to small (20 mg) food pellets in their home cages or encountered the food pellets for the first time during testing. Animals were tested for food pellet intake 120 min after injection with 10 μ g of SEA. The results are presented in Figure 7A. Statistical analysis revealed a main effect of pellet novelty/familiarity ($F_{(1,36)} = 180.0$; $p < 0.0001$), in that greater consumption was observed in animals that had been familiarized with the food pellets. In addition, there was a significant interaction between SEA treatment and pellet novelty/familiarity ($F_{(1,36)} = 13.3$; $p < 0.001$). Because we had observed previously that Prosobee consumption was reduced in response to SEA, a planned unpaired t test was conducted comparing SEA- and saline-treated animals in the novel pellet condition. As shown in Figure 7A, the difference between SEA- and saline-treated animals was found to be significant ($t_{(18)} = -2.71$; $p = 0.015$). These data support previous experiments using Prosobee consumption, in that exposure to unfamiliar food substances elicits a greater neophobic response in SEA-treated animals (Kawashima and Kusnecov, 2002).

An additional experiment was conducted using $TNF^{-/-}$ mice that had not been preexposed in their home cage to the small food pellets. As shown in Figure 7B, SEA- and saline-injected animals did not differ significantly in the amount of novel pellet ingestion. This supported the results of the Prosobee ingestion experiments involving $TNF^{-/-}$ mice (Fig. 6A).

Role of endogenous IL-1

Previously, we had reported an inability to detect (by ELISA) plasma IL-1 β in SEA-challenged mice (Kawashima and Kusnecov, 2002). However, mRNA for IL-1 β was increased in the spleen after 1–2 h (data not shown). Consequently, we explored the possibility that low concentrations of IL-1 β may still be neuromodulatory in SEA-induced hypophagia. The effects of SEA on food intake were tested in mice lacking IL-1RI (IL-1R $^{-/-}$), which mediates the behavioral effects of IL-1 β (Bluthe et al., 2000). The results showed that IL-1R $^{-/-}$ mice responded to 10 μ g of SEA with a marked reduction in consumption of Prosobee solution [mean consumption: saline ($n = 4$), 2.18 ± 0.29 g; SEA ($n = 5$), 0.43 ± 0.07 g; $t_{(7)} = -1.75$; $p < 0.001$]. Therefore, it is unlikely that reduced food intake after SEA involves engagement of IL-1RI.

Figure 6. Consumption of a novel liquid diet (Prosobee) in wild-type ($TNF^{+/+}$) and $TNF\alpha$ knock-out ($TNF^{-/-}$) mice. **A**, Mean intake ($\pm$ SE) of Prosobee in wild-type and knock-out mice after injection of 10 μ g of SEA ($n = 6$ per strain) or saline ($n = 5$ –7 per strain). **B**, Mean intake ($\pm$ SE) of Prosobee in wild-type and knock-out mice after injection of 100 ng of murine recombinant IL-1 β ($n = 6$ per strain) or saline ($n = 5$ per strain). **C**, Effect of pretreatment with hamster IgG (SEA, $n = 5$; saline, $n = 4$) or hamster anti-mouse $TNF\alpha$ antibody (SEA, $n = 6$; saline, $n = 4$) on mean intake ($\pm$ SE) of Prosobee in wild-type C57BL/6J mice that were given injections of 10 μ g of SEA or saline. * $p < 0.05$ (unpaired Student's t test) relative to corresponding saline control. Wt, Wild-type; ko, knock-out.

Figure 7. **A**, Consumption of novel and familiar food pellets after challenge of wild-type mice ($TNF^{+/+}$) with 10 μ g of SEA or saline ($n = 10$ /group). **B**, Effect of injection with SEA ($n = 8$) or saline ($n = 7$) on intake of novel food pellets by TNF knock-out mice ($TNF^{-/-}$). The knock-out mice were not tested for consumption of familiar food pellets, because SEA did not reduce consumption of familiar pellets in wild-type mice. In both experiments, testing commenced 2 h after injection. Data represents mean intake $\pm$ SE.

Corticosterone

Challenge with SEA activates the pituitary–adrenal axis, producing increased plasma levels of ACTH and corticosterone (Kawashima and Kusnecov, 2002; Kaneta and Kusnecov, 2005). To determine whether the lack of PVN c-Fos expression in $TNF^{-/-}$ mice (Figs. 4, 5) also resulted in corresponding endocrine changes, plasma corticosterone was measured 2 h after injection of 10 μ g of SEA into wild-type and knock-out mice. The results showed a significant increase in plasma corticosterone in wild-type mice challenged with SEA ($F_{(1,20)} = 20.68$; $p < 0.001$). This was attenuated in SEA-challenged $TNF^{-/-}$ mice, as supported by a genotype and toxin interaction ($F_{(1,20)} = 13.18$; $p < 0.01$) (Fig. 8A). Furthermore, it was shown that $TNF^{-/-}$ mice are not deficient in adrenocorticoid reactivity, as tested by injection of 100 ng of recombinant murine IL-1 β , which significantly elevated plasma corticosterone in both mouse strains (Fig. 8B).

Finally, the likelihood that SEA challenge increased plasma

corticosterone via hypothalamic release of CRH into the hypophyseal portal system was tested by pretreating wild-type mice with 500 μ g/kg astressin, a nonselective CRH receptor antagonist. Figure 8C shows that astressin significantly attenuated the increase in plasma corticosterone levels after SEA injection (toxin treatment: $F_{(1,20)} = 26.96$, $p < 0.0001$; astressin-by-toxin interaction: $F_{(1,20)} = 8.83$; $p < 0.01$). Therefore, it is likely that SEA activation of the PVN (Figs. 1, 4) results in an increased release of neurosecretory CRH.

T-cell activation by SEA in $TNF^{-/-}$ mice

It was possible that the failure of SEA challenge to increase neuronal c-Fos IR in $TNF^{-/-}$ mice was attributable to deficient

activation of T-cells. Therefore, mice were challenged with 10 μ g of SEA and killed 2 h later, and the spleens were removed for assessment of mRNA changes for IL-2 and IFN γ , cytokines exclusively produced by T-cells (Rosendahl et al., 1997). The results are presented in Table 3. Quantitation of mRNA revealed no difference in the amount of IL-2 nor IFN γ mRNA in the spleens of wild-type and mutant mice challenged with SEA. Interestingly, there was a difference in the GAPDH values between $TNF^{-/-}$ and $TNF^{+/+}$ animals, in that mutant mice (whether SEA or saline treated) had higher values than wild-type animals. However, within each strain, GAPDH did not change as a function of SEA challenge. This allowed amplified cytokine product to be compared accurately with GAPDH (Table 3, Normalization) without the possibility of distortion by SEA treatment. As seen in Table 3, the relationship of the normalizing gene (GAPDH) to the cytokine genes did not change the fact that spleens from SEA-treated animals had dramatically higher cytokine responses relative to saline-treated animals. Moreover, this was equivalent in mutant and wild-type mice.

The latter observation was reinforced by measurement of plasma for IFN γ (insufficient plasma was available for IL-2 determinations). This revealed a similar lack of difference between the two strains (Table 3). Therefore, the immune compartment of $TNF^{-/-}$ mice was competent in the ability to present SEA and activate T-lymphocytes, ruling out the possibility that these mice were immunologically unresponsive.

Discussion

Previous studies had confirmed that SEA, a T-cell SAg, exerts significant effects on pituitary–adrenal activation and neophobic reactivity to gustatory and contextual stimuli (Kawashima and Kusnecov, 2002; Kawashima et al., 2002). The present results suggest that these behavioral and endocrine effects involve underlying recruitment of brain nuclei that coordinate the CNS response to psychogenic and systemic stressors. Moreover, they suggest that production of $TNF\alpha$ is a necessary condition for these and other behavioral and endocrine effects to be observed after T-cell activation with SEA.

In so far as immune reactivity to SEA recruited key forebrain regions involved in stress, the hypothalamus was especially reactive in the PVN and arcuate subdivisions. Interestingly, c-Fos IR remained unchanged in the VMH and LH, areas traditionally implicated in appetite regulation (Elmquist et al., 1999; Schwartz et al., 2000). Therefore, SEA-induced hypophagia may use alter-

native neural substrates regulating food intake, such as the arcuate, which is pivotal to controlling food intake (Schwartz et al., 2000). Because the arcuate provides afferent connections to the PVN, it is plausible that populations of cells expressing anorexigenic peptides (e.g., CRH) were activated by SEA challenge. However, despite activation by nonimmunological (e.g., immobilization or restraint) and immunological (e.g., IL-1) stressors, the relevance of the arcuate to immune-derived anorexia has been questioned (Reyes and Sawchenko, 2002).

The activation of PVN neurons by SEA suggests possible involvement of CRH-positive neurons. Because the nonselective CRH receptor antagonist astressin (Guliyas et al., 1995) blocked the corticosterone response to SEA, it is likely that CRH-secreting neurons in the PVN were activated. Consideration should also be given to the BNST, another region activated by SEA that influences hypophagia via local CRH receptor engagement (Ciccocioppo et al., 2003). Indeed, central CRH receptor blockade abrogates anorexia induced by SEA (Kaneta and Kusnecov, 2005), although the relevant sites of CRH receptor engagement are not known.

The c-Fos data replicate the results of another study involving rats administered SEB (Wang et al., 2004), which stimulates a different subpopulation of T-cells than SEA (Gonzalo et al., 1993). Administration of SEB stimulated brain regions that we showed to be activated in mice given SEA (Wang et al., 2004). This is also in keeping with our previous data that SEB showed increased c-Fos and CRH mRNA expression in the PVN and ceA (Shurin et al., 1997; Kusnecov et al., 1999). Interestingly, the impact of SEB on central c-Fos expression in rats was attenuated by subdiaphragmatic vagotomy (Wang et al., 2004). This suggests that superantigenic effects on CNS function involve communication by vagal afferents, although it remains to be determined whether the effects of SEA in mice are similarly dependent on this mechanism. Indeed, given that the present results reveal a dependence on TNF α production, it is noteworthy that the neuroendocrine effects of exogenous TNF α rely on an intact vagus (Fleshner et al., 1998).

The hypophagia induced by SEA (as well as SEB) appears to be dependent on the novelty of palatable liquid diet (Kusnecov et al., 1999; Kawashima and Kusnecov, 2002). We extended this observation, demonstrating that SEA reduced voluntary ingestion of novel, but not familiar, food pellets, and it should also be noted that SEA does not affect operant responding for familiar food pellets (our unpublished observations). Therefore, illness-induced anorexia is unlikely to be an explanation for the reduction in food intake. More likely, the data suggest that anxiety/fear-like processes underlying neophobic reactivity may be augmented by SEA treatment, thereby promoting anorexia and/or greater avoidance of a novel diet. This may involve central CRH receptors (Kaneta and Kusnecov, 2005), because CRH also attenuates ingestion of novel foods (Heinrichs and Koob, 1992). Moreover, the SEA-induced activation of neural

Figure 8. Plasma corticosterone levels (nanograms per milliliter) in SEA- and saline-treated wild-type (TNF $^{+/+}$) and TNF α knock-out (TNF $^{-/-}$) mice. **A**, Mean plasma corticosterone ($\pm$ SE) in wild-type and knock-out mice after injection of 10 μ g of SEA ($n = 5$ –6 per strain) or saline ($n = 6$ per strain). **B**, Mean plasma corticosterone ($\pm$ SE) in wild-type and knock-out mice after injection of 100 ng of murine recombinant IL-1 β ($n = 5$ –6 per strain) or saline ($n = 6$ per strain). **C**, Effect of pretreatment with astressin (500 μ g/kg) or vehicle (0.2 ml of saline) on mean plasma corticosterone ($\pm$ SE) in wild-type C57BL/6J mice that were given injections of 10 μ g of SEA ($n = 5$ astressin; $n = 6$ vehicle) or saline ($n = 6$ astressin; $n = 6$ vehicle). Wt, Wild-type; ko, knock-out.

Table 3. SEA treatment induces T-cell cytokine mRNA and plasma IFN γ in TNF $^{+/+}$ and TNF $^{-/-}$ mice

	TNF $^{+/+}$ ($n = 4$ /treatment)		TNF $^{-/-}$ ($n = 4$ /treatment)	
	SEA	Saline	SEA	Saline
Real-time RT-PCR				
Target cDNA ^a				
IFN γ	54.93 $\pm$ 13.5	0.438 $\pm$ 0.11	115.0 $\pm$ 22.13	1.32 $\pm$ 0.52
IL-2	41.52 $\pm$ 11.8	0.11 $\pm$ 0.16	106 $\pm$ 40.4	0.11 $\pm$ 0.02
GAPDH	8.1 $\pm$ 0.98	6.76 $\pm$ 1.6	21.24 $\pm$ 6.4	18.3 $\pm$ 4.7
Normalization ^b				
IFN γ /GAPDH	651.9 $\pm$ 79.6	6.4 $\pm$ 0.4	624.5 $\pm$ 94.0	6.8 $\pm$ 1.0
IL-2/GAPDH	483.5 $\pm$ 81.7	1.8 $\pm$ 0.2	559.9 $\pm$ 164.4	2.7 $\pm$ 1.4
ELISA				
IFN γ (ng/ml) ^c	136.74 $\pm$ 18.5	27.0 $\pm$ 4.6	177.3 $\pm$ 28.0	16.8 $\pm$ 2.4

^aThe threshold cycle for each sample was converted to arbitrary units calculated using a standard curve as described in Materials and Methods.

^bFor each individual animal, the calculated arbitrary units for each gene of interest were expressed as a ratio of the corresponding GAPDH units for the same animal and multiplied by 100.

^cPlasma was assayed for IFN γ by commercial ELISA as described in Materials and Methods.

substrates for anxiety and/or fear, in particular the ceA, BNST, and lateral septum (Davis, 1998; Sheehan et al., 2004), also substantiates this hypothesis. Therefore, activation of these regions provides neuroanatomical support for the contention that the behavioral changes observed in response to SEA involve a neophobic and/or anxiogenic component.

The systemic impetus for SEA-induced CNS activation involves TNF α production, because SEA did not increase c-Fos expression in TNF α -deficient mice. This was not an intrinsic failure to activate the *c-fos* gene, because exposure to a psychogenic stressor (i.e., an open field) increased brain c-Fos in TNF α -deficient mice. Complementary experiments also showed that TNF α -deficient mice responded to IL-1 β with corticosterone and hypophagic responses. Therefore, endogenous TNF α is required to observe increased brain c-Fos IR after exposure to SEA, which is consistent with previous data showing that exogenous TNF α increases c-Fos IR in the PVN and ceA (Hayley et al., 2001). In additional experiments, TNF α -deficient mice failed to show corticosterone and hypophagic responses to SEA, and antibody to TNF α blocked SEA-induced reduction in food intake in wild-type mice. Collectively, these data strongly implicate TNF α as a critical mediator of SEA-induced neuromodulation.

How TNF α contributes to the neural effects of SEA is unknown but could involve transport of TNF α into the brain via TNF α receptor-mediated processes (Pan and Kastin, 2002). Indeed, the type I (p55) TNF α receptor is expressed in various circumventricular and parenchymal regions of the brain, includ-

ing the PVN (Nadeau and Rivest, 1999). Moreover, in p55 TNF α receptor-deficient mice, there is a significant attenuation of the corticosterone elevation induced by centrally administered TNF α (Benigni et al., 1996), whereas in rats undergoing turpentine-induced inflammation, the ACTH and corticosterone response is attenuated by central antagonism of TNF α (Turnbull et al., 1997). Consequently, circulating levels of endogenous TNF α may pass into the brain and stimulate p55 type I TNF α receptors on parenchymal cells, including those in the PVN. Whether this occurs in the hypothalamus remains to be determined. However, future studies can use double-label immunohistochemistry to determine whether c-Fos-positive cells also express TNF α receptors, as well as CRH.

The induction of secondary cytokines dependent on TNF α production cannot be ruled out, although in the present study, it does not appear that IL-1 is critical. For example, previous studies had suggested that TNF α exerts behavioral effects in part through IL-1 induction (Bluthe et al., 1991, 1994), although SEA injection does not result in measurable IL-1 β in plasma (Kawashima and Kusnecov, 2002). Although trace amounts of IL-1 could still exert biological effects, this was contraindicated by IL-1RI-deficient mice still showing hypophagia after SEA challenge. Because the anorectic effects of IL-1 rely on the presence of IL-1RI (Bluthe et al., 2000), these data question the involvement of IL-1 in the anorectic effects of SEA.

The absence of a corticosterone response to SEA in TNF α -deficient mice was consistent with the lack of PVN activation. Astressin treatment blocked corticosterone elevation in response to SEA, suggesting that CRH neurosecretory cells in the PVN are responsive to endogenous production of TNF α . This is consistent with reports of increased CRH IR in the PVN and median eminence after administration of TNF α to CD-1 mice (Hayley et al., 2001). Interestingly, use of the strong TNF α inducer LPS has implicated TNF α in HPA axis activation. However, in contrast to the present findings, the CNS effects of LPS do not rely entirely on TNF α . For example, blockade of TNF α only partially attenuated the ACTH and corticosterone increase induced by LPS (Perlstein et al., 1993; Ebisui et al., 1994; Turnbull and Rivier, 1998), and in TNF receptor I knock-out mice, attenuation of the corticosterone response to LPS occurred only at a lower dose (Hayley et al., 2004).

These observations raise questions concerning the relative neuromodulatory contributions of different cytokines elaborated by various bacterial toxins. In the case of SEA, and possibly other SAGs, TNF α is produced together with a range of other T-cell-derived cytokines, although potent neuromodulatory cytokines like IL-1 β are not elevated (Shurin et al., 1997; Kawashima and Kusnecov, 2002). In contrast, LPS induces not only TNF α but also IL-1 and IL-6, which all have considerable effects on the CNS. Hence, it can be hypothesized that the primacy of TNF α as a neuromodulator depends on the presence and/or absence of other coreleased neuromodulatory cytokines. Whereas any further discussion is speculative, the contrasting reactivity of the CNS to TNF α induced by LPS and SEA suggests that distinct patterns of cytokine production may influence how the brain responds to each individual cytokine. It is notable that circulating levels of TNF α achieved in response to SEA were in the subnanogram range, whereas that to LPS is tenfold higher (our unpublished observations). Furthermore, preliminary results have suggested that TNF α knock-out animals retain anorexia and central c-Fos induction in response to 1–5 μ g of LPS (our unpublished observations). Hence, the potency of endogenous TNF α may vary depending on concomitant production of other cytokines that may serve either restrictive and/or permissive roles in

CNS activation. This concept was already advanced through demonstrations of IL-1, TNF α , and IL-6 synergism (Brebner et al., 2000). In the case of LPS, the presence of other neuromodulatory cytokines may mitigate TNF α exerting an excessive influence. With SAGs, such as SEA, the induction of T-cell-derived cytokines and the relative lack of IL-1 may increase the likelihood that TNF α ensures optimal stimulation of the CNS. Indeed, it should be noted that increases in the number of brain c-Fos-immunoreactive cells were comparable between SEA- and LPS-challenged mice (Fig. 1), despite the fact that LPS induces much higher concentrations of TNF α .

In conclusion, the current results demonstrate that activation of T-lymphocytes by a SE elicits functional recruitment of brain areas devoted to the processing of stress-related information. This is dependent on the elaboration of TNF α , the presence of which is also necessary for the endocrine and anorectic responses to SEA. The precise pathway of influence exerted by TNF α remains to be determined. Multiple extant mechanisms are possible, such as the vagus, additional TNF α induction in the brain, and TNF α extravasation into brain parenchyma (Pan and Kastin, 2002). Whatever the relevant downstream processes, the present results strongly identify TNF α production as a necessary step in the alteration of brain and behavioral function after challenge with a T-cell SAG.

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