MECHANISMS OF THE NEURAL AND BEHAVIORAL EFFECTS OF STAPHYLOCOCCAL ENTEROTOXIN A AFTER ACUTE AND REPEATED EXPOSURE: THE ROLE OF TUMOR NECROSIS FACTOR-ALPHA

by

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ABSTRACT OF THE DISSERTATION

Mechanisms of the neural and behavioral effects of staphylococcal enterotoxin A after

acute and repeated exposure: The role of tumor necrosis factor-alpha

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Superantigens (SAgs) activate the immune system by stimulating excessive proliferation of T cells, resulting in the release of cytokines such as tumor necrosis factor-alpha (TNFα). As an adaptive feedback mechanism, SAgs can also activate the hypothalamic pituitary adrenal (HPA) axis by stimulating the release of corticotropin releasing hormone (CRH) from the hypothalamus, adrenocorticotropic hormone (ACTH) from the anterior pituitary, and ultimately corticosterone (CORT) from the adrenal gland. Behavioral consequences of SAg activation include increased gustatory neophobia, neophobia to inanimate non-gustatory objects, and heightened anxiety. Cytokines such as TNFα have been shown to mediate some of these behavioral consequences as well as the endocrine and neurobiological effects of SAg exposure.

The present experiments were designed to determine the role of TNF receptor I (TNFRI) and TNF receptor II (TNFRII) in mediating the effects of acute and repeated SEA. First, the *in vivo* repercussions of repeated stimulation with SEA were assessed.

Then TNFRI- and TNFRII-deficient animals were tested several hours after acute and secondary exposure to SEA, as well as several days after acute SEA exposure. These studies showed that TNFRI was important in mediating the anorexia and CORT response following acute SEA exposure, but not the increase in neophobia several days following exposure. In addition, TNFRI was also important in mediating the endocrine effects of repeated SEA.

Since TNFα was shown to regulate the endocrine effects of SEA, a set of experiments also confirmed that glucocorticoids play an important role in regulating TNFα tolerance following secondary SEA exposure. In addition, the consequences of glucocorticoid disruption on the effects of acute and repeated challenge with SEA were determined through the use of chronic restraint. The results showed that chronic restraint produced an overall increase in interleukin-10 (IL-10) and a blunted interleukin-2 (IL-2) response following immediate acute SEA exposure. However, when the acute exposure was given more distal to the end of restraint period there was an enhanced IL-2 and TNFα response following acute, but not secondary SEA exposure. Collectively, these studies demonstrate the reciprocal effect of TNFα and glucocorticoids after acute and repeated SEA exposure.

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List of Abbreviations

ACTH: adrenocorticotropic hormone

AMINO: aminoglutethimide

APC: antigen presenting cell

AVP: vasopressin

BBB: blood brain barrier

CNS: central nervous system

CORT: corticosterone

COX-II: cyclooxygenase II

CRH: corticotropin releasing hormone

DA: dopamine

DEX: dexamethasone

FADD: Fas associated death domain

GR: glucocorticoid receptor

HPA axis: hypothalamic-pituitary-adrenal axis

IFNγ: interferon gamma

IκB: inhibitor of κB

IL-1: interleukin-1

IL-2: interleukin-2

IL-4: interleukin-4

IL-6: interleukin-6

IL-10: interleukin-10

iNOS: inducible nitric oxide

LPS: lipopolysaccharide

MHC: major histocompatibility complex

MHC I: major histocompatibility complex molecule I

MHC II: histocompatibility complex molecule II

NAIP: neuronal apoptosis inhibitor protein

NE: norepinephrine

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO: novel object

OF: open field

PAMP: pathogen-associated molecular patterns

PI3K: Phosphoinositide 3-kinases

PRR: pattern recognition receptors

PVN: paraventricular nucleus of the hypothalamus

SAg: superantigen

SE: staphylococcal enterotoxin

SEA: staphylococcal enterotoxin A

SEB: staphylococcal enterotoxin B

SRBC: sheep red blood cells

sTNFRI: soluble tumor necrosis factor receptor I

sTNFRII: soluble tumor necrosis factor receptor II

TACE: tumor necrosis factor converting enzyme

TCR: T cell receptor

TGFβ: transforming growth factor beta

TLR: toll-like receptor

TLR4: toll-like receptor 4

TNFα: tumor necrosis factor-alpha

TNFRI: tumor necrosis factor receptor I

TNFRI$^{-/-}$: tumor necrosis factor receptor I knockout

TNFRI$^{+/-}$: tumor necrosis factor receptor I heterozygous

TNFRI$^{+/+}$: tumor necrosis factor receptor I wildtype

TNFRII: tumor necrosis factor receptor II

TNFRII$^{-/-}$: tumor necrosis factor receptor II knockout

TNFRII$^{+/-}$: tumor necrosis factor receptor II heterozygous

TNFRII$^{+/+}$: tumor necrosis factor receptor II wildtype

TRADD: tumor necrosis factor associated death domain

TRAF: tumor necrosis factor receptor associated factor

TSST: toxic shock syndrome toxin

Vβ: T cell receptor beta chain

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Chapter 1

General Overview of the Immune System

The primary function of the immune system is to protect the host from infection caused by foreign microorganisms (non-self), such as viruses and bacteria, and to provide restoration of health and long term immunity. In order to induce sufficient defense against pathogen exposure, the immune system has evolved from a fast-acting nonspecific immune response (innate immunity) to a specific and long-term response that includes memory and recall (adaptive immunity).

Aspects of innate immunity include physical, chemical, as well as microbiolgical barriers. If these barriers are not sufficient, granulocytes (neutrophils) as well as mononuclear phagocytes (monocytes and macrophages) can recognize microbes through pattern recognition receptors (PRRs) and engulf and eliminate the pathogen. Other granulocyte cells, including, neutrophils, eosinophils, and basophils, are also important for successful innate immunity.

To supplement and expand the innate immune response, the adaptive immune response generates antigen-specific effector cells that target and eradicate potential pathogens. Adaptive immunity depends on antigen presenting cells (APCs), such as dendritic cells, monocytes, and macrophages, to process and present the antigen to lymphocytes. The two major lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells) perform different, but complementary, functions. B cells are responsible for

humoral immunity, secreting antibodies into the circulation (e.g., blood and lymph fluids) while T cells mediate cellular immunity which can support humoral immunity or directly eliminate an infected cell.

T cells leave the bone marrow in an undifferentiated state (as hematopoietic cells of the lymphoid lineage) and migrate to the thymus gland where they mature. The criterion for maturation is the ability of the T cell to recognize self from non-self, which ultimately allows for self tolerance. Once mature, all T cells express the T cell antigen receptor (TCR), and either CD4 or CD8 molecules. Therefore there are $CD4^+/CD8^-$ or $CD4^-/CD8^+$ T cells. Induction of T cells requires a physical interaction step between the T cell and an APC, which expresses molecules of the major histocompatibility complex (MHC). In the presence of an antigen, the APC will digest the antigen and load fragments of that antigen onto the MHC molecule which will then be transported to the cell surface. The TCR on $CD4^+$ and $CD8^+$ T cells will recognize antigen peptides by variable regions of the alpha and beta chains specific to the antigens. CD8 T cells, also known as cytotoxic T cells, eliminate antigens that are found in the cytosol and recognize antigens bound to the MHC I molecule (MHCI). While the CD4 T cells recognize antigen bound by MHC class II and are subdivided into Th1 and Th2 subsets (also called helper T cells because of their ability to modulate B cells and produce antibody). The chief products of the CD4 T cells (as well as other cells including phagocytic cells) are cytokines, which serve a range of regulatory functions essential for optimal immune function.

Cytokines are protein molecules synthesized and secreted by cells in an autocrine and paracrine fashion. They are pleiotropic as they have the ability to stimulate or inhibit the activation, proliferation, and differentiation of various cells, and regulate the secretion

of antibodies or other cytokines. Some cytokines associated with T cell effector function include interleukin -2 (IL-2), interferon-gamma (IFNγ), and interleukin-10 (IL-10). Interleukin-2 stimulates B cells to produce antibody and is a T cell mitogen, which increases clonal expansion. Interferon-gamma is another important cytokine known to inhibit Th2 proliferation and increase MHC II expression on B cells and macrophages. In order to control the activities of such proinflammatory cytokines, anti-inflammatory cytokines such as IL-10 help dampen the immune response by inhibiting macrophage function and inhibiting proinflammatory cytokine production.

Immune to Brain Communication

It was once thought that the brain and the immune system were autonomous, but it is now understood that the central nervous system (CNS) and the immune system share bidirectional communication that encourages physiological and behavioral adaptations after exposure to a pathogen. The nervous system utilizes neuroendocrine agents, neurotransmitters, and neuropeptides to influence the immune system, while immunoregulatory agents such as cytokines can alter neuronal function. Over 20 years ago, it was discovered that glucocorticoids can modulate the immune system by altering the expression of major histocompatibility (MHC) molecules and cytokines (Snyder and Unanue, 1982). It was also demonstrated that immunosuppression and immuno-enhancement could be behaviorally conditioned, which further supports the idea that neuronal function can alter the immune system (Ader and Cohen, 1975; Kusnecov et al., 1989).

Alternatively, the immune system can modify the nervous system. An early example of CNS activation by the immune system came from Besedovsky et al. (1981), who discovered that supernatant from mitogen or antigen stimulated immune cells injected into animals resulted in activation of the HPA axis (Besedovsky et al., 1981). These studies were elaborated using a T cell dependent antigen, sheep red blood cells (SRBC), the administration of which to rats activated an immune response and the HPA axis (Besedovsky et al., 1985). These findings, along with the emerging literature addressing the immunosupressive properties of glucocorticoids, laid the groundwork for the concept of the immune hypothalamic-pituitary-adrenal (HPA) axis circuitry that is known today. Shortly thereafter, it was determined that cytokines such as IL-1β, TNFα, and IL-6 can activate the HPA axis directly (Besedovsky et al., 1986; Naitoh et al., 1988; Sharp et al., 1989).

The HPA axis is an essential component of the immunoregulatory neuro-endocrine mechanism. In response to an immunological stimulus (or psychogenic stress), the hypothalamus secretes corticotrophin releasing hormone (CRH) which promotes the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary. This release up-regulates the release of glucocorticoids (such as cortisol in humans and corticosterone (CORT) in mice) from the adrenal gland. The HPA axis can self-regulate by employing a negative feedback loop to ensure that circulating levels of glucocorticoids do not exceed homeostatic levels. The rise in glucocorticoids is hypothesized to result in the dampening of the immune response and provides a means for controlling what could be detrimentally high levels of proinflammatory cytokines. The mechanism through which this is achieved is through binding of glucocorticoids to the cytosolic glucocorticoid receptor (GR). Upon

binding, the GR is translocated to the nucleus where it can bind to DNA response elements to activate gene transcription.

Aside from activation of the HPA axis, cytokines have now been shown to have many other neuromodulatory functions. They have been shown to modify neurotransmitters by decreasing norepinephrine (NE) and increasing dopamine (DA) and serotonin (5-HT) metabolism in the brain, and can induce changes in behaviors like food intake and sleep (Blalock, 1994; Anisman and Merali, 1999; Brebner et al., 2000). These behavior alterations are collectively referred to as "sickness behavior", which is characterized by anorexia, lethargy, anhedonia, and alterations in sleep and sexual patterns (Danzer, 2001). They occur via neurotransmitter release, immediate early gene induction, and activation of the HPA axis (Dunn et al., 1999).

These studies indicate that peripheral cytokines serve as one of the main afferent signals from the immune system to the brain. Indeed, it has been demonstrated that peripherally injected cytokines can cross the blood brain barrier (BBB) or be actively transported (such as IL-1β, TNFα, and IL-6) (Gutierrez et al., 1993; Banks et al., 1994; Banks et al., 1995). Cytokines can also passively enter the brain through the circumventricular organs, vagal afferents, or be produced *de novo* by astrocytes and microglia (Hashimoto et al., 1991; Merill 1992; Dantzer et al., 1998). Additionally, peripheral cytokines can influence the CNS through the induction of other proteins, such as prostaglandins, that can cross the BBB (Banks et al., 1995; Faggioni et al., 1995).

Endotoxin Exposure

Much of our understanding regarding the actions of cytokines has come through the use of endotoxin exposure as a model for inflammation and sepsis. A particularly potent immunologic activator is the endotoxin lipopolysaccharide (LPS), which is the glycolipid component of the cell wall of gram-negative bacteria. Specific receptors called Toll-like receptors (TLR) recognize pathogen-associated molecular patterns (PAMPs) on bacteria and other pathogens (Medzhitov and Janeway, 2000). Toll-like receptor 4 (TLR4) on monocytes and macrophages is the critical receptor for LPS. Genetic knockout animals missing the gene for TLR4 are hyporesponsive to LPS administration (Hoshino et al., 1999). Upon recognition of LPS, signaling through TLR4 results in activation of transcription factors such as NF-κB, which results in the activation of proinflammatory cytokines, chemokines, and enzymes such as iNOS and COX-2 (Rivest, 2003; Lin and Yeh, 2005). LPS strongly stimulates circulating monocytes and tissue macrophages to produce a variety of proinflammatory cytokines, including IL-1β, IL-6, and TNFα. Ultimately, systemic exposure to LPS represents an acute phase reaction to a pathogen, resulting in fever, HPA activation, and a constituition of signs and symptoms collectively reffered to as "sickness behavior".

LPS has also been used to examine the neuromodulatory effects of cytokines. For example, systemic LPS has been shown to increase proinflammatory cytokines in the brain (Laye et al., 1994; Buttini and Boddeke, 1995). Interestingly, TNFα levels can remain elevated for up to ten months after a single injection of LPS (Qin et al., 2007). Systemic LPS has also been shown to induce gliosis, microglial cell activation, and apoptosis in the brain (Semmler et al., 2005; Qin et al., 2007). Additionally, this

endotoxin model has been critical in demonstrating the involvement of the neuroendocrine system after infection. For example, both adrenalectomy and the use of glucocorticoids receptor antagonists, such as RU-486, have been shown to be lethal after exposure to LPS (Hawes et al., 1992; Beishuizen and Thijs, 2003).

While cytokines each play an important role in mediating the effects of LPS, they also possess a number of redundant pathways, which allow for compensation. For example, IL-1 knockout mice respond normally to systemic LPS, and IL-6 does not seem to be involved in NF-κB activation after exposure to LPS (Bluthe et al., 2000; Rivest, 2003). Inhibition of TNFα only partially attenuates the ACTH and CORT response after LPS, and the same effect can be seen with TNFRI knockout animals as well (Ebisui et al., 1994; Turnbull and Rivier, 1998). Further, it has been hypothesized that IL-1 receptor I knockout animals continue to respond to the LPS due compnesation by TNFα (Bluthe et al., 2000). Therefore, the pleiotropic properties of cytokines allow for plasticity within the cytokine network, thereby permitting compensation.

Superantigens and the CNS

While LPS has enhanced the understanding of neural-immune interactions, it is also important to determine the impact of T cell derived cytokines on CNS function. Models of immunization with benign proteins, such as keyhole limpet hemocyanin (KLH), have been utilized, although they have been quite limited. The discovery of Superantigens (SAg), however, has allowed for further investigation of the consequences of exposure to T-cell-dependent pathogens on the CNS.

While LPS increases cytokines through macrophages, SAgs are powerful immunological activators that are considered macrophage-independent, causing the proliferation of T cells and massive cytokine production, including IL-1β, IL-2, TNFα, and IFNγ (Gonzalo et al., 1993; Sundstedt et al., 1994; Goehler et al., 2001). Superantigens are potent immunologic stimuli that originate from bacteria and viruses (Proft and Fraser, 2003; Wang et al., 2004b) and possess the capacity to stimulate in an MHC-dependent manner up to 10-20% of all T cells in a given host (Zamoyska, 2006). This major recruitment of T cells is independent of clonal specificity, and results in substantial proliferation and cytokine production (Florquin et al., 1994; Gonzalo et al., 1994). It is of note that the circulating concentration of cytokines (eg., IL-2, IFNγ and TNFα) achieves easily detectable levels, far exceeding the capacity of regular, benign protein antigens (eg., hemocyanin) to generate similar amounts that can be detected *in vivo* without resorting to limiting dilution procedures (Troutt et al., 1992). The latter is a reflection of the lower frequency of T cell recruitment, but does speak to the potential clinical impact that SAgs can exert on the host by virtue of committing so many more T cells into a cytokine-producing state. That is, as a systemic condition, exposure to superantigenic molecules can pose considerable risk due to the sustained production of cytokines that are normally tightly regulated to prevent excessive inflammation and immunopathological disease. For example, it has been well established that exposure to SAgs can result in shock and increased mortality (Sriskandan and Altmann, 2008), with some suggestion that SAgs can promote increased vulnerability to autoimmune responses (Samarkos and Vaiopoulos, 2005; Matsubara and Fukaya, 2007). Therefore, the

extraordinary nature of the T cell response to SAg molecules poses a significant threat to health.

Given the protective nature of the immune response against infectious pathogens, it is unusual that in the case of SAgs, the immediate response bypasses the basic tenets of adaptive immune reactivity, such as antigen processing and peptide presentation to T cells, with associated promotion of B cell antibody responses and T cell cytotoxicity. Typically, this canonical progression of the adaptive immune response takes place over a 24-48 hr period prior to the appearance of antigen-binding antibody responses. However, the unique nature of the SAg stimulation of the T cell receptor results in massive proliferation and cytokine production in a matter of hours (Bette et al., 1993). The protective function of this response has remained elusive, while the suggestion that it benefits more the pathogen producing the toxin, rather than the host, has not been empirically tested (Sriskandan and Altmann, 2008).

Superantigens were first identified by Marrack and Kappler (Kappler et al., 1989; Dellabona et al., 1990; Herman et al., 1991), with the largest number attributed to the gram positive bacteria *Staphylococcal aureus and Streptococci* (Proft and Fraser, 2003; Wang et al., 2004b). Of these, the best characterized are the staphylococcal enterotoxins, for which an appreciable amount of information exists in terms of their ability to stimulate specific subsets of mouse, rat and human T cells (Bode et al., 2007; Emmer et al., 2008; Ferry et al., 2008; Rajagopalan et al., 2008). In addition, SAgs have been identified for B cells (Silverman and Goodyear, 2006), although much of the present discussion will focus on T cell superantigens. The superantigenic properties of these agents are a reflection of their ability to stimulate 10^4 fold more T cells than conventional

antigens. The term antigen refers to any stimulus that initiates an immune response and has the capability of inducing the production of antibodies. In so far as the latter is typically dependent on the cooperation of T cells, most antigenic molecules engage T cells subsequent to processing and MHC-dependent presentation by antigen-presenting cells (eg. dendritic cells). This is achieved in a clonally specific manner, such that the inner peptide-binding groove of the T cell receptor is the site for specific recognition of the multitude of different antigenic determinants (or epitopes) that can be presented by MHC molecules. Therefore, for each peptide sequence representing an epitope of some larger protein antigen, there exists a given T cell clone whose TCR recognizes that particular epitope. The net result of this specificity is that of the entire pool of mature T cells in a mammalian organism, the frequency of responsiveness to epitopes from processed proteins derived from foreign sources (eg., bacteria or viral envelopes) is estimated to be approximate 0.002% (Zamoyska, 2006). Therefore, the considerably greater number of T cells stimulated by SAgs represents an extraordinary activation of the immune system, and consequently higher levels of cytokine production. This over-abundance of circulating cytokines may have a profound impact on biological functions, including those of the central nervous system.

The abundant numbers of T cells activated by SAgs is a reflection of oligoclonal stimulation and reflects the selectivity of SAgs for unique motifs on the variable region of the TCR beta chain (Vβ) (Kappler et al., 1989; Dellabona et al., 1990; Herman et al., 1991). However, the molecular characteristics of superantigenic stimulation of T cells are best understood in terms of the recruitment of T cells carrying specific Vβ genes (e.g., Vβ1, Vβ2, Vβ3 etc; see Proft & Fraser, 2003 for a detailed description)(Proft and Fraser,

2003). Briefly, relatively invariant amino acid sequence motifs can be present on multiple clonally-specific TCRs that are encoded by the same Vβ gene. The net result is that T cells bearing clonal specificity towards different antigenic peptides can still be categorized according to the same Vβ gene coding for this common, invariant motif on the TCR. Consequently, T cells can be classified, for example, as $Vβ3^+$ T cells, and still be further differentiated according to their responsiveness to different antigenic peptides (Gomez et al., 2000).

Staphylococcal Enterotoxins

Perhaps the greatest understanding of the immunological effects of SAgs has come from studies of the secreted toxins of *Staphylococcal aureus*, a gram positive bacteria long recognized as a major pathogen responsible for infections and food poisoning (Thomas et al., 2007). The major exotoxins of *S.aureus* have been classified as cytotoxins, pyrogens, and exfoliative toxins, with the superantigenic toxins falling into the pyrogenic class (Lowy, 1998). These include the staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST). With regard to the SEs, serological distinctions have been made, with each toxin coded by separate genes, and with some amino acid homology found across different toxins (Lowy, 1998). These variations are reflected by the application of an alphabetic nomenclature for the SEs (viz., SEA [for staphylococcal enterotoxin A], SEB, SEC, and so on), with some being further categorized into subtypes (eg., SEC_1, SEC_2 and SEC_3). Indeed, as reported in previous reviews, at least 15 different SEs have been identified along with their Vβ specificity (Sundberg et al., 2002; Petersson et al., 2004). Analysis of this Vβ specificity has revealed considerable heterogeneity in

their affinity to the full range of known Vβ genes in a number of different species including human and non-human primates, as well as rats and mice (Petersson et al., 2004). In mouse studies, it is important to note that the relative percentage of T cells bearing any one particular member of the Vβ family can differ between inbred mouse strains. This can result in variations in the composition of T cells bearing different Vβ phenotypes in mice exposed to particular SEs (Liang et al., 1994). For example, many studies have used C57BL/6 and BALB/c mice, and in these strains, the magnitude of T cell reactivity varies according to which Staphylococcal SAg has been administered. Therefore, it is commonly observed that in BALB/c mice, exposure to staphylococcal enterotoxin B (SEB) will preferentially engage Vβ8 T cells, whereas in C57BL/6 mice these cells are not as readily recruited; alternatively, injection of C57BL/6 mice with SEA produces marked activation of Vβ3 T cells. These biases and the ensuing dramatic effects on cytokine production have been exploited by different investigators, resulting in good evidence that SEA and SEB produce neurobiological and behavioral effects, as summarized in Table 1, and further discussed below (Kusnecov and Goldfarb, 2005).

Superantigens and the Hypothalamic Pituitary Adrenal (HPA) Axis

As noted earlier, the staphylococcal enterotoxins are classed as pyrogenic toxins, suggestsing the activation of central thermoregulatory brain systems, such as those controlled by the hypothalamus. Indeed, it has been demonstrated that rats injected with SEB show febrile responses, which was pursuant to an initial activation of hypothalamic neurons, as well as elevations in plasma corticosterone (Goehler et al., 2001). Corticosterone is a glucocorticoid produced by cells of the adrenal cortex, and has long

been regarded as an endocrine measure of physiological and/or psychological stress (McEwen, 2007). Additionally, elevations in corticosterone are generally a result of upstream activation of the hypothalamic-pituitary axis which results in the release of ACTH, the pituitary hormone responsible for the adrenocortical release of glucocortioid hormones (McEwen, 2007). The observation of increased corticosterone release in response to SEB administered to rats (Goehler et al., 2001) was an extension of earlier work in mice first reported by Gonzalo et al (1993). This latter study demonstrated that the increase in CORT production was important for the early clonal deletion of activated Vβ3 and Vβ8 cells after SEA or SEB exposure, respectively, and inhibition of the CORT response enhanced mortality in animals treated with SEA and SEB (Gonzalo et al., 1993). In this regard, it was clear that glucocorticoid responses to bacterial SAgs were a critical adaptive feedback mechanism, as has been shown in the endotoxin (or LPS) shock model (Berczi, 1998; Beishuizen and Thijs, 2003). These observations of SEB-induced corticosterone release were corroborated by studies investigating steroid regulation of T cell apoptosis (Williams et al., 1994), but without determining whether central mechanisms were driving the elevations in corticosterone. This latter issue was addressed by Shurin et al (1997) who found in BALB/c mice that the elevated corticosterone response to SEB was associated with ACTH production (Shurin et al., 1997). This effect of SEB on the corticosterone response has consistently been demonstrated in rats and mice (Del Rey et al., 2000b; Pacheco-Lopez et al., 2004).

To the extent that concordant changes in ACTH and corticosterone constitute evidence for activation of the pituitary-adrenal axis, this suggested the activation of neurally controlled ACTH secretagogues, such CRH (also known as CRF) and arginine

vasopressin (AVP) (Tilbrook and Clarke, 2006). The plausibility of upstream neural events being involved in increased pituitary-adrenal activation was provided by evidence for SEB induced activation of the paraventricular nucleus (PVN) of the hypothalamus, as measured by immunohistochemistry and *in situ* hybridization for the immediate early gene, *c-fos* (Goehler et al., 2001; Bette et al., 2003; Wang et al., 2004a). In contrast, a more recent study found that exposure of rats to SEB did not produce an appreciable activation of the PVN (Serrats and Sawchenko, 2006). However, this latter study did observe elevated plasma concentrations of corticosterone and ACTH, as well as central activation of other brain regions important to emotional regulation. Moreover, in mice it was found that challenge with SEB not only elevated corticosterone levels, but also increased norepinephrine levels, suggesting activation of the sympathetic nervous system (Del Rey et al., 2000b), which is associated with increased activation of the PVN (Del Rey et al., 2000b; Schlenker, 2005).Consequently, at least for SEB, the bulk of the evidence in rats and mice is supportive of a central basis for the stimulatory effects of this SAg on pituitary-adrenal hormones.

In addition to SEB, another staphylococcal superantigen, SEA, has been shown to produce activation of the HPA axis. In the original report by Gonzalo et al (1993) injection of C57BL/6 mice with SEA increased plasma corticosterone. This was confirmed by Shurin et al (1997), this effect being associated with elevated ACTH (Kaneta and Kusnecov, 2005). Additional investigations showed that the pituitary-adrenal effects of SEA occurred at a minimum dose of 1 μg per mouse (approx. 40 μg/kg), with a peak elevation of plasma corticosterone measured at 2 hrs (Kawashima and Kusnecov, 2002). Interestingly, in the same study, administration of SEB to C57BL/6 mice produced

a modest increase in plasma corticosterone; however, this was relatively short-lived, but suggested that the small proportion of Vβ8+ T cells in the C57BL/6 mouse could be stimulated by the appropriate SAg to produce a neuroendocrine effect.

It should be noted that most studies on the effects of SEA or SEB on HPA axis activation have assessed hormone levels after only a single injection. Generally, in cases such as endotoxin (i.e. LPS) challenge, it is difficult to assess the neurobiological effects of repeated challenges since an immunologic form of tolerance develops, and which accounts for a loss in corticosterone elevations to LPS. Similarly, in the case of SAgs, and in particular SEA and SEB, repeated exposures can result in T cell anergy (Gonzalo et al., 1993). Since nothing was known about the corticosterone response after repeated injections of SEA, a recent study with C57BL/6 mice (Urbach-Ross et al., 2008) (See Chapter 3) administered SEA up to four times at intervals of 3-5 days. In contrast to studies with LPS, it was found that the corticosterone response to SEA was still evident in response to the third injection, although by the fourth challenge this was no longer the case (Urbach-Ross et al., 2008). In an unpublished observation, the corticosterone response of BALB/c mice to a second injection of SEB was still evident when the toxin was administered 7 days after the first (unpublished observations, Kusnecov laboratory). In summary, there appears to be little dispute that SEA and SEB activate the pituitary-adrenal axis in mice and rats, and that this can be reenlisted with repeated exposures to these SAgs.

It was established some time ago that SEA and SEB stimulate T lymphocytes through co-dependent binding to MHC Class II molecules (Kappler et al., 1989; Herman et al., 1991). However, some evidence also exists that Staphylococcal enterotoxins

stimulate non-T cells (Bright et al., 1999; Yoon et al., 2001), and this may be relevant to

the pituitary-adrenal effects of SEA and SEB. However, it was shown earlier that athymic

nude mice, which lack mature T lymphocytes, failed to show a corticosterone response

following SEB injection (Williams et al., 1994); conversely, the corticosterone response

became evident if mice were reconstituted with T cells, suggesting a dependence on the

presence of functional T cells capable of responding to SEB (ibid). Pharmacologic

suppression of T cell function using cyclosporine was also shown to inhibit the

corticosterone response to SEB (Shurin et al., 1997), but not to SEA (Kawashima and

Kusnecov, 2002). In both cases, cyclosporine completely suppressed T cell proliferation

and IL-2 production, and also did not affect the ability of LPS (a predominantly

monocyte/macrophage stimulus) to activate the pituitary-adrenal axis (Shurin et al., 1997).

Moreover, in the case of SEB-treated BALB/c mice, depletion of macrophages did not

affect the corticosterone response (ibid). Consequently, in the case of SEA the results

suggests that non-T cell mechanisms may be responsible for increased corticosterone

levels. However, an additional experiment using T cell deficient Rag-1 knockout mice

failed to induce a corticosterone response to SEA, but not to LPS (Kawashima and

Kusnecov, 2002). Therefore, while the cyclosporine results for SEA remain perplexing,

there appears to be good evidence that the corticosterone-elevating effects of SEA and

SEB require the presence of mature and functional T cells.

The Role of Corticotropin-Releasing Hormone (CRH)

Pituitary ACTH release is under the influence of various neuropeptide hormones,

including CRH and AVP. The relative primacy of these peptides in exerting their effects

on ACTH release varies under different stress conditions, although it is generally agreed that in response to acute stressors, CRH is the main peptide driving ACTH secretion (Steckler and Holsboer, 1999; Lolait et al., 2007a; Lolait et al., 2007b). In contrast, the contribution of AVP appears to be incorporated during chronic stress conditions (Makara et al., 2004; Volpi et al., 2004). It has been known for some time that immunologic stimuli activate the HPA axis, which ultimately led to confirmation that central CRH release was associated with pituitary-adrenal responses to cytokines, such as interleukin-1 (Turnbull and Rivier, 1999). In addition, the ACTH response to an injection of LPS was shown to be attenuated by CRH receptor antagonism (Rivier et al., 2003).

As in the case of endotoxin and IL-1β challenge, a number of studies suggest that in mice, CRH may be involved in the effects of acute SAg injection on HPA axis activation. Mice challenged with SEB showed increased CRH mRNA levels in the PVN and central nucleus of the amygdala, and immunoneutralization of CRH significantly reduced the ACTH response to SEB (Kusnecov et al., 1999). Similarly, since pituitary ACTH-secreting cells express the R1 subtype of CRH receptors (Rivier et al., 2003), mice challenged with SEA were systemically administered the selective CRH-R1 antagonist, astressin; the results showed a significant attenuation of the corticosterone response to SEA (Rossi-George et al., 2005). These data demonstrate that the full extent of the HPA axis is activated by SAg administration, with initial recruitment of CRH producing neurons in the PVN serving as the initial stimulus within the neuroendocrine system. However, this conclusion applies to responses induced by acute SAg injections. As noted earlier, it has been documented that the corticosterone response to SEA continues to be evident after 2-3 injections (Urbach-Ross et al., 2008). Additional

information is required as to whether this increase in corticosterone is dependent on CRH on each occasion of repeated SEA exposure, or whether recruitment of other ACTH secretagogues is involved. Indeed, it has been noted that the initial early phase of the pituitary-adrenal response to a single LPS injection is dependent on the AVP 1b receptor (Lolait et al., 2007b). Whether a similar early dependence on AVP occurs after SAg injections remains to be determined.

Effects of Superantigens on Behavior

There is now a large literature on the behavioral effects of immunological activation, which has led to the concept of 'sickness behavior' as a behavioral syndrome emerging from cytokine-induced activation of the CNS (Dantzer et al., 2008). The behavioral changes observed are typical of organismic reactions to stress, and generally reflect anorexia, anhedonia, impaired somnolence, and disruption of cognitive processes (Anisman et al., 2005; Dantzer et al., 2008), and may represent the alignment of behavioral goals with the effector state of the immune system. For example, removal of pathogens by immunological cells and antibody requires a general systemic adjustment which includes not only increased endocrine activity but also restriction of behaviors that otherwise would compromise neutralization and elimination of infection.

Indeed, increased activation of the HPA axis has been hypothesized to regulate ongoing immune responses (Besedovsky and del Rey, 2000a), and it is well known that higher-order neural structures that provide afferent input to the hypothalamus are involved in controlling ingestive, emotional and cognitive processes. Through immediate early gene mapping studies (eg., c-Fos immunohistochemistry), it has been confirmed

that such areas include not only the cortex and hypothalamic nuclei, but also the

hippocampus, septum, bed nucleus of the stria terminalis, and amygdala, in addition to

central autonomic nuclei such as the locus coeruleus and nucleus of the solitary tract

(Ericsson et al., 1994; Gaykema et al., 2007). Consquently, while animals may display

inhibited movement and exploration, there is little reason to suspect that sickness

behavior reflects a suspension of cognitive and emotional processing. Surprisingly, mice

acutely administered bacterial SAgs intraperitoneally do not show overt evidence of

malaise, such as piloerection and diarrhea (unpublished observations, Kusnecov

laboratory), although in rat studies, pyrogenic effects have been observed (Goehler et al.,

2001). In contrast, enteric delivery of staphylococcal enterotoxins or injection in the

presence of D-galactosamine (D-gal), a liver toxin, will produce malaise and/or septic

shock and increased mortality (Harris et al., 1993; Gonzalo et al., 1994; Aoki et al., 1995).

These latter experimental manipulations are unique or do not correlate with T cell

activation, and are therefore difficult to relate to those studies reporting CNS effects after

bolus intraperitoneal injections of SEA or SEB in the absence of any further treatments

(eg., d-galactosamine). In the studies already discussed showing HPA axis activation,

SEA or SEB treatment increases circulating IL-2 and TNFα, but does not affect mobility

nor subsequent 24 hr body weight loss, which stands in contrast to a reduction in the

ingestion of food other than that normally provided in the home cage (viz., regular

laboratory chow) (Kusnecov et al., 1999; Rossi-George et al., 2005). However, if animals

were preexposed to the irregular food (liquid diet or commercial food pellets) the SAg-

induced hypophagia (or anorexia) was significantly attenuated (Kusnecov et al., 1999;

Kawashima and Kusnecov, 2002; Rossi-George et al., 2005). These manipulations were

designed to test the neophobic reactions of the animals to novel food, and suggested that the T cell response to SEA or SEB augments food neophobia. Furthermore, after familiarization with a given food in an operant chamber where nose-pokes deliver food pellets, there was no disruption of performance or ingestion of food pellets in response to SEA treatment (Kusnecov laboratory, unpublished observations). Therefore, acute systemic injections of staphylococcal enteroxins at minimal doses that activate the HPA axis do not produce dramatic signs of malaise that might otherwise lead to an interpretation of anorexic behavior due to illness or motoric impairment.

Further assessment of behavior following SAg challenge has revealed enhanced neophobia in the presence of inanimate, non-gustatory objects (Kawashima and Kusnecov, 2002). An open field-novel object test was used, since it was demonstrated to be an index of anxiety-like behavior in mice (Dulawa et al., 1999; Henry et al., 2006). Animals were observed initially exploring an empty open field environment, after which an unfamiliar cylindrical object was placed in the central region of the field. As reported by Kawashima and Kusnecov (2002), there was no impact of SEA challenge on exploration of the open field, which was in keeping with points made earlier concerning the absence of frank malaise and continued maintenance of motor behavior. However, the introduction of a novel object resulted in greater arrest of ongoing behavior and physical interaction with the object in SEA treated animals. This likely reflected increased anxiety and/or neophobic behavior (Kawashima and Kusnecov, 2002), and provided additional evidence, that as shown later for SEB, there is no fundamental suppression of locomotor behavior, unless provoked by novel stimuli (Kawashima and Kusnecov, 2002; Rossi-George et al., 2004).

It was thought that the suggestion of increased anxiety-like changes produced by the novel object test in SEA treated animals could be generalized to other more traditional tests of anxiety-like behavior. One such test is the elevated plus maze (EPM), long considered as a useful index of modified anxiety state in rats and mice. However, it was paradoxically observed that when C57BL/6 and BALB/c mice were challenged with SEA or SEB, respectively, exploratory behavior was in the direction of less, rather than more, anxiety (Rossi-George et al., 2004). That is, animals showed greater preference for entering the open arms of the EPM, which is generally interpreted as a sign of reduced fear/anxiety. Furthermore, testing for anxiety-like behavior in the light-dark box, another commonly used test of anxiety (Ballaz et al., 2007), failed to show any influence of SEA or SEB treatment. For example, administration of SEB to male BALB/c mice did not affect latency to exit from the dark compartment, nor the number of light–dark transitions and total time spent exploring the illuminated arena (Rossi-George et al., 2004). Interestingly, in the EPM, BALB/c mice given SEB spent more time spent in the open arms, which at least demonstrates a behavioral effect otherwise unseen in the light-dark box test. It should be acknowledged, however, that the light–dark box and EPM may not assess similar underlying "emotional" processes (Holmes et al., 2001), since the stimulus conditions of each test are different, and may not be engaging and/or interfering with relevant neurobiological processes that one might be attributable to anxiety-like states. For example, while the increased open arm exploration due to SEA or SEB treatment might otherwise suggest less "anxiety," an alternative interpretation could easily attribute this behavior to increased impulsiveness. Such an interpretation is highly speculative, however, and indeed raises an important problem regarding what constitutes "anxiety" in

these tests. This problem is compounded by the failure of others to successfully identify

anxiety-like behavior in the EPM or light-dark box following ostensibly anxiogenic

treatments, as discussed elsewhere (Rossi-George et al., 2004). The hypothesized

anxiogenic properties of SAgs are supported, however, by neuronal activation studies that

show an increase in activation in areas involved in mediating the stress response. For

instance, exposure to SEB has been shown to activate limbic areas in the brain such as

the lateral septum (LS), CeA, and PVN (Goehler et al., 2001; Wang et al., 2004a).

Nonetheless, the range of behavioral assessments that could be conducted on animals

treated with SEA or SEB has not been fully exhausted since nothing is known at present

concerning cognitive behaviors, and within this category, learning and memory. However,

at the very least, perhaps the most reliable change is that of reduced food intake, and

therefore, has been used to determine the central and peripheral mechanisms by which

SEA exerts its effect on behavior.

Role of CRH

Contextual novelty can alter the quantity of food and/or water consumed, and this

has been shown to be CRH-dependent, since this highly versatile peptide has long been

regarded as anxiogenic (Koob and Heinrichs, 1999). However, the arousing or anxiety-

regulating properties of CRH are considered the result of differential engagement of two

major CRH receptors, CRH-R1 and CRH-R2 (Liebsch et al., 1999). The anxiogenic

effects of CRH are believed to be mediated by CRH-R1(Steckler and Holsboer, 1999) ,

which was also shown to be the mechanism by which SEA injection led to increased

pituitary-adrenal activation. As for the anorexic effects of CRH, either receptor may be

involved, although the hypophagia may be mediated by increased arousal or a non-arousal based inhibition of food ingestion, where animals simply fail to show a motivation to consume food. Therefore, it was proposed that CRH-R1 mediated anxiety-based suppression of food intake, whereas basic appetite regulation occurred through CRH-R2 (Zorrilla et al., 2003). Moreover, while CRH showed greater selectivity for CRH-R1, CRH-R2 was shown to be more selectively engaged by the more recently discovered peptide, Urocortin (UCN) (Zorrilla et al., 2003).

A test of which CRH receptor mediated the effects of SEA on food intake in a novel situation was assessed by Kaneta and Kusnecov (2005), using two different CRH receptor antagonists administered intracerebroventricularly. Use of the non-selective antagonist α-helical CRF led to attenuation of SEA-induced anorexia, with no effect observed after infusion of the selective CRH-R2 antagonist, astressin-2B (Kaneta and Kusnecov, 2005). This supported the hypothesis that SEA treatment increases central release of CRH, which acts mainly through CRH-R1 to inhibit food intake. Moreover, given the view that CRH-R1 may suppress food intake under conditions of stressor exposure (Zorrilla et al., 2003), this data supports the hypothesis for an increased anxiety-like state induced by SEA challenge.

The Mediating Role of Cytokines

A key property of SAgs is their ability to increase the production of cytokines. Cytokines constitute the soluble mediators of intercellular communication within the immune system, although it is recognized that the cellular origins of cytokines extend beyond the immune system to include endothelial cells, endocrine tissue, and the brain.

Within the immune system, cytokine functions include promotion of cellular proliferation, differentiation and implementation of effector functions such as cytotoxicity and antibody production. These effects are consistent with the protective aspects of pathogen-directed immunological responding. Further regulatory functions supported by cytokines include suppression of cytokine production and cellular function in and of itself, as well as reduction of leukocyte numbers through apoptosis.

The two major cytokines produced in response to an acute injection of SEA or SEB are IL-2 and TNFα, which have both been shown to exert neurobiological effects. The acute effects of IL-2, however, have not been documented in relation to gustatory behavior, but rather dopamine-related changes and behavioral activity in the presence of novelty (Zalcman et al., 1998), and disruption of intracranial self-stimulation, a measure of hedonic activity (Anisman et al., 1998). However, TNFα has been shown to produce anorexia and sickness behavior, as well as activation of the HPA axis (Hayley et al., 1999; Hayley et al., 2001). Consequently, it has proven relevant as a potential mechanism for SEA-induced behavioral changes in the context of gustatory neophobic behavior. Indeed, recently it was shown that SEA challenge increased central *c-fos* induction in limbic brain regions, and this was absent in animals deficient for TNFα production (TNF-knockout mice) (Rossi-George et al., 2005). Furthermore, it was shown that TNFα knockout mice failed to display anorexic behavior and a corticosterone response to SEA (Rossi-George et al., 2005). The role of TNFα was further corroborated using immunoneutralization of systemic TNFα, in that antiserum for TNFα blocked the corticosterone response and anorexia in wildtype mice given SEA (ibid). Therefore, it was evident from these results that an important mediational role exists for TNFα in the

behavioral, endocrine and neurobiological effects of SEA treatment. Whether the same is true for SEB remains to be determined.

This latter conclusion regarding TNFα was conducted after an acute injection of SEA. More recent work showed persistent corticosterone responses and anorexic behavior after 2-3 SEA injections, which still produced significant TNFα production (Urbach-Ross et al., 2008) (See Chapter 3). However, the magnitude of the TNFα response was reduced by close to 40-50% after two and three injections. Moreover, anorexic behavior was no longer evident after three injections, while the corticosterone response persisted after the third injection; this suggested potentially separate immunological mechanisms provoking behavioral and endocrine changes due to SEA challenge (Urbach-Ross et al., 2008). Whether TNFα is an important mediator even after repeated exposures to SEA remains to be determined, although there does not appear to be a strong case for IL-1β as an important mediator of SAg effects. That is, IL-1β levels increased substantially after repeated injections of SEA and were, in fact, quite elevated after four injections of SEA, when both endocrine and anorexic effects of SEA were no longer present (Urbach-Ross et al., 2008). Moreover, in IL-1 receptor knockout mice, the level of anorexia after SEA and SEB challenge did not differ from wildtype animals (Kusnecov et al., 1999; Rossi-George et al., 2005).

Effects of repeated Exposure to Bacterial Endotoxins and SAgs

Beeson (1946) was one of the first to define endotoxin tolerance when he discovered that repeated injections of typhoid bacterial pyrogen attenuated the febrile response. It has also been shown that pretreatment with a sublethal dose of LPS can

protect against a secondary lethal dose of LPS (Ziegler-Heitbrock, 1995). This phenomenon of endotoxin tolerance is now well established and is thought to involve both the immune system and the HPA axis. Subsequent challenge with LPS has been shown to decrease peripheral levels of IL-1β, TNFα, IL-6, and IFNγ (Urbach-Ross and Kusnecov, 2007). This is consistent with findings in our laboratory demonstrating that repeated exposure to LPS attenuated the splenic IL-1β, TNFα, and IL-6 response after subsequent exposure. The HPA axis also becomes less responsive with repeated LPS exposure, showing a reduction in ACTH and CORT production following LPS exposure (Grinevich et al., 2001; Beishuizen and Thijs, 2003; Urbach-Ross and Kusnecov, 2007). In fact, it has been shown that a single injection of LPS can attenuate the CORT response to a secondary exposure four weeks later (Valles et al., 2002).

The mechanism through which endotoxin tolerance occurs has not been fully elucidated. Macrophages from LPS-tolerant mice fail to respond to further stimulation with LPS (Mathison et al., 1990). It has been suggested that this inability of monocytes to respond may be due to the downregulation of the transcription for TLR4 and upregulation of NF-κB1 (p50), which can block proinflammatory gene activation (Poltorak et al., 1998; Kastenbauer and Ziegler-Heitbrock, 1999; Medvedev et al., 2000). The production of anti-inflammatory agents such as CORT, IL-10, and TGF-β has also been suggested to play a role in the induction of LPS tolerance (Frankenberger et al., 1995; Medvedev et al., 2000). Further, it has been noted that injection of TNFα and IL-1β produced a hyporesponsiveness to a lethal dose of LPS (Wallach et al., 1988).

Although the mechanism for LPS induced tolerance may be different, repeated exposure to SAgs can also produce tolerance to further stimulation with the same SAg.

While acute exposure to SAgs induces a strong T cells response, it has been demonstrated that repeated exposure to SAgs induces the development of T cell unresponsiveness (Feunou et al., 2003; Miller et al., 1999). The initially expanded population of T cells after acute exposure undergoes apoptosis, and those that do not undergo apoptosis become unresponsive to subsequent SAg exposure (Kawabe and Ochi, 1991; MacDonald et al., 1991). This tolerance may be induced by T cell anergy and a shift towards a Th2-type response (Miller et al., 1999). It has been noted that memory CD4 T cells, but not naïve T cells, become anergic after exposure to SEB (Watson et al., 2003). One suggested mechanism for T cell anergy is a Vβ-specific internalization of the T cell receptor (TCR) (Makida et al., 1996). Regulatory cytokines may also play a role in the induction of T cell anergy. For example, Sunstedt et al. (1997) demonstrated that there is an inverse relationship between the amount of IL-10 and the production of IL-2 in the serum after SAg re-stimulation (Sundstedt et al., 1997). Indeed, IL-10 has been shown to inhibit the production of IL-2 as well as IFNγ in human peripheral blood T cells (Taga and Tosato, 1992). Interleukin-10 has also been shown to inhibit antigen-specific T cell proliferation due to diminishing the antigen-presenting capacity of monocytes via the downregulation of MHC class II molecule expression (de Waal Malefyt et al., 1991).Therefore, it is generally agreed that stimulation with SAg prevents the production of proinflammatory cytokines after re-stimulation.

The increase in glucocorticoids after SAg exposure provides another means for inducing SAg tolerance. Glucocorticoids have many immunosuppressive properties, including inhibiting of macrophage function, decreasing the synthesis of proinflammatory cytokines, and switching the T cell response to a Th2 response (Elenkov, 2004). It has

also been suggested that glucocorticoids play a role in mediating T cell clonal deletion after SAg exposure (Gonzalo et al., 1993). Gonzalo et al. (1993) demonstrated that treatment with the glucocorticoid receptor antagonists RU-486 or adrenalectomy made animals more susceptible to the mortality induced by SEB and SEA, while treatment with the synthetic glucocorticoid dexamethasone reversed these effects. It was determined that the CORT increase after SAg exposure was important for the early clonal deletion of activated Vβ3 or Vβ8 T cells. Therefore, blockade of CORT could lead to uncontrolled T cell activation and ultimately septic shock.

Tumor Necrosis Factor-alpha

In the 1970s TNFα was originally characterized for its anti-tumor capabilities *in vivo* (Carswell et al., 1975). A few years later it was discribed that TNFα was identical to a molecule found by a separate group, referred to as Cachectin (Beutler et al., 1985). As of today there are many roles for TNFα that have been discovered, ranging from the mediation of septic shock, apoptosis, leukocyte migration, and clearance of infections in the periphery to serving dual neuroprotective and degenerative roles in the CNS (Sedgwick et al., 2000). It is a member of a family of 19 different signaling molecules produced in multiple cell types, including monocytes, macrophages, T and B cells, neurophils, and mast cells. It can also be produced in the brain by astrocytes and macrophages. When it is expressed it is released as a 26-kDa transmembrane precursor protein that is subsequently cleaved by TNFα converting enzyme (TACE) into a 17-kDa stable homotrimer (Kriegler et al., 1988). While both the cell-associated form and the secreted form are biologically active, it is the soluble form that is responsible for

endotoxin-induced lethality and it is the binding to its receptors that mediate most of the biological properties of TNFα (Josephs et al., 2000).

Tumor Necrosis Factor-alpha Signaling

Many of the different functions of TNFα seem to be regulated by its two separate receptors: TNFRI (p55, p60, CD120a) and TNFRII (p75, p80, CD120b) which mediate apoptosis via caspase activation and cell survival, respectively (Shohami et al., 1999). Binding of TNFα to both receptors results in receptor aggregation and signaling through divergent signal transduction pathways. Binding to TNFRI results in the association of TNF-receptor-associated death domain (TRADD) with other binding partners, which can transduce signals though FADD (Fas associated death domain) to initiate apoptosis. Tumor necrosis factor receptor 1 also has the ability to interact with TNFα associated factors, TNF-receptor-associated protein 1 (TRAF1), TNF-receptor-associated protein 2 (TRAF2), and TNF-receptor associated protein 6 (TRAF6) to activate NF-κB and increase the transcription of many genes (Bazzoni and Beutler, 1996; Sedgwick et al., 2000; Kruglov et al., 2008). Signaling through TNFRII, on the other hand, primarily results in interactions with TRAF1 and TRAF2 to activate NF-κB along with many other map kinases. What ultimately defines the end result of protection versus cell death has not been completely determined, however, it is understood that the activation of NF-κB can ultimately increase the transcription of many beneficial or detrimental proteins depending on the activation of other proteins (Shohami et al., 1999). Although TNFRII is known at the protective pathway, it has been suggested to work in concert with TNFRI to promote apoptosis. Tartaglia et al., (1993) found that blocking TNFRII with monoclonal

antibodies resulted in a partial inhibition of TNFα toxicity independent of TNFRII signaling. This study also determined that TNFRII had a higher affinity and faster dissociation kinetics than TNFRI. These data taken together lead to the "ligand passing" hypothesis, suggesting that TNFRII can enhance association of TNFα to TNFRI, thereby indirectly contributing to cell death (Tartaglia et al., 1993).

Consequences of TNFα

Administration of LPS results in the induction of many cytokines including TNFα, and it is thought that many of the behavioral and endocrine outcomes of LPS exposure are partially mediated by TNFα. Central administration of TNFα alone results in sickness behavior including reduction in locomotion, activation of the HPA axis, increased anxiogenic like behavior in the EPM, and anorexia (Sharp et al., 1989; Connor et al., 1998; Hayley et al., 1999). Transgenic mice that over express TNFα have been shown to have decreased body weight, alterations in exploratory behavior, retardation in the acquisition of passive avoidance, and an increase in analgesia (Fiore et al., 1996). As discussed previously, it has been hypothesized that tolerance to LPS is associated with the reduction in TNFα (Porter et al., 1998). While TNFα may mediate some of the effects of LPS and neutralization of TNFα using antibodies has been shown to reduce the ACTH response after LPS, it appears as though the effects of LPS are not fully dependent on the presence of TNFα (Turnbull and Rivier, 1998). Studies from our laboratory have shown that TNFα knockout animals continue to display anorexia after LPS administration (data not shown). This is consistent with findings that TNFα knockout animals displayed a normal cytokine response after LPS administration, and that clinical treatment with TNFα

antibodies did not benefit patients with septic shock (Fisher et al., 1996; Marino et al., 1997). These findings are contrary to those found for SEA, in that TNFα was necessary for HPA activation, neuronal activation, and anorexia after SEA administration (Rossi-George et al., 2005).

As to the different receptors, animals deficient in TNFRI appear to be resistant to LPS induced mortality and toxicity but prone to infection from *Listeria monocytogenes*, while TNFRII knockouts are resistant to LPS toxicity and to the lethality of TNFα itself (Pfeffer et al., 1993; Rothe et al., 1993; Erickson et al., 1994). Double knockout mice do not display alterations in gross brain morphology and show normal hippocampal-dependent learning (Bruce et al., 1996). Simen et al. (2006) demonstrated that both TNFRI and TNFRII knockout animals showed a non-depressed phenotype in the forced swim test, while TNFRII knockouts showed increased sucrose consumption (Simen et al., 2006). This suggests that TNFα may influence depressive like symptoms even under nonpathological conditions.

An increase in TNFα in the brain is associated with a wide range of neuropathological conditions including ischemia, trauma, multiple sclerosis, Parkinson's disease, and excitotoxicity (Pan et al., 1997; Viviani et al., 2004). While inflammation has traditionally been linked in exacerbating neuropathology, there are also cases in which it may be neuroprotective. Tumor necrosis factor-alpha has been shown to be both protective (Cheng et al., 1994; Barger et al., 1995) and detrimental to neurons (Zhao et al., 1999). For example, TNFα was shown to contribute to ischemic injury, increase oxidative stress, and increase neuronal sensitivity to nitric oxide (Barone et al., 1997; Han et al., 2001; Hemmer et al., 2001). Tumor necrosis factor-alpha has also been shown to increase

extracellular concentrations of glutamate, which may be one of the contributing factors for the synergistic role in mediating cell death (Sitcheran et al., 2005; Zou and Crews, 2005). Moreover, TNFα can increase the expression of the AMPA receptors permeable to calcium, thereby enhancing the likelihood of excitotoxic damage (Beattie et al., 2002; Stellwagen et al., 2005). Conversely, TNFα has been shown to protect against excitotoxicity through the TNFR2-PI3K-Akt-NF-κB pathway (Marchetti et al., 2004). In addition, it has been suggested that TNFα can protect against excitotoxic damage by increasing the expression of neuronal apoptosis inhibitor protein (NAIP) and increasing the number of neurons expressing calbindin-D_{28k}, thereby facilitating calcium homeostasis (Cheng et al., 1994; Thompson et al., 2004).

The amount of the cytokine present may be one of the determining factors in the shift from neuroprotection to neurotoxicity. It has been demonstrated that high doses of TNFα potentiated AMPA-induced toxicity, while lower doses were protective (Bernardino et al., 2005). Tumor necrosis factor-alpha has also been shown to be protective against neuronal death after nitric oxide excitotoxicity (Turrin and Rivest, 2006) and it has been demonstrated that TNFα -/- mice showed a significant reduction in remyelination (Arnett et al., 2001). Within the brain, TNFα can also stimulate microglial cells to activate the transcription of other cytokines such as IL-1β, as well as the cytokine-regulated transcription factor, NF-κB. Both IL-1β and NF-kB have been implicated in neurodegenerative, as well as protective, functions in the brain (Nguyen et al., 2002). Therefore, excessive production of TNFα may result in downstream changes that can modulate cell survival in the brain.

Clinical Significance

Bacterial superantigens have been implicated in the pathogenesis of many diseases ranging from toxic shock syndrome, food poisoning, atopic dermatitis, Kawasaki syndrome, and sepsis. The use of SAgs and any other toxin (eg., LPS), is based on the need to model infection-related circumstances that might shed light on neural-immune interactions. Infection is a dynamic process that has a localized origin (eg., lung, gut, wound) and spreads as a result of pathogen replication, followed by ongoing interactions with innate and adaptive components of the immune apparatus. Therefore, bolus injection models serve only to provide important information about the potential mechanisms and sets of interactions that may be generated by immunological, endocrine and neurobehavioral processes solicited by introduction of the isolated bacterial toxin. Further research is required to examine within the temporal framework of a progressive infection how neural-immune interactions may be similar or different. At the very least, body weight loss and appetite reduction are typical of chronic infections, and in this regard the bolus injection models accurately reflect metabolic and motivational changes that individuals may undergo during infectious illness. Indeed, efforts are underway to develop specific antagonists for superantigenic molecules. The efficacy of these antagonists may potentially reduce many of the severe pathological effects of staphylococcal and streptococcal infections, where T cell activation by superantigenic exotoxins is likely to occur. Moreover, should antagonists only partially reduce the capability of SAgs to stimulate T cells, further antagonism of TNFα, and possibly other cytokines, will serve to reduce the neurobiological effects that ensue from infection,

thereby reducing changes in body weight, and other motivational, emotional and cognitive alterations are likely to result from activation of stress systems in the brain.

Aims

Staphylococcal enterotoxin A activates the HPA axis and produces gustatory neophobia as well as other indicators of sickness behavior. Data from our laboratory have shown that TNFα mediates these effects, and TNFα knockout animals fail to display an increase in corticosterone (CORT), anorexia, and neuronal activation in response to SEA administration. While the effects of acute SEA are well documented, the behavioral and endocrine consequences of repeated SEA have not been fully established. Further, the role of TNFRI and TNFRII in mediating the effects of acute and chronic SEA has not been explored. Therefore, the current project has addressed the consequences of repeated SEA as well as the role of TNFRI and TNFRII in mediating its effects. Additionally, the upregulation of glucocorticoids after SAg exposure has been shown to protect animals from lethal shock and has been suggested to play a role in endotoxin tolerance. To understand the impact of glucocorticoids in regulating tolerance to secondary SEA or LPS this study determined the immunological effect of CORT inhibition to acute and secondary exposure to SEA or LPS. Finally, while there is considerable work characterizing the effect of stress on macrophage-mediated inflammation, there is little addressing the immunological effect following a T cell mediated immune response. This study aimed to determine the effect of glucocorticoid disruption through the use of chronic restraint on the response to both acute and repeated SEA.

Working Hypothesis

Our studies have confirmed that TNFα is one of the main signals for behavioral and neural effects of acute SEA. We now seek to determine which receptors are primarily responsible for mediating the effects of TNFα after acute SEA. While the cytokine profile is well established after acute SEA, alterations in cytokine induction of repeated SEA administration have not been fully explored. Therefore, we will first attempt to determine the effects of repeated SEA on the cytokine profile, CORT response, and sickness behavior. Then, we will examine whether a lack of TNFRI or TNFRII will have an effect on the consequences of acute and repeated SEA exposure. Studies have shown that TNFRI is the more significant mediator of TNFα and LPS (Tracey et al., 1987; Rothe et al., 1993; Benigni et al., 1996; Peschon et al., 1998). Therefore, it was hypothesized that TNFRI, but not TNFRII deficient animals, would show blunted endocrine and behavioral effects following both acute and repeated exposure to SEA.

A normal glucocorticoid response following SAg and endotoxin exposure has been suggested to be necessary for the induction of tolerance. Therefore, it was hypothesized that interfering with the glucocorticoid response by inhibiting glucocorticoids or by administering chronic stress would interfere with SAg and endotoxin tolerance.

Chapter 2

General Methodology

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 under 12:12 hr light:dark illumination (lights on 0600h). This mouse strain was used because the T cell receptor Vβ3 phenotype, which reacts strongly to SEA, is highly expressed on T cells from C57BL/6J mice. For this reason, C57BL/6 mice have been the strain of choice for studies with SEA (Kusnecov and Goldfarb, 2005). Animals were fed milled mouse chow *ad libitum* for five weeks. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Rutgers University.

Generation of knockout animals

TNFRI-deficient and TNFRII-deficient male mice bearing a C57BL/6 background were obtained from The Jackson Laboratory along with C57BL/6 females. The $TNFRI^{-/-}$ and $TNFRII^{-/-}$ population was expanded by backcrossing with C57BL/6J mice, followed by maintenance of breeding between F1 heterozygous ($TNFRI^{-/+}$ and $TNFRII^{-/+}$) males and females, with the Het, KO, and WT 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.

Genotyping of TNFRI-deficient and TNFRII-deficient mice

Tail tissue (2-3 mm) from each mouse cut with a fresh razor blade and stored at -70 °C until DNA extraction. Upon extraction the tissue was digested with 200μl of lysis buffer (100mM Tris-HCl, pH 8.5, 5 mM EDTA, 200mM NaCl, 0.2% SDS, 1mg/ml proteinase K) and incubated at 54 °C overnight. The following morning samples were spun at 1100 rpm for 10 minutes after which the supernatant was collected. Precipitation of the DNA was achieved by adding 200μl of isopropyl alcohol to the supernatant after which the DNA was removed and washed in 70% EtOH. The DNA pellet was then resuspended in 100μl of Tris-EDTA buffer (pH 8). After resuspension 1μl of the DNA was used in a 50μl polymerase chain reaction (PCR; TAQ PCR Master Mix Kit, Qiagen, Valencia, CA). The primer sequences and PCR cycling for TNFRI TNFRII genotyping conditions were obtained from Jackon Laboratories (Bar Harbor, ME) (TNFRI - http://jaxmice.jax.org/strain/002818.html; TNFRII - http://jaxmice.jax.org/strain/002620.html) and the oligonucleotides were synthesized by the DNA synthesis facility at the University of Medicine and Dentistry of New Jersey. The sequences can be found in Table 2. 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). Once the animals were genotyped, they were housed four per cage corresponding to their genotype and allowed to acclimate for at least a week before testing. All knockout animals were tested along with their heterozygous and wildtype littermate controls.

Reagents

Staphylococcal enterotoxin A was purchased from Sigma-Aldrich (St. Louis, MO) or

Toxin Technology (Sarasota, FL). All injections of SEA were given intraperitoneally (IP)

at a dose of 5 μg/mouse in pyrogen-free physiological saline in a volume of 0.2 ml.

Behavioral Testing for Liquid Consumption

Prosobee is a substance that is readily consumed by mice and can therefore be used as a

measurement of sickness induced anorexia (Kaneta and Kusnecov, 2005; Rossi-George et

al., 2005). The Prosobee solution was made following the manufacturer's instructions.

For testing, animals were placed in a white opaque box along with a preweighed bottle of

Prosobee. After one hour the animals were returned to their home cage and the bottles

were reweighed in order to measure the total consumed by each animal. When there was

consumption testing at different time points the animals remained in the opaque cages

until the next session.

Blood and Tissue Collection

Animals were sacrificed by decapitation two hours after the last injection of SEA or

saline and blood and tissue collected. This time point was previously shown to be optimal

for increased pituitary-adrenal activation and cytokine production (Kawashima and

Kusnecov, 2002). Plasma CORT exhibits diurnal rhythmicity, in that CORT

concentrations rise as the dark cycle approaches. Therefore, animals were always

sacrificed between 11:00 A.M. and 2:00 P.M. during the light cycle to prevent an overall

ceiling effect. Trunk blood was collected by rapid decapitation into heparin-treated

vacutainer tubes (Becton Dickinson, Rutherford, NJ). The blood was centrifuged

immediately at 2000 rpm for 15 min, and the plasma was collected and stored at -70°C.

Tissue was flash frozen in 2-methylbutane and stored at -70°C until protein extraction.

Protein extraction and quantification

The spleens were dissected and placed in 1 ml of 1mM phenylmethanesulfonyl fluoride

(PMSF) in 0.1M phosphate buffer to inhibit protease activity. The tissue was

homogenized and centrifuged at 4000 RPM for 30 minutes after which the supernatant

was collected. Total protein was quantified using the BCA protein assay kit (Pierce,

Rockford, IL). Absorbance was read at 562 nm and concentrations were calculated off a

standard curve generated using bovine serum albumin (BSA). Total protein was

expressed as $\mu g/ml$.

Cytokine enzyme linked immunosorbent assay (ELISA)

Spleen homogenates were centrifuged and the supernatant assayed for immunoreactive

IL-2, IFNγ, TNFα, IL-1β, IL-10, IL-4, and IL-6 using OptEIA ELISA kits according to

the manufacturer's instructions (BD Biosciences, San Diego, CA). Portions of plasma not

dedicated to CORT assays were also assayed for select cytokines. Plasma was diluted at

1:4 dilutions for all cytokines except IL-2 which was run at a 1:40 dilution. All standards

were run in duplicate. The absorbance was read at 450 nm using EL800 universal BioTek

microplate reader. Concentrations were calculated off a standard curve using KC Junior

software (Biotek).The cytokine data were expressed as a ratio of total protein (pg of

cytokine/μg of protein).

Corticosterone Radioimmunoassay (RIA)

Plasma corticosterone was measured by ImmunoChem™ Double Antibody Corticosterone ^{125}I kit following the manufacturer's instructions (MP Biomedicals, Irvine, CA). All standards and samples were run in duplicate and counted using a Cobra II Auto Gamma counter. The corticosterone data were expressed as ng/ml.

Adrenocorticotropic Hormone Radioimmunoassay (RIA)

Plasma corticosteone was measured by ImmunoChem™ Double Antibody ACTH ^{125}I kit following the manufacturer's instructions (MP Biomedicals, Irvine, CA). All standards and samples were run in duplicate and counted using a Cobra II Auto Gamma counter. The ACTH data were expressed as pg/ml.

Reverse Transcription and Real Time PCR

Relative quantitation of CRH, TNFRI, TNFRII, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 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). Primer sequences can be found in Table 3. Total RNA was isolated using Trizol® Reagent (Invitrogen, Carlsbad, CA) following manufacture's instructions. Briefly, tissue was homogenized in 1 ml of TRIzol® reagent for every 100 mg of tissue after which samples were incubated at room temperature for five minutes. After incubation, 0.2 ml of chloroform were added per 1 ml of TRIzol® and samples were vortexed, incubated at room temperature, and spun at

12,000 x *g* for ten minutes. Following centrifugation, the aqueous phase was removed and transferred to a new tube. Precipitation of the RNA was then achieved by adding 0.5 ml of isopropyl alcohol for every 1 ml of TRIzol® reagent. The preparation was then spun at 12,000 x *g* for ten minutes after which the supernatant was removed, leaving behind the RNA precipitate. The precipitate was washed with 75% ethanol and allowed to air dry for five minutes before resuspension in sterile DEPC-treated water. Samples were quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and samples with a 260/280 ratio less than 1.6 were excluded. Generation of cDNA from 1 µg of RNA was accomplished by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). 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 twofold dilution series of cDNA from spleen RNA obtained from an animal that was given an injection of SEA (undiluted control cDNA, 200 arbitrary units) or from hypothalamus of an LPS treated animal (undiluted control cDNA, 100 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.

Immunocytochemistry

For perfusion, animals were lethally anesthetized with 50 mg/Kg sodium pentobarbital and sacrificed via transcardial perfusion for 5 min with isotonic saline, followed by 10 min with 4% paraformaldehyde, and a 5-min wash with isotonic saline. Brains were post-fixed overnight in 4% paraformaldehyde and then placed in 30% sucrose solution until sectioning. Coronal brain sections of 30 μm thickness were made on a freezing microtome and cryopreserved at -20 °C as free-floating sections.

Tissue sections were incubated initially for 72 h with rabbit anti-mouse c-Fos (1:15,000; Oncogene Science, Cambridge, MA), at 4 °C in 0.4% Triton X-100 in KPBS, pH 7.2. For verification of the staining specificity of the antibody, rabbit IgG (Sigma, St. Louis, MO) was used at 1:1000 dilution to serve as an isotype control. After rinsing in Potassium phosphate buffered saline (KPBS), the tissues were incubated for 2 h at room temperature in KPBS/0.4% Triton X-100 containing a 1:500 final dilution of biotinylated goat anti-rabbit IgG or goat anti-rat IgG (Vector Laboratories, Burlingame, CA). After an additional rinse, tissues were treated for 1 h at room temperature with a 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 diaminobenzadine (DAB) substrate solution consisting of 0.175 M NaOAc containing 25 mg/ml $NiSO_4$, 0.2 mg/ml DAB, and 0.28% H_2O_2. Termination of the enzyme-substrate reaction was achieved by rinsing the tissues in 0.175 M NaOAc and then in KPBS, after which the tissues was mounted on Superfrost Plus slides (Fisher Scientific), dehydrated in a graded series of alcohols, and clarified and coverslipped using Histoclear and

Histomount (VWR Scientific, Westchester, PA). The sections were counterstained with 1% neutral red. For quantitation of 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. Four sections per animal were counted, with each representing points 0.25 mm apart along the anterior to posterior extent from these Bregma points. The minimum pixel size for a cell to be counted was five pixels and the maximum size was 35 pixels. The threshold for each image was determined separately for each individual image. Each section was counted three times and a total of four sections per animal were counted and averaged. Because thresholding the image was different for each image, sections were randomly hand counted for verification. One section from each of six animals was randomly chosen for hand counting and correspondence to the software was established. Confirmation of software accuracy was conducted by two observers, blind to treatment, hand-counting selected regions of interest. Variation in number between human and software counts were 1–2%.

Statistical Analysis

Most experiments conformed to a factorial design and were therefore analyzed by ANOVA using Statview, a statistical software package. In the event of significant main effects, appropriate post hoc comparisons were conducted with the inbuilt protection for Type I error. 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 published data were warranted (Rossi-

George et al, 2005), an unpaired t test was conducted.

Chapter 3

Relationship of Varying Patterns of Cytokine Production to the Anorexic and Neuroendocrine Effects of Repeated Staphylococcal Enterotoxin A Exposure

Introduction

It has been shown that the pronounced stimulatory effects of acute SAg exposure on T cell activation are distinct from those seen after repeated exposure, whereby the initially expanded population of T cells either undergoes apoptosis or becomes unresponsive to subsequent SAg exposure (Kawabe and Ochi, 1991; MacDonald et al., 1991; Miller et al., 1999; Feunou et al., 2003). This tolerance may be induced by T cell anergy and a shift towards a Th2-type response (Miller et al., 1999; Watson et al., 2003). The mechanism for this is not fully understood, although regulatory cytokines may play a role. For example, Sunstedt et al. (1997) demonstrated an inverse relationship between the concentration of serum IL-10 and IL-2 after SEA restimulation. Therefore, given the well known anti-inflammatory effects of IL-10 (Strle et al., 2001), it is possible that repeated exposure to SEA leads to a progressive inhibition of proinflammatory cytokines, and coincident loss of neuromodulatory changes associated with SEA exposure.

While acute exposure to SAgs has repeatedly been shown to induce tolerance to subsequent exposure, there are far fewer studies addressing repeated SAg exposure. Morever, there is no evidence for the effects of repeated SEA exposure on HPA-axis activation and alterations in sickness behavior. If repeated SEA exposure continues to exert neuromodulatory effects, it is important to determine the relative pattern of cytokine responses, since the nature of the cytokine response to initial and subsequent exposures to

SEA may vary. Where studies have looked at repeated SEA exposure, most have focused on endpoint cytotoxic and proliferative *in vitro* measures subsequent to *in vivo* injections (Sundstedt et al., 1994; Kuroda et al., 1996; Miller et al., 1999). However, little is known about *in vivo* variations after repeated SEA in the composition and magnitude of different cytokines initially induced by an acute SEA injection.

In the present study, the endocrine, behavioral and cytokine-inducing effects of acute and repeated injections of SEA were determined under conditions that varied the temporal parameters between succeeding SEA injections. This was based on previous reports that the nature of the T cell and cytokine responses to SEA varied as a function of different time intervals (measured in days) between initial and subsequent challenges with SEA (Sundstedt et al., 1994). These previous observations were conducted in T cell transgenic mice, but have yet to be repeated in normal C57BL/6 mice. Moreover, given that dynamic T cell alterations (eg., proliferation) persist after a single injection of SEA, it is conceivable that further introduction of SEA will impact on these and possibly other non-activated lymphocyte populations, resulting in altered patterns of cytokine production. Furthermore, the persistence of the neuroendocrine and behavioral responses to SEA, in the context of changing patterns of cytokine production has not previously been determined**.** Therefore, given that repeated exposure to SAgs results in T cell tolerance (Kawabe and Ochi, 1991; MacDonald et al., 1991; Miller et al., 1999; Feunou et al., 2003), it was important to determine whether tolerance occurs also to the neurobehavioral effects of SEA after repeated treatment. To this end, the present study sought to determine the persistence of plasma corticosterone and anorexic effects in response to repeated challenges with SEA. Moreover, splenic and plasma cytokine

measures were conducted to determine whether loss of endocrine and behavioral effects of SEA was associated with a similar cessation of cytokine reactivity.

Materials and Methods

Animals

See "General Methods".

Reagents

See "General Methods".

<u>Experimental Procedure: Schedules for SEA Injection</u>

Experiment 1.1: Two Injections of SEA two and three Days Apart

In this experiment, the effects of two SEA exposures separated by two and three days was tested. Sundstedt et al. (1994) demonstrated that a single injection of SEA resulted in proliferation of spleen cells, however secondary *in vitro* exposure within a short time interval (7days) resulted in T cell anergy (Sundstedt et al., 1994). Further, it has also been reported T cell expansion peaked at two days after a single injection of SEA and continued to decline thereafter (McCormack et al., 1993; Kuroda et al., 1996). Consequently, this particular study chose to look at the effect of a second injection of SEA that coincided with a time when T cells were actively dividing, as based on the literature.

The design of the experiment and the temporal distribution of injections over a four

day period are shown in Table 4. Although no group was given more than two injections

of SEA, some groups received saline injections to control for handling and injection of

SEA in other groups subjected to different temporal parameters. Subjects were given an

acute injection of SEA (Total of N = 11), two injections of SEA spaced two days apart

[SEAx2 (2d)] (N = 6), and two injections of SEA spaced three days apart [SEAx2 (3d)]

(N = 5). As already noted, there were two different acute SEA and saline groups to

control for the different injection schedules.

Experiment 1.2: Injections of SEA three and five days apart

In contrast to Experiment 1.1, this experiment assessed the effects of different

regimens of SEA exposure on the anorexic response. As reported in the literature,

repeated exposure to SEA significantly reduces T lymphocyte reactivity (Sundstedt et al.,

1994; Miller et al., 1999), although the nature and magnitude of the cytokine responses

after repeated *in vivo* SEA is less well known. However, it could be hypothesized that the

refractory nature of the T cell response after repeated SEA treatment may underlie a loss

in behavioral reactivity to SEA. Therefore, in this current experiment, the schedule of

injections involved spacing two SEA injections three or five days apart, in order to

capitalize on the effects of the three day separation in Experiment 1.1, as well as

determine whether SEA still has a behavioral effect if administered after the proliferative

peak reported to occur in response to an initial SEA injection (McCormack et al., 1993;

Kuroda et al., 1996).

Table 5 summarizes the injection schedule. All animals received a total of three injections. Control animals received injections of saline on days 0, 2, and 5. Animals given a single SEA injection (Acute SEA) were injected with saline on days 0 and 2 and given an injection of SEA on day 5 (N = 8). The group of animals that were given two injections of SEA spaced three days apart [SEAx2 (3d)] was given saline on day 0 and SEA on days 2 and 5 (N = 8). The other group that received two injections of SEA spaced five days apart [SEAx2 (5d)] received SEA on days 0 and 5 with saline on day 2 (N = 8). Finally, a group of animals were given three injections of SEA on days 0, 2, and 5 (SEAx3) (N = 8). On day 5 of the experiment, and two hours after injection, animals were tested for anorexia, by assessing the consumption of a commercially available and highly palatable baby liquid formula (Prosobee, Mead Johnson, Evansville, IN).

Experiment 1.3: Repeated SEA injection protocol

In order to extend the findings of Experiment 1.2, the animals from that experiment were not sacrificed on the day of consumption testing, but were given further injections of SEA. This allowed for the assessment of the cytokine profile and the CORT response in animals given two injections of SEA spaced five days apart (which was not measured in Experiment 1.1). In addition it was possible to examine the influence of three and four injections of SEA on these parameters. Table 6 summarizes this extended regimen of treatment, and for the most part, the treatment schedule is self-explanatory (See Table 6). Briefly, for the animals that were sacrificed after the final SEA injection, the SEA 5d group represented two injections of SEA given 5 days apart, with the

corresponding control receiving a single injection of SEA on day 5 and saline on day 10 (Control 5d). Groups designated as SEAx3a and SEAx4 received three and four injections respectively with the controls receiving a saline injection on the day of sacrifice instead of SEA (Controlx3a and Controlx4 respectively). The SEAx3b group also received three injections of SEA, but the spacing of the three injections for this group was every 5 days (see Table 6). The control for this group received SEA injections as shown in Table 6, but was given saline on the day of sacrifice (see Table 6, Controlx3b). For ease of interpretation, all control groups that had either only received Saline or SEA from D0-D5, but were injected with Saline on the kill day (viz., D10), are indicated with shading. For all of the post-hoc tests each group was compared to their respective control as indicated above. Finally, there were N = 4 animals in each group. This reduction in N was inevitable, since this experiment involved splitting each of the groups in Experiment 1.2. As we have repeatedly demonstrated, SEA is a powerful stimulant of corticosterone and cytokine production ((Kawashima and Kusnecov, 2002; Rossi-George et al., 2004; Kaneta and Kusnecov, 2005; Rossi-George et al., 2005), and therefore does not require exceedingly large numbers of animals to demonstrate a clear difference between saline and SEA-treated groups. Considerable care was taken to minimize the presence of non-specific stress to ensure that endocrine parameters were not unduly confounded. Moreover, for at least cytokine determinations, four animals per group provided sufficient statistical power, which was borne out by the results. Moreover, as the results showed, this experiment revealed the expected increases in corticosterone that is a routine feature of SEA challenge.

Blood and Tissue Collection

See "General Methods".

Protein extraction and quantification

See "General Methods".

Cytokine enzyme linked immunosorbent assay (ELISA)

See General Methods".

Corticosterone Radioimmunoassay (RIA)

See "General Methods".

Statistical Analysis

See "General Methods".

Results

Experiment 1.1

Table 4 presents the design for this experiment. It should be noted that both groups of animals that received saline injections were included in the ANOVAs as separate groups. However, for purposes of clarity, they were collapsed in the figures, since in all cases animals sacrificed after a saline injection (whether pretreated with saline or SEA) did not show any differences in cytokine or corticosterone production. This was a universal observation, being the case also for experiments 1.2 and 1.3.

Splenic Cytokines

Figure 3.1 shows the cytokine concentrations for TNFα, IL-1β, IL-2 and IFNγ as

measured in the non-dissociated spleen. It is evident that for all cytokines, except IFNγ,

there was a marked increase in response to an acute injection of SEA. Variations in

response, however, were noted in response to the second injection of SEA given two or

three days after the initial treatment. These analyses are summarized below.

Tumor Necrosis Factorα. Treatment with SEA significantly increased the level of TNFα

($F_{(3,29)}$ = 66.314, *p* <.0001). However, both secondary injections of SEA showed an

attenuated TNFα response compared to the first injection (SEA 2d, *p* < .0001; SEA 3d, *p*

< .0001).

Interleukin-1β. There was a significant increase in IL-1β after injection with SEA in all

of the groups ($F_{(3,28)}$ = 26.433, *p* < .0001). Interestingly, there was no attenuation of the

IL-1β response to another SEA injection two days after initial SEA exposure (SEA 2d, p

= .1694), although a significant reduction relative to the acute response was observed if

SEA was given again three days later (SEA 3d, *p* = .0203).

Interleukin-2: Acute or secondary administration of SEA increased the level of IL-2

($F_{(3,29)}$ = 48.615, *p* < .0001). Neither of the secondary injections of SEA attenuated the

IL-2 response, but rather appeared to increase the level of IL-2 above that of an acute

injection. (SEA 2d, *p* < .0001; SEA 3d, *p* = .0428).

Interferon-γ. There was no significant production of IFNγ after a single injection of SEA,

although a second injection of SEA given two days after the initial injection caused a

significant increase in IFNγ (SEA 2d, $p < .0001$). This was no longer evident, however, if the second injection was given three days after the initial SEA injection.

Corticosterone: Figure 3.2 shows the plasma corticosterone concentrations associated with the above treatments and cytokine responses. It can be seen that there was a significant increase in the corticosterone response after treatment with SEA ($F_{(3,26)}$ = 5.998, $p = .0030$). This was also the case after secondary exposure to SEA two or three days after the initial administration.

Experiment 1.2

Table 5 shows the design for this experiment. As described in the materials and methods, the rationale for this experiment was to extend the cytokine and corticosterone data from Experiment 1.1 to a confirmation of whether the well-documented anorexic response to SEA is retained after two or more SEA treatments. Testing for anorexia involved exposure to a palatable liquid diet (Prosobee), which is consumed without the need for water or food deprivation. Previous studies have shown that this reveals significant anorexic or hypophagic effects subsequent to a single SEA injection. In the present experiment, the retention of this anorexic response was determined after multiple injections of SEA. Figure 3.3 shows that treatment with SEA decreased consumption of Prosobee ($F_{(4,34)}$ = 5.060, $p = .0026$), this being evident after an acute injection of SEA (p = .0010). Interestingly, two injections of SEA either three or five days apart continued to decrease consumption of Prosobee ($p = .0151$; $p = .0130$). However, three injections of SEA abrogated the anorexia, showing no difference to controls ($p = .7303$). Therefore,

while two injections spaced at different intervals continue to exert a behavioral effect, the effect of a third injection is lost, in spite of the three injections in this particular group occurring within the same five-day period. Whether this is related to changes in cytokine production and loss of a corticosterone response after the third injection was assessed in the next experiment.

Experiment 1.3

Table 6 shows the design for this experiment, which extended the treatments of surviving animals in Experiment 1.2. It is acknowledged that the effects of additional SEA treatments may have interacted with prior behavioral testing. However, it was anticipated that this would be without major effect, given that animals were already habituated to multiple handling and intraperitoneal injections. In any case, all groups from Experiment 2 were subdivided into those that received a Saline or SEA injection, and sacrificed two hours later and corticosterone, and splenic and plasma cytokines were then determined. For all ANOVAs, the respective control groups for each regimen of SEA treatment were included in the analysis, and are also indicated in the figures.

Corticosterone: Figure 3.4 shows the plasma corticosterone concentrations for the various SEA treatment groups. A significant SEA treatment effect was obtained ($F_{(9,30)} = 4.667$, $p = .0006$), with the acute SEA treatment showing the strongest response ($p = .0009$). Although significantly attenuated from acute SEA, two and three injections of SEA continued to increase the level of CORT (SEA 5d, $p = .0452$; SEAx3a, $p = .0149$).

Interestingly, three injections spaced at five day intervals (SEAx3b) and four injections

(SEAx4) of SEA failed to increase CORT.

Splenic Cytokines

Figure 3.5 shows the cytokine concentrations for TNFα, IL-1β, IL-2 and IFNγ as

measured in the non-dissociated spleen.

Interleukin-2: There was a significant effect of SEA treatment on the concentration of

splenic IL-2 ($F_{(9,27)}$ = 56.754, p = <.0001). All of the animals that received SEA on the

day of sacrifice showed an increase in IL-2 when compared to their respective controls.

Acute exposure to SEA and two injections of SEA showed the greatest increases of

splenic IL-2 compared to their respective controls (p = <.0001), while three injections

(SEAx3a and SEAx3b)) and four injections of SEA showed increased, but attenuated, IL-

2 levels (p <.0001; p = .0040; p = .0003). Therefore, as the number of exposures to SEA

exceeds two, the IL-2 response to additional SEA challenges is substantially diminished.

Interferon-γ: Irrespective of the type of SEA regimen, there was a main effect of SEA

treatment on splenic IFNγ concentrations ($F_{(9, 27)}$ = 9.585, p = <.0001). Post hoc analysis

revealed that acute SEA, along with two and three injections of SEA all increased the

level of splenic IFNγ compared to their respective controls (p = .0099, p <.0001; p

= .0017; p = .0412). Four injections of SEA did not increase IFNγ (p = .0844).

Interestingly, two injections of SEA seemed to exacerbate the level of splenic IFNγ

compared to acute exposure to SEA (p = .0015) and seems to represent the time point in

which there is a maximum increase due to the fact that two injections of SEA show a

significantly higher level of IFNγ compared to three and four injections of SEA (SEAx3a, $p = .0075$; SEAx3b, $p = .0002$; SEAx4, $p = <.0001$). It should also be noted that in Experiment 1, a similar augmenting effect of two injections, spaced two days apart, was also observed for IFNγ.

Interleukin-1β: There was a significant effect of the differential regimen of SEA on IL-1β production ($F_{(9,27)} = 9.585$, $p = <.0001$). Post hoc analysis revealed that acute SEA did not cause an elevation in IL-1β ($p = .5294$). After the second injection of SEA, however, IL-1β was drastically increased in all treatment groups when compared to their respective controls (all groups: $p < .0001$). While not as extreme as IFNγ, the trend suggests that three and four injections of SEA did not elevate IL-1β as much as two injections (SEAx3a, $p = .1085$, SEAx3b, $p = .0003$; SEAx4, $p = .0575$).

Tumor Necrosis Factorα: There was a significant effect on the level of TNFα after treatment with SEA ($F_{(9,30)} = 35.190$, $p <.001$). Acute SEA significantly increased the level of TNFα compared to the respective control ($p <.0001$). While two or three injections of SEA (SEAx3a) continued to show an elevated level of TNFα compared to their controls (SEA 5d: $p = <.0001$; SEAx3a: $p = .0139$), the levels were significantly attenuated compared to an acute injection of SEA (SEA5d, $p <.0001$: SEAx3a, $p <.0001$). Interestingly, three injections of SEA every 5 days (SEAx3b) and four injections of SEA (SEAx4) did not increase splenic TNFα.

Interleukin-10: There was a significant SEA treatment effect on the levels of IL-10 ($F_{(9,29)}$ = 30.983, $p <.0001$), which was associated with a progressive increase in IL-10 output as the number of SEA injections increased (SEA5d, $p = .0010$; SEAx3a, $p <.0001$; SEAx3b, $p = .0270$; SEAx4, $p < .0001$). Interestingly, there was a marked difference between the SEAx3a and SEAx3b groups, suggesting that the timing of injections was critical in determining whether a third injection of SEA would induce a strong IL-10 response.

Interleukin-4: There was no significant SEA treatment effect on the IL-4 response ($F_{(9,30)}$ = .957, $p = .4931$).

Plasma cytokines

Figure 3.6 summarizes the plasma IL-1β and IFNγ concentrations measured in response to different numbers of SEA injection. These cytokines showed augmented splenic responses secondary to injections of SEA. Therefore, it was important to determine whether this also reflected considerable extracellular release of the cytokines. IL-1β was of particular interest since it plays an important role in promoting anorexic responses and HPA axis activation.

Interleukin-1β: The plasma IL-1β results were surprising in that they failed to parallel those obtained for the spleen, there being no significant effect of SEA treatment ($F_{(9,24)}$ = 2.085, $p = .0730$). Perhaps the most notable suggestion of an increase was after four injections of SEA, which was actually significant using a post hoc comparison (SEAx4, p

= .0026). However, the relevance of this to neuromodulation by SEA cannot be determined, since the plasma corticosterone response was not elevated in this group.

Interferon-γ: In contrast to IL-1β, there was a significant effect of SEA treatment on plasma IFNγ after differential regimens of SEA ($F_{(9,28)}$ = 17.786, p <.0001). Post hoc analysis revealed that acute SEA, along with two, three, and four injections of SEA, increased the level of splenic IFNγ compared to their respective controls (Acute SEA, p = .0063; SEA5d, p <.0001; SEAx3a, p = 0019; SEAx3b, p = .0225; SEAx4, p = .0024). This mirrored findings for the spleen, especially the augmenting effect of a second injection of SEA (p < .0001).

Discussion

Previous studies have confirmed that systemic SEA challenge produces significant neural, behavioral and endocrine effects. These include induction of c-Fos immunoreactivity in limbic brain regions, activation of the HPA axis, and augmentation of neophobic behavior (Gonzalo et al., 1993; Kawashima et al., 2002; Kawashima and Kusnecov, 2002; Rossi-George et al., 2004). These effects have been observed only in relation to a single challenge of SEA, there being no evidence for dynamic changes in the relationship between cytokine production and neurobehavioral alterations after multiple exposures to SEA. This question is important in light of evidence that superantigens can induce immunological tolerance, which may result in the elimination of any neurobehavioral effects subsequent to SEA challenge. Consequently, the present study

sought to determine the persistence of plasma corticosterone and anorexic effects in response to repeated challenges with SEA.

Since acute injection of SEA increases plasma corticosterone in a CRH-dependent manner (Rossi-George et al., 2005), the current study focused on this glucocorticoid as a measure of HPA axis activation. It is understood, however, that in relation to multiple exposures to SEA, the central mechanisms responsible for the corticosterone response may shift, as has been demonstrated for chronic psychogenic stressors. As shown in the results, repeated SEA treatment continued to elevate plasma corticosterone, suggesting that this endocrine effect is not confined to acute SEA exposure. However, the interval between SEA challenges may be an important determining factor of the magnitude of this effect. In Experiment 1, challenge with SEA two or three days after an initial priming injection, produced a corticosterone response that was equal in magnitude to a single exposure. In contrast, Experiment 3 demonstrated that when the priming injection was given five days earlier (SEA 5d group, Table 6), the CORT response to the second SEA challenge was reduced by approximately 50%. Interestingly, this 5 day interval between injections did not affect the anorexic response to the second SEA challenge, as tested in Experiment 1.2 [see Fig 3, SEAx2 (5d)]. This suggests a possible dissociation between the behavioral effects of SEA and its capacity to affect the neuroendocrine system.

This dissociation is also implied by the results obtained for three injections of SEA. Two three-injection regimens were instituted in Experiment 1.3. For one group (SEAx3a), a reinterpretation of Table 6 indicates that SEA injections essentially occurred on time point days 0, 3 and 8, while for the second group (SEAx3b), SEA exposures occurred on days 0, 5 and 10 (as actually indicated in Table 6). Under these two separate

regimens, the latter group failed to show a significant increase in the corticosterone response to the final SEA injection. This was not the case for the SEAx3a group which showed a significant increase in plasma corticosterone (see Figure 3.4). Therefore, if three injections are spaced at 5 day intervals, the neuroendocrine effect is lost. However, this does not appear to be a function of there being a 5 day interval between the final two injections, but rather a function of the interval between the initial two injections, which varied between these two groups. That is, a second injection given 5 days, rather than 3 days, after the first priming dose, enables refractoriness to the neuroendocrine effects of a third SEA injection. Whether this third injection needs to be given 5 days later or can be delivered earlier does not appear to be critical. This is suggested by the behavioral effects of Experiment 1.2, where it was demonstrated that a third injection of SEA given 3 days after the second injection failed to elicit an anorexic response. It is not known whether this was also associated with a deficient corticosterone response. These data suggest that an accumulation of SEA exposures within 2-3 days of a priming injection may facilitate continued CNS reactivity to subsequent challenges with SEA. However, as the interval between the first two injections increases, the facilitatory influence of these initial injections dissipates.

The immunological mechanisms underlying these effects remain elusive, but are likely to be dependent on the kinetics of the T cell proliferative response after an acute injection to SEA. The primary cells responding to SEA bear the T cell receptor $V\beta 3$ and $V\beta 11$ phenotypes, and display peak proliferative capacity two days after injection (McCormack et al., 1993; Kuroda et al., 1996), after which expansion declines and enters a phases of clonal deletion and T cell anergy (Kawabe and Ochi, 1991; MacDonald et al.,

1991). In the present study, *in vivo* measures of cytokine production were taken to determine whether the endocrine and behavioral effects of SEA injections before, during and after these stages of T cell activity could be related to shifting patterns of cytokine output. In particular, it was of interest to monitor the TNFα response, since the CNS effects of an acute SEA challenge were shown to be dependent on the presence of TNFα (Rossi-George et al., 2005).

As expected, an acute injection of SEA induced a strong TNFα response, which was significantly attenuated after secondary exposure to SEA. In contrast, IL-1β, IFNγ, and IL-2 all remained elevated after secondary exposure, which for IFNγ was consistently augmented if given two days after priming (see results for Experiment 1.1 and 1.3). The augmentation of the IL-2 response observed in Experiment 1.1 was not observed in Experiment 1.3, although it did not change in magnitude from that to a single injection of SEA. However, three or four injections of SEA led to substantially attenuated IL-2 and IFNγ responses, which is consistent with the literature (Gaus et al., 1994; Sundstedt et al., 1994; Florquin et al., 1995; Noel et al., 2001). There is evidence to suggest that the source of IFNγ may be CD8+ T cells, which, unlike CD4$^+$ T cells, fail to enter an anergic state subsequent to superantigenic exposure (Kawabe and Ochi, 1991; Sundstedt et al., 1994; Hoiden and Moller, 1996). However, in the context of the current study, there is little evidence that IFNγ is a primary stimulus for the HPA axis and modulation of behavior. Nonetheless, this has yet to be tested in the superantigen model, and in the present case may represent a viable factor that affects the CNS. Indeed, it has been demonstrated that shock induced by a secondary injection to SEB is IFNγ dependent (Plaza et al., 2007). Whether this implies a role for IFNγ in CNS signaling, is not known.

The anergic response that is observed after SEA exposure is partially mediated by the anti-inflammatory cytokine IL-10. For example, IL-10 has been shown to inhibit production of IL-2, TNFα, and IFNγ and directly inhibit T cell proliferation (de Waal Malefyt et al., 1991; Taga and Tosato, 1992; Sundstedt et al., 1997). The current findings showed that primary exposure to SEA led to a progressively enhanced IL-10 response after each successive exposure to SEA. This suggests that repeated exposure to SEA produces a shift from a Th1 to a Th2 cell response, although it might be expected that other Th2 cytokines, such as IL-4, might also show greater prominence with repeated SEA exposure. This was not the case, since the splenic concentration of IL-4 after acute or repeated SEA remained unchanged. This lack of an IL-4 response to an acute SEA challenge is not inconsistent with others superantigen models (Krakauer, 1995; Rink et al., 1996; Assenmacher et al., 1998), but contrasts with other findings of an increase in IL-4 after a single SEA or SEB exposure (Lagoo et al., 1994). Still others have found enhanced IL-4 responses after repeated exposure to SEA or SEB, although this is not a consistent finding (Florquin et al., 1995; Sundstedt et al., 1997; Miller et al., 1999). Consequently, the role of IL-4 is still an unresolved issue, but at least in the present study does not appear to be of relevance to the corticosterone and behavioral effects that were observed in response to acute or repeated SEA.

The pattern of cytokine responses obtained in the current study is simultaneously compelling and potentially complex. Tumor necrosis factor, which plays an important role in mediating the behavioral and endocrine effects of acute SEA exposure (Rossi-George et al., 2005) is substantially reduced (by 50%) in response to secondary and tertiary injections of SEA, which still activate corticosterone, but not necessarily anorexia

(recall that in Experiment 2, three injections of SEA failed to reduce food intake). Therefore, TNFα may be more relevant to gustatory reactions to SEA, while the increased corticosterone may be still promoted by additional cytokines.

Among neuromodulatory inflammatory cytokines, IL-1β has been the best characterized. However, in relation to superantigenic effects on CNS function it has not been considered a prominent factor, given the difficulty to detect plasma IL-1β after acute SEA or SEB injections (Kawashima and Kusnecov, 2002; Rossi-George et al., 2005). In contrast to plasma, Experiment 1.1 showed that an acute injection of SEA increased splenic IL-1β although this was not replicated in Experiment 1.3. A possible explanation for this may be due to the greater amount of handling, behavioral testing and ip injections imposed on animals prior to the acute challenge in Experiment 1.3 (see Table 6). Indeed, repeated handling has been shown to modify immune responses and under the current circumstances may have inhibited the IL-1β response to SEA (Moynihan et al., 1989; Moynihan et al., 1990; Moynihan et al., 1992). While this explanation would require further empirical testing, it was evident that splenic IL-1β concentrations increased in response to repeated injections of SEA, and this also occurred in plasma. The capacity for IL-1β to be synthesized in response to additional SEA administration suggests that the lack of an increase to acute SEA was an inherent feature of the differences in experimental design between Experiments 1.1 and 1.3 (eg., amount of handling and testing). These differences notwithstanding, it was evident that up to two, three and four exposures to SEA were capable of driving up IL-1β responses to a significantly greater extent than a single SEA treatment. To our knowledge, we are the first to report these observations. Further, it is interesting to note that while TNFα levels were inversely

related to IL-10 production, splenic IL-1β was not inversely related to IL-10. It has been shown that Interleukin-10 can inhibit both IL-1β and TNFα, and that IL-10 deficient mice treated with SEB show enhanced TNFα, IL-1β, and IL-2 responses (Hasko et al., 1998; Moore et al., 2001). The current results, however, suggest that IL-1β may have escaped IL-10 regulation after repeated treatment with SEA. Additionally, while IL-1β spleen and plasma levels remained elevated, this was observed when the corticosterone response to SEA was attenuated or absent. Although IL-1β is a strong activator of the HPA-axis, this suggests that IL-1β may not be mediating the HPA axis activation after repeated SEA challenge.

In conclusion, secondary exposure to SEA continued to produce CNS reactivity, in that animals continued to display anorexia and an increase in corticosterone. Repeated exposure to SEA attenuated TNFα while other cytokines such as IL-1β, IFNγ, and IL-10 remained elevated. While TNFα has previously been shown to be important in mediating the effects of SEA, it remains to be determined which cytokines are mediating the behavioral and endocrine effects during repeated conditions. While TNFα remains a likely candidate, its impact may occur in an additive and/or synergistic fashion. Moreover, it is possible that the somewhat attenuated, but still significant levels of TNFα may still have neuromodulatory effects, given that there is evidence for TNFα mediated sensitization of behavioral and endocrine effects (Hayley et al., 2002). Similarly, IL-2 has been shown to alter behavior and activate the HPA axis, may therefore be a significant contributor to the continued corticosterone and anorexia to secondary and tertiary SEA challenge (Karanth et al., 1994; Pauli et al., 1998; Zalcman et al., 1998; Zalcman, 2001).

Chapter 4

The Role of TNFRI and TNFRII in Mediating the Behavioral

and Endocrine Effects of Acute SEA

Introduction

A substantial amount of data has pointed to TNFα as being responsible for many of the toxic and behavioral consequences of macrophage mediated endotoxin shock (LPS) (Bluthe et al., 2000; Rossi-George et al., 2005). Our laboratory has demonstrated that TNFα can also play an important role in mediating the behavioral and endocrine effects of a T cell mediated response (SEA) (Rossi-George et al., 2005). For example, one study showed that animals lacking TNFα exhibit an attenuated c-Fos response in brain regions involved in mediating fear and anxiety, a blunted CORT response, and abrogated sickness behavior after a single exposure to SEA (Rossi-George et al., 2005). Moreover, TNFα immunoneutralization *in vivo* through administration of anti-TNFα antibody caused abrogation of the sickness effect of SEA (Rossi-George et al, 2005). While this suggests that systemic TNFα is important in mediating the behavioral effects of SEA, it does not explain whether these effects are dependent on TNFRI or TNFRII. Therefore, it was important to clarify these previous findings by assessing the roles of the TNFα receptors TNFRI and TNFRII in the behavioral and endocrine effects of acute SEA. Many previous studies have shown that TNFRI is the more significant central mediator of the CORT response to central TNFα and LPS and that it also mediates septic shock (Tracey et al., 1987; Rothe et al., 1993; Benigni et al., 1996; Peschon et al., 1998). Therefore, it was expected that TNFRI deficient animals would show a greater attenuated CORT response and sickness behavior after challenge with SEA. Because we were

hypothesizing the importance of TNFRI in mediating the consequences of SEA challenge, this study also examined changes in the mRNA expression of CRH in the hypothalamus of the TNFRI deficient animals to determine if changes in the CORT response were mediated through a downregulation of CRH. In addition, the mRNA expression of TNFRII in the hypothalamus was also measured to establish if there was a compensatory upregulation of this receptor in the absence of TNFRI. Finally, the hypothalamic TNFRI mRNA expression in the WT animals was assessed to clarify whether exposure to acute SEA altered the expression of this receptor.

Material and Methods

Animals and Genotyping

See "General Methods" for details.

Reagents

Staphyloccal enterotoxin A was purchased from Toxin Technology (Sarasota, FL). All injections of SEA were given intraperitoneally (IP) at a dose of 5 µg/mouse in pyrogen-free physiological saline in a volume of 0.2 ml.

Experimental Procedure

In this experiment the effect of acute SEA in TNFRI deficient (TNFRI$^{-/-}$) (Experiment 2.1) and TNFRII deficient (TNFRII$^{-/-}$) (Experiment 2.2) was assessed in both males and females. All knockout animals were tested along with their heterozygous (TNFRI$^{+/-}$ and

TNFRII$^{+/-}$) and wildtype (TNFRI$^{+/+}$ and TNFRII$^{+/+}$) littermate controls [(Experiment 2.1: Male, N = 10/group; Female, 7-8/group) (Experiment 2.2: Male, N = 8-9/group, Female, N = 7-9/group)]. Animals were sacrificed two hours after SEA exposure to examine the CORT, splenic TNFα, and hypothalamic mRNA changes of CRH, TNFRI, and TNFRII. To determine the anorexic response after SEA exposure, a second set of animals were put into a consumption test. Animals were placed into the consumption test two hours after the injection of SEA for one hour (Test 1). At the end of the hour the Prosobee was removed from the cage and the animals remained in the consumption test cages for one hour until the second consumption test began (Test 2). Therefore animals were tested for consumption at two (Test 1) and four (Test 2) hours after SEA treatment. These animals were allowed to survive and used for experiment 3 and 4 to determine the behavioral effects of SEA four days after exposure and to clarify the effects of repeated SEA exposure in TNFRI$^{-/-}$ and TNFRII$^{-/-}$ animals. It is important to note that the first consumption test took place two hours after the injection of SEA and corresponded to the first sacrifice time point for CORT and TNFα analysis. The second consumption test, however, did not start until four hours after SEA treatment. Therefore, male TNFRI$^{-/-}$ and TNFRI$^{+/+}$ in Experiment 2.3 were sacrificed at specific time points to determine the CORT and TNFα response before and after the consumption test. One set of animals was never placed into the consumption test and sacrificed four hours after SEA exposure [4 Hr Home Cage] (N = 5-7). Another group was given the fist consumption test and sacrificed right before the second consumption test started [4 Hr + Prosobee] (N = 5-7/group) and the third group was sacrificed right after the second consumption test [5 Hr

+ Prosobee] (N = 5-7/group). This allowed us to assess the CORT and TNFα response in the animals throughout the entire consumption test.

Blood and Tissue Collection

See "General Methods".

Protein Extraction and Quantification

See "General Methods".

Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

To ensure that any changes in the TNFRI and TNFRII$^{-/-}$ animals in response to SEA were not due to altered TNFα production, splenic TNFα was measured in all animals. See General Methods".

Corticosterone Radioimmunoassay (RIA)

See "General Methods".

Reverse Transcription and Real Time PCR

See "General Methods".

Statistical Analysis

See "General Methods".

Results

Experiment 2.1

TNFRI Deficient Males

Corticosterone: Statistical analysis revealed that TNFRI$^{-/-}$animals failed to display an increase in CORT after treatment with SEA as compared to TNFRI$^{+/+}$ and TNFRI$^{+/-}$ animals ($F_{(2, 54)}$ = 20.822, $p <$ .0001). Interestingly, although the TNFRI$^{+/-}$ animals continued to show a CORT response, it was significantly attenuated compared to TNFRI$^{+/+}$ animals ($F_{(1, 36)}$= 5.445, p = .0253). Therefore, a 50% reduction in TNFRI was sufficient to attenuate the CORT response after treatment with acute SEA in males. See Figure 4.1.

Splenic Tumor Necrosis Factor-alpha: To ensure that these animals showed similar TNFα levels after SEA exposure, splenic TNFα was measured. Figure 4.2 demonstrates that all genotypes showed similar increases in the production of TNFα after treatment with acute SEA ($F_{(1, 54)}$ = 165.950, $p <$ .0001).

Consumption: The effects of SEA on consumption in male TNFRI$^{+/-}$, TNFRI$^{-/-}$, and TNFRI$^{+/+}$mice can be found in Figure 4.3. As revealed by ANOVA there was a significant genotype effect whereby the TNFRI$^{-/-}$ animals, regardless of treatment, were consuming more than the other animals during the first consumption test ($F_{(2, 68)}$ = 3.802, p = .0272). This is consistent with reports that TNFRI may be involved in mediating depressive like symptoms (Simen et al., 2006). Although there was not an interaction effect during the first consumption test, there was a significant reduction in consumption

that appeared to be more prominent in the TNFRI$^{+/+}$ and TNFRI$^{+/-}$ animals ($F_{(1, 68)}$ = 8.995, p = .0038). This is consistent with the CORT data, in that the TNFRI$^{-/-}$ animals failed to produce a CORT response after treatment with SEA. Interestingly, the second test showed that irrespective of genotype there was a reduction in consumption after treatment with SEA ($F_{(1, 68)}$ = 49.433, p < .0001). Therefore, by four hours all genotypes appear to be similar in their anorexic response to SEA.

Hypothalamic mRNA

CRH: The TNFRI$^{-/-}$ animals showed an attenuated expression of CRH regardless of treatment with SEA or saline ($F_{(2, 54)}$ = 4.667, p = .0135). There was not, however, a change in CRH expression in any of the genotypes after treatment with SEA. See Figure 4.4.

TNFRII: Figure 4.5 shows that regardless of genotype or treatment, there were no changes in TNFRII mRNA expression.

TNFRI: The TNFRI$^{+/+}$ animals were assessed for TNFRI mRNA expression to determine if SEA would augment the expression of TNFRI. SEA did not augment the mRNA expression of TNFRI in the hypothalamus. This can be seen in Figure 4.6.

In summary, treatment with SEA did not augment the mRNA expression of hypothalamic CRH, TNFRII, and TNFRI. Interestingly, the TNFRI$^{-/-}$ animals showed an overall decrease in the expression of CRH. The decrease in CRH mRNA could be linked to the increase in consumption observed above.

TNFRI Deficient Females

Corticosterone: As revealed by ANOVA, females showed a similar response to males. Analysis revealed a significant genotype by treatment interaction, in which TNFRI$^{-/-}$ animals showed a blunted CORT response compared to TNFRI$^{+/-}$ and TNFRI$^{+/+}$ animals after treatment with SEA ($F_{(2, 40)} = 11.054$, $p = .0002$). Unlike the males, however, the TNFRI$^{+/-}$ animals did not show an attenuated CORT response compared to the TNFRI$^{+/+}$ animals. See Figure 4.7.

Splenic Tumor Necrosis Factor-alpha: Figure 4.8 shows that all genotypes obtained similar increases in the production of TNFα after treatment with acute SEA ($F_{(1, 40)} = 263.085$, $p < .0001$). Therefore, there were no differences in the TNFα production after treatment with SEA in these three genotypes.

Consumption: The effects of SEA on consumption in female TNFRI$^{+/-}$, TNFRI$^{-/-}$, and TNFRI$^{+/+}$ mice can be found in Figure 4.9. Similar to the males, it appeared as though irrespective of treatment, the female KO subjects consumed more Prosobee compared to the TNFRI$^{+/+}$ and TNFRI$^{+/-}$ animals ($F_{(2, 73)} = 9.566$, $p = .0006$). There was also a significant treatment effect, however it appeared to be driven primarily by the TNFRI$^{+/-}$ subjects displaying a reduction in consumption after treatment with SEA ($F_{(1, 73)} = 9.566$, $p = .0028$). By the second test all genotypes showed a reduction in consumption after treatment with SEA ($F_{(1, 73)} = 100.512$, $p < .0001$).

Experiment 2.2

TNFRII Deficient Males

Corticosterone: Figure 4.10 demonstrates that all genotypes showed an increase in CORT after treatment with SEA ($F_{(1, 48)} = 42.108$, $p < .0001$). Interestingly, regardless of treatment, the TNFRII$^{-/-}$ animals showed an exaggerated level of CORT ($F_{(2, 48)} = 3.946$, $p = .0259$).

Splenic Tumor Necrosis Factor-alpha: Irrespective of genotype, all subjects showed an increase in TNFα after treatment with SEA (($F_{(1, 46)} = 231.910$, $p < .0001$). See Figure 4.11.

Consumption: Statistical analysis showed a significant treatment effect, whereby subjects showed a reduction in consumption after treatment with SEA during the first test ($F_{(1, 48)} = 9.397$, $p = .0036$). Interestingly, this effect was primarily driven by the large reduction in consumption in the TNFRII$^{+/-}$ animals after treatment with SEA. Removal of the TNFRII$^{+/-}$ subjects from the analysis showed that the TNFRII$^{-/-}$ and TNFRII$^{+/+}$ did not show much of a reduction in consumption after SEA ($F_{(1, 28)} = .512$, $p = .1266$). The second test revealed that all genotypes showed a reduction in consumption after SEA during the second consumption test ($F_{(1, 48)} = 37.539$, $p < .0001$). See Figure 4.12.

TNFRII Deficient Females

Corticosterone: Figure 4.13 demonstrates that regardless of genotype, all subjects showed an increase in CORT after treatment with SEA ($F_{(1, 48)} = 37.539$, $p < .0001$).

Splenic Tumor Necrosis Factor-alpha: Irrespective of genotype, statistical analysis revealed that all subjects showed an increase in TNFα after treatment with SEA ($F_{(1, 45)} =$ 65.195, $p < .0001$). See Figure 4.14.

Consumption: Analysis by ANOVA demonstrated a significant treatment effect for both consumption tests, whereby all genotypes showed a reduction in consumption after treatment with SEA ($F_{(1, 47)} = 14.467$, $p = .0004$); $F_{(1, 47)} = 67.237$, $p < .0001$). This is demonsrated in Figure 4.15.

Experiment 2.3

4 hr HCC Compared to 4 hr + Prosobee:

Figures 4.16-4.19 presents CORT, splenic TNFα, splenic IL-2, and hypothalamic CRH and TNFRII in male TNFRI$^{-/-}$ and TNFRI$^{+/+}$ subjects 4 hours after treatment with SEA or saline directly from the home cage or from the consumption test.

Corticosterone: Treatment with SEA and exposure to the Prosobee test significantly increased CORT in both TNFRI$^{+/+}$ and TNFRI$^{-/-}$ animals ($F_{(1, 39)} = 27.669$, $p < .0001$; $F_{(1, 39)} = 66.306$, $p < .0001$). Contrary to what was expected, analyses by ANOVA revealed that there was not a significant attenuation of the CORT response after exposure to SEA in the TNFRI$^{-/-}$ animals ($F_{(1, 39)} = .031$, $p = .8603$). See Figure 4.16.

Splenic Tumor Necrosis Factor- alpha: Treatment with SEA significantly elevated splenic TNFα levels compared to saline treated animals ($F_{(1, 39)} = 131.155$, $p < .0001$).

Surprisingly, there was a significant genotype by treatment interaction, showing that

TNFRI$^{-/-}$ animals maintained an exaggerated level of splenic TNFα after treatment with

SEA compared to their TNFRI$^{+/+}$ counterparts ($F_{(1, 39)}$ = 4.206, p = .0471). See Figure

4.17.

Spenic Interleukin-2: Statistical analysis showed a significant increase in splenic IL-2

after treatment with SEA regardless of genotype and testing situation ($F_{(1, 39)}$ = 159.743, p

< .0001). See Figure 4.18.

Hypothalamic mRNA

CRH: There were no significant alterations in CRH mRNA expression. See Figure 2.19.

TNFRII: There were no significant changes in TNFRII mRNA expression. See Figure

4.19.

TNFRI: Figure 4.20 shows that there was a trend to suggest an upregulation of TNFRI

mRNA after exposure to SEA in the TNFRI$^{+/+}$ animals ($F_{(1, 18)}$ = 3.682, p = .0710).

However, this was significantly decreased after exposure to the Prosobee test ($F_{(1, 18)}$ =

5.953, p = .0253).

4 hr + Prosobee Compared to 5 hr + Prosobee:

Figures 4.21-4.24 presents CORT, splenic TNFα, splenic IL-2, and hypothalamic CRH

and TNFRII in male TNFRI$^{-/-}$ and TNFRI$^{+/+}$ subjects after removal from the consumption

test at 4 or 5 hours following SEA treatment.

Corticosterone: Treatment with SEA increased the CORT response as compared to saline treated animals ($F_{(1, 42)}$ = 8.956, p < .0046). To assess the changes in CORT within the test, the data showed that there was a decline in the CORT response in the five hour time point as compared to the 4 hour time point regardless of genotype or treatment with SEA or saline ($F_{(1, 39)}$ = 12.038, p < .0012). See Figure 4.21.

Splenic Tumor Necrosis Factor- alpha: Irrespective of genotype, treatment with SEA increased splenic TNFα after treatment with SEA ($F_{(1, 42)}$ = 95.683, p < .0001). After treatment with SEA, there was a significant reduction of TNFα in the 5 hr + Prosobee subjects compared to the 4 hr + Prosobee subjects ($F_{(1, 42)}$ = 27.695, p < .0001). See Figure 4.22.

Splenic Interleukin-2: Regardless of genotype, treatment with SEA increased splenic IL-2 after treatment with SEA ($F_{(1, 42)}$ = 139.438, p < .0001). After treatment with SEA, there was a significant reduction of IL-2 in the 5 hr + Prosobee subjects compared to the 4 hr + Prosobee subjects ($F_{(1, 42)}$ = 21.338, p < .0001). See Figure 4.23.

Hypothalamic mRNA

CRH: There were no significant alterations in CRH mRNA expression. See Figure 4.24.

TNFRII: There were no significant changes in TNFRII mRNA expression. See Figure 4.24

TNFRI: Figure 4.25 showed that the TNFRI$^{+/+}$ animals did not display any significant differences in the TNFRI mRNA expression regardless of treatment or the testing situation.

Discussion

Both male and female TNFRI$^{-/-}$ animals showed an increase in Prosobee consumption regardless of treatment during the first testing session, while the TNFRII$^{-/-}$ subjects did not show any difference in consumption when compared to the controls. Simen et al., (2006) found the opposite effect, reporting that TNFRII$^{-/-}$ but not TNFRI$^{-/-}$ animals showed enhanced consumption of a 1% sucrose solution. However, it is important to note significant methodological differences between these two studies. Aside from the different highly palatable substance chosen, the Simen study water-deprived the animals for four and fourteen hours before testing. Water deprivation has been shown to be highly stressful to animals, increasing c-Fos expression in the PVN and activating the HPA axis (Kovacs and Sawchenko, 1993; Wotus et al., 2007). In addition, the animals in that study were placed through a battery of highly stressful behavioral tests such as the forced swim test, a hot-plate test, and fear conditioning before the consumption test. Therefore, the methodological differences make the direct comparisons between these two studies difficult. Nevertheless, the findings that TNFRI$^{-/-}$ animals consumed more than the controls is consistent with the notion that TNFRI is important in mediating depressive like behavior (Simen et al., 2006). The decrease in depressive like behavior in the TNFRI$^{-/-}$ animals could be linked to the lower CRH mRNA expression in these animals.

To determine the role of TNFRI and TNFRII in mediating sickness behavior after SEA treatment, both TNFRI$^{-/-}$ and TNFRII$^{-/-}$ males and females were assessed for consumption after SEA challenge. It was determined that consumption in the males was reduced after treatment with SEA. Although there was not a significant genotype by

treatment interaction effect, the reduction in consumption seemed to be driven by the TNFRI$^{+/-}$ and TNFRI$^{+/+}$ animals and not the TNFRI$^{-/-}$ animals. Therefore, it did appear as though the TNFRI$^{-/-}$ animals showed an attenuated anorexia. For the females, it is interesting to note that while the TNFRI$^{+/+}$ animals showed no reduction in consumption, the TNFRI$^{+/-}$ subjects did, which seems to mean that the significant treatment effect was driven by the TNFRI$^{+/-}$ animals. Perhaps 50% loss of TNFRI made the females more susceptible to the effects of SEA. By the second test session all genotypes showed a reduction in consumption after treatment with SEA.

Consistent with the attenuated anorexia in the males, both male and female TNFRI$^{-/-}$ animals showed a blunted CORT response after challenge with SEA as compared to the TNFRI$^{+/+}$ and TNFRI$^{+/-}$ animals. These changes were not due to changes in T cell reactivity or cytokine production because all the genotypes showed equal enhancement of TNFα after treatment with SEA. While the TNFRI$^{-/-}$ subjects showed a blunted CORT response and attenuated anorexia, there were no changes in CORT, TNFα, or consumption in the TNFRII$^{-/-}$ animals as compared to the TNFRI$^{+/+}$ and TNFRII$^{+/-}$ subjects. These findings are consistent with previous studies that point to TNFRI being the more significant central mediator of the CORT response to central TNFα and LPS (Benigni et al., 1996; Peschon et al., 1998). Moreover, TNFα induced sickness behaviors such as weight loss, change in social exploration, and immobility were not exhibited in TNFRI deficient mice as they were in TNFRII deficient mice (Palin et al., 2008). It is important to note that regardless of genotype, the TNFRII male cohort showed enhanced CORT production. This effect, however, was not observed in the females. Given that central TNFRI has been shown to be the primary pathway for CORT production, it was

surprising that TNFRII^{-/-} males showed an enhanced CORT production after SEA exposure. The counterbalancing nature of TNFRII may suggest that TNFRII can dampen the CORT response, which could be accomplished by modifying signaling through TNFRI, thereby exaggerating CORT levels in its absence. Future studies should focus on determining the role of TNFRII in mediating the CORT response to both immunologic and psychogenic stressors.

It is important to note that the TNFRII^{-/-} females did not show the enhanced CORT production as the males did. However, both male and female TNFRII^{-/-} animals showed similar elevated TNFα and CORT in response to SEA. If we had seen a sexually dimorphic different response to SEA, it would have been difficult to interpret because we did not track the estrous cycle of the females. However, there is a vast amount of literature addressing sexually dimorphic differences between males and females in their immune responsiveness. For example, females have been found to be much more susceptible to superantigen shock than males (Faulkner et al., 2007). The high concentrations of estrogens, especially during proestrus, has been correlated with the enhanced immune responsiveness (Schwartz et al., 2004). Intriguingly, Faulkner et al., (2007) found that superantigen-sensitive HLA class II transgenic (HLA-DQ8) female mice were more susceptible to sepsis induced by *M1 S. pyogenes strain H305*, which was associated with a more prominent increase in TNFα and IL-6 as compared to the males. In addition, the enhanced susceptibility to SEB/D-galactosamine (Dgal) lethality in a different strain of superantigen-sensitive HLA class II transgenic female mice (HLA-DRI) also corresponded to the enhanced TNFα production. In this latter study it was discovered that female mice had a significantly lower level of soluble TNFRI (sTNFRI) and soluble

TNFRII (sTNFRII) compared to males. These soluble receptors are released after an inflammatory insult to bind excess circulating TNFα thereby limiting excessive TNFα and restricting its bioactivity (Van Zee et al., 1992). Therefore, females may be more sensitive to superantigens due to the reduction in sTNFRs, which could ultimately lead to excess circulating TNFα (Faulkner et al., 2007). This was confirmed by showing that the females were more susceptible to TNF/Dgal. Interestingly, tamoxifen treatment partially attenuated the effects of SEB/Dgal and TNF/Dgal treatment. This protection appeared to be induced by the increase in sTNFRI in the tamoxifen treated females.

Notably, the data presented in the current study did not show the females being more sensitive to SEA. The animals in this study, however, were not superantigen-sensitive HLA class II transgenics and they were not co-administered Dgal with SEA. There were, however, sexually dimorphic differences in the overall level of CORT production, in that the $TNFRII^{-/-}$ males showed excessive production and the females did not. Also, in Experiment 2.1 the female $TNFRI^{+/+}$ animals did not show a reduction in consumption as did the males. Therefore, in contrast to what Faulkner et al., (2007) found, the females appeared to be less susceptible to the anorexic effects of SEA. This did not replicate in experiment 2.2, where both male and female $TNFRII^{+/+}$ subjects showed comparable anorexia after SEA exposure. The animals in experiment 2.2, however, were bred from $TNFRII^{+/-}$ animals. This strain difference could help explain the discrepancy between the reactivity of the $TNFRI^{+/+}$ and $TNFRII^{+/+}$ females to SEA between Experiment 2.1 and 2.2. Future studies are needed to assess differences in sTNFRI and sTNFRII in the wildtype animals to explain some of the sexually dimorphic differences.

Experiment 2.1 showed TNFRI to be important in mediating the CORT response and sickness behavior after acute SEA challenge. While the CORT response was markedly attenuated two hours after SEA challenge, the TNFRI$^{-/-}$ animals no longer showed attenuated anorexia during the second consumption test. While the first consumption test took place two hours after the injection of SEA and corresponded to the time point for the CORT data, the second consumption test did not start until four hours after SEA treatment. Therefore, the data from Experiment 2.3 helped to clarify the magnitude of the CORT response throughout the testing situation. The majority of the previous effects were only observed in the TNFRI$^{-/-}$ males, and so only TNFRI$^{-/-}$ and TNFRI$^{+/+}$ males were used for Experiment 2.3. The data from Experiment 2.3 showed that by four hours the TNFRI$^{-/-}$ animals showed a normal CORT response to SEA. This was important because it showed that the CORT response reflected the consumption data in that both TNFRI$^{-/-}$ and TNFRI$^{+/+}$ animals displayed a similar decrease in consumption after SEA. Furthermore, it demonstrated that TNFRI may only partially mediate the endocrine and behavioral response to SEA. Other cytokines, such as IL-2, were also found to be upregulated and may have contributed to the CORT response in the absence of TNFRI. Indeed, IL-2 has been shown to alter behavior and activate the HPA axis, and may therefore be a significant contributor to the continued effects of SEA exposure at the four hour time point (Karanth et al., 1994; Pauli et al., 1998; Zalcman et al., 1998; Zalcman, 2001). Immuno-neutralization studies with IL-2 in TNFRI$^{-/-}$ animals may help to clarify the role of IL-2 in mediating the endocrine effects of SEA.

It was also hypothesized that TNFRII may compensate for the lack of TNFRI, thereby maintaining a delayed, although significant, increase in CORT. Analysis of the

mRNA for TNFRII did not show any changes in the expression of TNFRII, although the time point of two and four hours after SEA exposure may not have been sufficient to observe the difference. Therefore, additional time points may identify changes in the expression of TNFRII. Additionally, mRNA levels do not always correlate with changes in protein levels. Therefore, future studies should determine posttranscriptional modifications to the receptor that might take place after SEA exposure in TNFRI^{-/-} animals. Moreover, the use of double knockout animals for TNFRI and TNFRII may help to resolve whether the delayed CORT response was a result of TNFRII compensating for the lack of TNFRI. Finally, it is also important to note that exposure to SEA did not change the expression of TNFRI mRNA after SEA exposure in the TNFRI^{+/+} animals. These observations corroborate previous studies that found that unlike LPS, treatment with SEB did not influence the expression of TNFα, TNFRI, or TNFRII in the brain at 1, 4, 8, 12, and 24 hours after injection (Bette et al., 2003).

Experiment 2.3 also demonstrated that the TNFRI^{-/-} animals showed an exaggerated level of TNFα after exposure to SEA four hours after the challenge. This increase could be due to many factors. For example, it may be related to a heightened responsiveness to the stress of injection in the TNFRI^{-/-} animals. As mentioned previously, repeated handling has been shown to modify immune responses, which under the current circumstances may have been exaggerated in the TNFRI^{-/-} animals in response to a single injection of SEA (Moynihan et al., 1989; Moynihan et al., 1990; Moynihan et al., 1992). Indeed, severe stressors such as exposure to tail shock have been shown to increase cytokines ((Nguyen et al., 1998; Nguyen et al., 2000). In this case, the absence of TNFRI may enhance an animal's sensitivity to even moderate stress, such as an injection, which

increases splenic TNFα after SEA exposure. However, this seems unlikely, due to reports that TNFRI$^{-/-}$ animals display similar levels of anxiety in the elevated plus maze, open field, and light dark box (Simen et al., 2006). Also, by five hours the TNFα levels showed a reduction regardless of genotype or treatment. Therefore, the genotype difference in the levels of TNFα was not consistent, as it was not observed at two hour or at five hours after SEA exposure. Alternatively, the exaggerated TNFα may have been a consequence of the blunted CORT response observed at the two hour time point. Because the anti-inflammatory properties of glucocorticoids include the ability to inhibit the transcription of proinflammatory cytokines such as TNFα, blockade of the CORT response results in excessive inflammation. Therefore, the attenuated CORT response may have allowed for exaggerated TNFα levels at the four hour time point. By four hours, however, the CORT response in the TNFRI$^{-/-}$ animals was identical to that of the TNFRI$^{+/+}$ animals. This suggests that the normalized TNFα levels at the five hour time point was a result of the reinstated CORT response at four hours.

Previous studies have confirmed that SEA exerts its behavioral and endocrine effects through TNFα (Rossi-George et al., 2005). The present results suggest that TNFRI partially mediates the HPA activation, along with enhanced gustatory neophobia. Both male and female TNFRI$^{-/-}$ animals showed a significant attenuation of the CORT response and showed modified anorexia two hours after treatment. Importantly, by four hours the TNFRI$^{-/-}$ animals showed an elevated CORT response comparable to the TNFRI$^{+/+}$ animals. By this time point, all genotypes also showed a marked decrease in consumption. To understand the mechanism by which the CORT response increased at the four hour time point, mRNA expression of CRH and TNFRII were assessed. It was

discovered that, regardless of genotype, the TNFRI$^{-/-}$ animals showed a reduction in the mRNA expression of CRH. This reduction could be attributed to the reduction in depressive like behavior in these animals. However, there were no changes in CRH after treatment with SEA, nor was there any alteration in the mRNA expression of TNFRII or TNFRI after treatment with SEA. Future studies should aim at determining the role of other cytokines, such as IL-2, in mediating the effects of SEA.

Chapter 5

The Role of TNFRI and TNFRII in Mediating the Behavioral

Effects of SEA Four Days after Exposure

Introduction

While considerable evidence has shown that SAg administration increases

peripheral cytokine levels, there is little evidence that it increases cytokines in the brain.

For example, it has been reported that exposure to SEB does not produce an increase in

the brain mRNA levels of IL-1β or TNFα (Del Rey et al., 2000a; Bette et al., 2003).

However, although SAg exposure may not alter central cytokine levels, it does produce

behavioral consequences. Studies from our laboratory have demonstrated that there are

specific adjustments in behavior that differ from classic "sickness behavior". Unlike with

exposure to LPS, administration of SEA and SEB did not produce conditioned taste

aversion, and reduction in consumption was only produced in a novel environment

(Kusnecov et al., 1999; Kawashima and Kusnecov, 2002). Therefore, exposure to SAgs

may not produce classical anorexia, but may produce a neophobic effect instead. This

neophobic effect can be extended to other behaviors as well, in that exposure to SEA did

not produce alterations in behavior in the open field until a novel object was placed inside

(Kawashima and Kusnecov, 2002). Behaviors in the elevated plus maze (EPM) after SAg

exposure seem difficult to interpret, because exposure to SEA or SEB produced increased

exploration in the open arms of the apparatus (Rossi-George et al., 2004). This would

suggest an anxiolytic effect of SAg exposure, although the increase in exploration could

be attributed to heightened arousal. Additionally, SEA and SEB administration did not

alter behavior in the light-dark box, although animals placed into a novel consumption test prior to the light-dark box test did show anxiogenic like behavior after SEA or SEB administration (Rossi-George et al., 2004).

Studies of neuronal activation have revealed increased activation in areas that mediate the stress response, supporting the hypothesis that SAgs have anxiogenic properties. For example, exposure to SEB has been shown to activate limbic areas in the brain such as the lateral septum (LS), the central nucleus of the amygdala (CeA), and the paraventricular nucleus of the hypothalamus (PVN) (Goehler et al., 2001; Wang et al., 2004a). Additionally, recent work from our laboratory has shown that SEA induces an increase in neuronal activation in these limbic regions as well as a reduction in food intake (Rossi-George et al., 2005). The changes in behavior and CNS function appear to be mediated by endogenous TNFα, and animals deficient in TNFα showed an attenuated c-Fos response in brain regions involved in mediating fear and anxiety, a blunted CORT response, and abrogated sickness behavior after a single exposure to SEA (Gonzalo et al., 1993; Rossi-George et al., 2005). Interestingly, IL-1β did not appear to mediate any of these effects. Plasma levels of IL-1β were undetectable after a single SEA exposure and IL-1 receptor knock-out animals continued to show a reduction in food intake after a single injection of SEA (Kawashima & Kusnecov 2002; Ross-George et al., 2005).

To our knowledge, the long-term behavioral consequences after SEA exposure have not previously been demonstrated. Therefore, the purpose of this experiment was to determine the effect of SEA challenge given several days before exposure to a novel environment containing a novel object. Moreover, Chapter 4 described the importance of TNFRI in mediating the anorexic effects of acute SEA. Therefore, this study assessed the

long-term non-appetitive behavioral consequences of SEA in TNFRI$^{-/-}$ (Experiment 3.1)

and TNFRII$^{-/-}$ (Experiment 3.2) males and females. Four days after treatment with SEA,

animals were tested for anxiety-like behavior in the open field with a novel object placed

inside. To our knowledge, this is the first study addressing the behavioral outcome of

SAg exposure several days after SEA exposure in wildtype or in TNFRI$^{-/-}$ and TNFRII$^{-/-}$

animals.

Material and Methods

Animals and Genotyping

See "General Methods" for details.

Reagents

Staphyloccal enterotoxin A was purchased from Toxin Technology (Sarasota, FL). All

injections of SEA were given intraperitoneally (IP) at a dose of 5 μg/mouse in pyrogen-

free physiological saline in a volume of 0.2 ml. Animals were tested four days after

exposure to SEA.

Experimental Procedure

This experiment examined the effect of SEA four days after the initial exposure in the

open field /novel object (OF/NO) test in TNFRI- (Experiment 3.1) and TNFRII-deficient

(Experiment 3.2) males and females along with their wildtype and heterozygous

littermate controls. The animals used in this experiment were the animals previously

treated with SEA or saline for the consumption test in Chapter 4. Four days after

treatment with SEA and the consumption test the animals were placed in the OF/NO test. These animals were then used for the next study (Chapter 6) in which they received a second injection of SEA and were killed the following day (Day 5), two hours after the final exposure.

Simultaneous Open Field (OF)/ Novel Object (NO) exploration (OF/NO test)

Four days after treatment with SEA or saline animals were monitored for exploratory behavior and behavioral reactivity to a novel object in the OF/NO test. The open field measured 63 x 57 x 28 cm (L x D x H), demarcated by a grid of 30 equally sized squares. In the center of the OF was a novel cylindrical object (6.5 cm in diameter and 11 cm in height). An overhead CCD camera recorded all animal movements and the animal's behavior was monitored over a five minute period, after which it was returned to its home cage. Behavior was recorded using a videotracking system (SMART: San Diego Instruments, San Diego, CA) that recorded the location of the animal over the 5 min test period. For the purposes of analysis by the SMART software, the NO/OF test was divided up into 30 zones. These zones were then included into associations to include the center (where the NO was located), the middle (area in between the periphery and the center), and the periphery. The parameters that were chosen for analysis from the SMART data were: (i) percent time in the periphery, (ii) percent time in the middle, (iii) total distance traveled, (iv) total number of entries, (v) latency to enter the center, (vi) percent time in center, and (vii) total number of entries to center. Videotape replays of specific behaviors were also recorded by an observer blind to experimental treatments. These specific behaviors included (i) number of nose contacts with NO, (ii), latency to

contact the NO, (iii), number of rears in the periphery, and (iv) number of rears on the NO.

Results

Experiment 3.1 OF/NO Behavior Four Days After SEA Exposure in TNFRI Deficient Mice

Males

Figures 5.1-5.3 presents evidence for the effects of SEA treatment in the OF/NO test. Statistical analysis revealed that treatment with SEA four days before testing in the OF/NO test resulted in a reduction in total activity level as reflected by a significant reduction in the total distance traveled ($F_{(1, 68)} = 5.885$, $p = .0179$) and a significant decrease in the number of total entries ($F_{(1, 68)} = 5.954$, $p = .0173$) (See Figures 5.1a and 5.1b). Figure 5.1c shows that when the total number of entries was split into one minute intervals and expressed as a repeated measures ANOVA there was a decrease in the total number of entries over time ($F_{(1, 68)} = 5.835$, $p = .0184$). As can be seen in Figures 5.1a-5.1c there were no genotype effects on these specific behaviors.

Figure 5.2-5.3 shows that treatment with SEA induced anxiogenic like behavior in the NO/OF test. For example, regardless of genotype, exposure to SEA increased the latency to enter the center ($F_{(1, 68)} = 5.010$, $p = .0285$) (See Figure 5.2a) and increased the latency to contact the NO ($F_{(1, 68)} = 9.532$, $p = .0029$) (See Figure 5.2b). Treatment with SEA, however, did not change the time spent in specific areas of the OF/NO test as reflected by the percent time in the periphery, the percent time in the middle, and the percent time in the center. Further analysis into the percent time in the center, however,

revealed some interesting results. Figure 5.3b shows that when the data was split into one minute intervals and expressed as a repeated measures ANOVA there was a trend to suggest that there was a genotype by treatment effect ($F_{(2, 68)}$ = 2.805, p = .0675). This trend stemmed from the behavior of the animals during "minute one" and "minute two". A separate one-way ANOVA was performed at each one minute interval and showed that at "minute one" the statistical trend suggested that the TNFRI$^{+/-}$ animals treated with SEA spent more percent time in the center than any of the other animals ($F_{(2, 68)}$ = 2.804, p = .0676). This became more evident at "minute two" where the TNFRI$^{-/-}$ and TNFRI$^{+/+}$ animals treated with SEA showed a decrease in the percent time spent in the center while the TNFRI$^{+/-}$ subjects showed an increase ($F_{(1, 68)}$ = 3.574, p = .0334). In general, the one minute interval repeated measures ANOVA also revealed that over time there was a progressive increase in the total percent time spent in the center ($F_{(4, 272)}$ = 27.304, p < .0001).

Although treatment with SEA increased the latency to enter the center and the latency to contact the NO, there were no changes in the total number of entries to the center (See Figure 5.4a), the number of contacts with the NO, the number of rears in the periphery, and the number of rears on the NO. This data is summarized in Table 7. As can be seen in Figure 5.4b, a repeated measures ANOVA showed that while treatment with SEA or genotype did not affect the total number of entries to the center throughout the five minute trial, there was an increase in the total number of entries to the center over time ($F_{(4, 272)}$ = 21.084, p < .0001).

Females

Figures 5.5-5.8 presents evidence for the effects of SEA treatment in the OF/NO test. Statistical analysis revealed that treatment with SEA four days before testing in the OF/NO test resulted in a reduction in total activity level as reflected by a significant reduction total distance traveled ($F_{(1, 73)} = 9.789$, $p = .0025$) and a decrease in the total number of entries ($F_{(1, 73)} = 8.964$, $p = .0038$) (See Figures 5.5a and 5.5b). The genotype of these animals did not modify the effect of SEA on these parameters. Figure 5.5c shows that when the data was split into one minute intervals and expressed as a repeated measures ANOVA there was a significant decrease in the total number of entries after treatment with SEA ($F_{(1, 73)} = 9.130$, $p = .0035$). The repeated measures ANOVA also demonstrated that regardless of treatment or genotype, the total number of entries decreased over time ($F_{(4, 292)} = 5.858$, $p = .0002$).

Figure 3.6-3.8 shows that treatment with SEA induced anxiogenic like behavior in the OF/NO test, whereby regardless of genotype, SEA decreased the total number of entries to the center ($F_{(1, 73)} = 5.431$, $p = .0225$) (See Figure 5.6a). As shown in Figure 5.6b, further analysis revealed that regardless of treatment or genotype, there was an increase in the total number of entries over time ($F_{(4, 292)} = 31.070$, $p < .0001$). Another measurement of anxiety is the percent time spent in the center of the testing apparatus. Figure 5.7 illustrated that treatment with SEA did not augment the percent time spent in the center, however there was a significant genotype effect ($F_{(2, 73)} = 3.541$, $p = .034$). This genotype effect was a result of the TNFRI$^{+/+}$ animals spending more time in the center of the OF/NO. A more detailed analysis in Figure 5.7b revealed that when the data was split into one minute intervals and expressed as a repeated measures ANOVA there

was a significant decrease in the percent time spent in the center after treatment with SEA ($F_{(1, 73)}$ = 5.072, p = .0273). This data also demonstrated that there was an increase in the percent time spent in the center over the five minute test ($F_{(4, 292)}$ = 31.070, p < .0001). Therefore, as the test progressed the animals became more comfortable with the environment and increased the exploration to the center. An increase in the latency by which an animal enters the center is another indication of heightened anxiety. Although there was not a treatment effect, Figure 5.8 reveals that there was a genotype effect for the latency to enter the center, whereby the TNFRI$^{+/-}$ animals showed an increase in the latency to enter the center ($F_{(2, 73)}$ = 3.135, p = .0494).

There were no changes in the percent time in the periphery, percent time in the middle, number of contacts with NO, and latency to contact the NO. There were also no changes in exploratory behavior such as the number of rears in the periphery or the number of rears on the NO. This data is summarized in Table 8.

Experiment 3.2 OF/NO Behavior Four Days after SEA Exposure in TNFRII Deficient Mice

Males

Figures 5.9-5.11 presents the data for the effects of SEA treatment and genotype in the OF/NO test. Statistical analysis revealed that treatment with SEA or genotype did not alter activity levels in the OF/NO test. This is demonstrated in Figures 5.9a and 5.9b, whereby there were no changes in total distance traveled and total number of entries. Further analysis of the total number of entries over each one minute interval, however,

revealed that there was a significant genotype by interval interaction ($F_{(8, 192)} = 2.154$, $p = .0327$). This was due to the TNFRII$^{+/-}$ animals increasing their total number of entries over time compared the TNFRII$^{+/+}$ and TNFRII$^{-/-}$ animals. Therefore, the TNFRII$^{+/-}$ animals appeared to be more active over time in the OF/NO test. See Figure 5.9c.

Just as SEA did not augment activity levels, treatment with SEA or genotype did not change the emotional state of these animals. For example, there were no changes in the latency to enter the center, total number of entries to the center, number of contacts with the NO, and latency to contact the NO. There were also no changes in the percent time spent in specific areas of the OF/NO test as reflected by the percent time in the periphery, percent time in the middle, and percent time in the center (Data summarized in Table 9). For example, Figure 5.10 shows that all animals showed an increase in the percent time spent in the center as the test progressed ($F_{(4, 192)} = 32.349$, $p < .0001$) with genotype or treatment not influencing this parameter. Interestingly, the only parameter that SEA augmented was exploratory behavior such as the number of rears in the periphery ($F_{(1, 48)} = 4.313$, $p = .0432$) (See Figure 5.11); however, SEA did not have an effect on the number of rears on the NO.

Females

Figures 5.12-5.15 presents evidence for the effects of SEA treatment in the OF/NO test. Statistical analysis revealed that treatment with SEA four days before testing in the OF/NO test resulted in a reduction in total activity level as reflected by a significant reduction in the total distance traveled ($F_{(1, 47)} = 6.329$, $p = .0154$) and a trend to suggest a decrease in the number of total entries ($F_{(1, 47)} = 3.039$, $p = .0878$) (See

Figure 5.12a and 5.12b). Figure 5.12c shows that the repeated measures ANOVA for the total number of entries also revealed a trend to suggest that treatment with SEA decreased the total number of entries ($F_{(1, 47)}$ = 3.201, p = .0800). Over time all animals showed an increase in the total number of entries ($F_{(4, 188)}$ = 3.689, p = .0064).

Treatment with SEA increased the behavioral reactivity of these animals in the OF/NO test. A significant genotype by treatment interaction was obtained for the percent time in the center, whereby the TNFRII$^{+/-}$ animals failed to decrease the percent time spent in the center after treatment with SEA compared to the TNFRII$^{+/+}$ and TNFRII$^{-/-}$ animals ($F_{(2, 47)}$ = 3.318, p = .0449) (See Figure 5.13a). Therefore, while both the TNFRII$^{+/+}$ and TNFRII$^{-/-}$ animals were showing heightened anxiety in the OF/NO test after SEA exposure, the TNFRII$^{+/-}$ animals remained unaffected. The repeated measures ANOVA revealed a significant genotype by treatment effect by interval effect for the percent time in the center ($F_{(8, 188)}$ = 2.723, p = .0073) (See Figure 3.13b). Although just approaching significance, the attenuated total number of entries to the center supports the increase in anxiety observed previously ($F_{(1, 47)}$ = 3.783, p = .0578). A more detailed repeated measures ANOVA in Figure 5.14b verified that the SEA treated animals decreased the number of entries to the center ($F_{(1, 47)}$ = 4.101, p = .0486). It also demonstrated that all animals showed an increase in the total number of entries to the center as the test progressed ($F_{(4, 188)}$ = 17.565, p < .0001), however genotype or treatment did not affect this parameter.

There were no changes in the percent time in the periphery, percent time in the middle, latency to enter the center, number of contacts with NO, and latency to contact the NO (See Table 10). Interestingly, Figure 5.15 demonstrates that there were changes in

exploratory behavior as exemplified by a significantly attenuated number of rears on the NO after SEA exposure ($F_{(1, 47)}$ = 4.195, p = .0461). There were no changes, however, in the number of rears in the periphery.

Discussion

The use of SAgs is based on the need to model infection-related circumstances that might shed light on neural-immune interactions. This study aimed to determine the behavioral consequences of SEA in wildtype, TNFRI$^{-/-}$, and TNFRII$^{-/-}$ animals four days after exposure. For the TNFRI male cohort, regardless of genotype, SEA reduced overall activity and increased anxiogenic like behavior in the OF/NO test. This was exemplified by an increase in the latency to enter the center, an increase in the latency to contact the NO, and a decrease in the percent time spent in the center of the test. Interestingly, although the TNFRI$^{-/-}$ males showed a blunted CORT response to SEA in the previous study, there was no major genotype differences in any of the parameters tested. The only difference emerged during a separate one-way ANOVA for the percent time in center at each one minute interval. At "minute one", the trend suggested that the TNFRI$^{+/-}$ males treated with SEA spent more percent time in the center than any of the other males. This became more noticeable at "minute two", when the TNFRI$^{-/-}$ and TNFRI$^{+/+}$ males treated with SEA showed a decrease in the percent time spent in the center, while the TNFRI$^{+/-}$ animals showed an increase. Therefore, the TNFRI$^{+/-}$ males appeared to be differentially sensitive to the anxiogenic like properties of SEA.

Although the females did not exhibit the identical behavioral changes induced by SEA that were observed in the males, they did display a similar behavior profile, showing

a decrease in overall activity and an increase in anxiety like behavior. The TNFRI$^{+/-}$ females were also shown to be differentially sensitive, exhibiting an increase in the latency to enter the center.

In contrast to the TNFRI male cohort, the TNFRII male cohort had very little response to SEA, showing only a change in the number of rears in the periphery. This was unexpected because both cohorts had a C57BL/6J background with a 129S2 donor strain. Therefore, it was expected that the TNFRII$^{+/+}$ animals would be behaviorally similar to the TNFRI$^{+/+}$ in the OF/NO test. However, although their backgrounds were the same, the animals in our colony were generated using heterozygous by heterozygous breeding. This breeding showed inherent differences between the two strains, with the TNFRI$^{+/-}$ breeders yielding nearly twice as many pups as the TNFRII$^{+/-}$ mice. Therefore, it is possible that maternal influences could account for the discrepancy in behavior. Nevertheless, in response to SEA, the TNFRII female cohort showed a decrease in overall activity and an increase in anxiogenic like behavior that was very similar to the TNFRI female cohort. Overall, there were no major genotype differences in these animals, although the TNFRII$^{+/-}$ animals did show an increase in the percent time in the center after treatment with SEA as compared to the TNFRII$^{+/+}$ and TNFRII$^{-/-}$ animals. This is consistent with the findings regarding the TNFRI$^{+/-}$ animals. Therefore, both strains of heterozygous animals appeared to be differentially sensitive to the anxiogenic properties of SEA as compared to the wildtype and the knockout animals. This data was consistent with other studies that have found TNFRI$^{-/-}$ and TNFRII$^{-/-}$ animals to be similar to wildtype animals in tests of anxiety such as the OF and the EPM (Simen et al., 2006; Quintana et al., 2007).

This data demonstrated that SEA continued to induce anxiogenic like behavior from animals several days after exposure, regardless of their genotype. We are hypothesizing that the decrease in general activity was not due to a continued sickness response but rather an enhanced neophobic reaction. This is supported by the observation in our laboratory that exposure to SEA does not increase general malaise or decrease locomotor behavior. Furthermore, Kawashima and Kusnecov (2002) reported that SEA challenge did not impact activity in the open field as measured by total line crossings. However, the introduction of a novel object resulted in an increase in the activity of the control animals and a reduction in activity in the SEA treated animals. Therefore, the reduction in total distance traveled and total number of entries may have been a reflection of enhanced neophobia, as opposed to an increase in general malaise. Ultimately, exposure to a T cell stimulus may prime or sensitize the animals to be more behaviorally reactive in a stressful situation such as exposure to a novel object in a novel environment.

Indeed, sensitization to a psychogenic stressor can occur upon re-exposure to the same or a different stressor. Anisman et al. (1993), found that cytokines can alter central neurotransmitter functioning, which can then affect lymphocyte functioning (Anisman et al., 1993). Hence, it has been suggested that an immunological challenge may change behavior precisely because it is interpreted by the brain as a stressor. This is supported by findings by Schmidt et al. (1995), who discovered that exposure to IL-1β caused an upregulation of AVP in the median eminence (Schmidt et al., 1995). This resulted in an increased HPA response to both tail shock and secondary exposure to IL-1β (Schmidt et al., 1995). A separate study found that challenge with IL-1β one to two weeks before exposure to a novel environment resulted in an exaggerated HPA response (Tilders and

Schmidt, 1999). This effect was also attributed to an increase in the co-expression of CRH and vasopressin (AVP) in the median eminence. Additionally, upregulation of AVP is also observed under conditions of chronic stress and therefore provides further support for the similarities between a psychogenic stressor and an immunologic stressor (Bartanusz et al., 1993). Hayley et al., (1999) showed that TNFα can exert a cross-sensitization to itself by demonstrating exaggerated anorexia and CORT levels as well as an increase in norepinephrine release after secondary exposure to TNFα (Hayley et al., 1999). Hence, it was hypothesized that TNFα may work similarly to IL-1β, by increasing AVP expression, which sensitizes the HPA response. Future studies should assess the role of AVP in mediating the enhanced neophobia observed after challenge with SEA.

Tumor necrosis factor-alpha has been shown to be one of the principal mediators of the behavioral and endocrine response to SEA (Rossi-George et al., 2005). Interestingly, while TNFRI has been shown to be important for mediating the CORT response to acute SEA it did not seem to play a role in mediating the long term behavioral consequences. The TNFRII pathway did not seem to play a role either, as there were no major differences in the TNFRII^{-/-} animals as compared to the controls. Although TNFα may be important for the initial behavior and endocrine response, other cytokines and pathways may compensate. Indeed, cytokines have multiple redundant pathways, which allow one to compensate in the absence of another. For example, IL-1β deficient mice respond normally to systemic LPS, and IL-6 does not seem to be involved in NF-κB activation after exposure to LPS (Bluthe et al., 2000; Rivest, 2003). Inhibition of TNFα only partially attenuates the ACTH and CORT response after LPS, and the same effect can be seen with TNFRI^{-/-} animals as well (Ebisui et al., 1994; Turnbull and Rivier,

1998). Further, it has been hypothesized that IL-1 receptor I knockout animals do not respond to the LPS, due to the replacement of the cytokine by TNFα (Bluthe et al., 2000). Regarding SEA, cytokines such as IL-2 and IFNγ are also upregulated, and may perhaps contribute to the increased neophobia. Interleukin-2 has been shown to alter behavior and activate the HPA axis, and may therefore be a significant contributor to the continued behavioral effects of SEA exposure (Karanth et al., 1994; Pauli et al., 1998; Zalcman et al., 1998; Zalcman, 2001). In addition, the compensatory mechanism of the receptors may take place as well. For example, it may be necessary to remove both TNFRI and TNFRII from the animals in order to see an effect. This may have been the case in Experiment 2.3, where by four hours the CORT response in the TNFRI$^{-/-}$ animals was similar to the TNFRI$^{+/+}$ animals. Removal of both receptors may have allowed for a complete blockade of the CORT response and an altered behavioral phenotype in the OF/NO test. This experiment also confirmed previous findings that TNFRI$^{-/-}$ and TNFRII$^{-/-}$ animals do not display differences in tests of anxiety (Simen et al., 2006).

Exposure to SAgs has been shown to induce anxiety like behavior and increase neophobia (Kawashima and Kusnecov, 2002; Rossi-George et al., 2004). The current study demonstrated that SEA continued to modify behavioral reactivity to a mild stressor such as exposure to an OF/NO test several days after the initial exposure. This behavioral sensitization was revealed as an enhanced neophobia in the OF/NO test. The absence of TNFRI and TNFRII did not modify the anxiety like behavior in the OF/NO test, demonstrating that signaling through TNFα may not be the primary mediator for the long term anxiogenic properties of SEA. The compensation of the other receptor or other

cytokines after treatment with SEA may have played a role in mediating the increased

neophobia in the TNFRI$^{-/-}$ and TNFRII$^{-/-}$ animals.

Chapter 6

The Role of TNFRI and TNFRII in Mediating the Endocrine

Effects of Repeated SEA Exposure

Introduction

The data from Experiment 1 demonstrated that the pattern of TNFα after repeated SEA exposure mirrored the pattern of the CORT response. Experiment 2 confirmed that signaling through TNFRI is responsible for mediating the anorexia and the CORT response after exposure to acute SEA. Therefore, TNFα may continue to be the driving force of the CORT response, even under repeated conditions. Moreover, while the data from Chapter 4 showed that signaling through TNFRII may not be important for mediating the response to acute SEA, it still may be important in mediating the endocrine effects of re-exposure to SEA. Therefore, this study addressed the importance of TNFRI and TNFRII in mediating the CORT response after secondary exposure to SEA. Because TNFRI may be a critical mediator of the CORT response to acute SEA, this study also examined changes in the mRNA expression of CRH in the hypothalamus to determine whether the CORT response was associated with regulation of CRH. In addition, the mRNA expression of TNFRII in the hypothalamus was measured to establish whether there was a compensatory upregulation of transcription in the absence of the TNFRI gene. Finally, the hypothalamic TNFRI mRNA expression in the TNFRI$^{+/+}$ animals was assessed to clarify whether exposure to secondary SEA altered the expression of this gene.

Material and Methods

Animals and Genotyping

See "General Methods".

Reagents

Staphyloccal enterotoxin A was purchased from Toxin Technology (Sarasota, FL). All injections of SEA were given intraperitoneally (IP) at a dose of 5 μg/mouse in pyrogen-free physiological saline in a volume of 0.2 ml.

Experimental Procedure

This study determined the effects of repeated SEA in TNFRI (Experiment 4.1) and TNFRII (Experiment 4.2) deficient males and females. Corticosterone, splenic TNFα, and hypothalamic mRNA changes for CRH, TNFRI, and TNFRII were assessed to elucidate the consequences of repeated SEA exposure in these knockout animals. Prior to the final injection and sacrifice, animals were put through a series of behavioral tests. On day 0 the animals were given an initial injection of SEA and exposure to a consumption test (Experiment 2.1 and 2.2). To measure differences in behavior several days after SEA exposure the same animals were placed in the OF/NO test four days after the initial exposure (Experiment 3.1 and 3.2). The following day (Day 5) animals were given a second injection of SEA or saline and sacrificed two hours after the final exposure. Experiment 1.3 confirmed that there was no residual effect of SEA on CORT five days after treatment. Therefore, it was not necessary to include a group of animals receiving SEA on day 0 and saline on day 5.

Blood and Tissue Collection

See "General Methods".

Protein Extraction and Quantification

See "General Methods".

Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

See General Methods".

Corticosterone Radioimmunoassay (RIA)

See "General Methods".

Reverse Transcription and Real Time PCR

See "General Methods".

Statistical Analysis

See "General Methods".

Results

Experiment 4.1 The Role of TNFRI in Mediating the Endocrine Effects of Repeated SEA

Males

Figures 6.1-6.4 present mean CORT, splenic TNFα, and hypothalamic CRH and TNFRII mRNA in male TNFRI$^{+/-}$, TNFRI$^{-/-}$, and TNFRI$^{+/+}$ animals treated with secondary SEA.

Corticosterone: Analysis by ANOVA revealed a significant genotype by treatment effect, whereby TNFRI$^{-/-}$ animals showed a blunted CORT response after secondary SEA exposure compared to TNFRI$^{+/-}$ and TNFRI$^{+/+}$ animals ($F_{(2, 68)} = 3.561$, $p = .0338$). Importantly, the TNFRI$^{+/-}$ animals also showed an attenuated CORT response after secondary SEA exposure compared to the TNFRI$^{+/+}$ animals ($F_{(1, 49)} = 4.301$, $p = .0434$). See Figure 6.1.

Splenic Tumor Necrosis Factor-apha: Statistical analysis revealed a significant treatment effect, whereby all subjects showed an increase in splenic TNFα after exposure to SEA ($F_{(1, 68)} = 17.844$, $p < .0001$). See Figure 6.2.

Hypothalamic mRNA

CRH: The results did not show a significant treatment or genotype effect on the expression of hypothalamic CRH mRNA expression. See Figure 6.3.

TNFRII: Analysis by ANOVA revealed a significant main effect ($F_{(2, 66)} = 3.526$, $p < .0351$), whereby the TNFRI$^{-/-}$ animal failed to increase hypothalamic TNFRII mRNA expression after treatment with SEA compared to the TNFRI$^{+/-}$ and TNFRI$^{+/+}$ animals. See Figure 6.4.

TNFRI: Analysis by ANOVA in Figure 6.5 demonstrates that the TNFRI$^{+/+}$ animals did not show a significant change in hypothalamic TNFRI mRNA expression after secondary SEA exposure.

Females

Figures 6.6-6.7 presents mean CORT and splenic TNFα, in female TNFRI$^{+/-}$, TNFRI$^{-/-}$, and TNFRI$^{+/+}$ animals treated with secondary SEA.

Corticosterone: Analysis by ANOVA revealed a significant treatment by genotype effect, whereby the TNFRI$^{-/-}$ animals showed an attenuated CORT response after secondary treatment with SEA ($F_{(2, 74)} = 5.658$, $p = .0052$). See Figure 6.6.

Splenic Tumor Necrosis Factor-apha: Treatment with SEA significantly increased splenic levels of TNFα in all genotypes ($F_{(1, 51)} = 98.219$, $p < .0001$). See Figure 6.7.

Experiment 4.2 The Role of TNFRII in Mediating the Endocrine Effects of Repeated SEA

Males

Figures 6.8-6.9 presents mean CORT and splenic TNFα, in male TNFRII$^{+/-}$, TNFRII$^{-/-}$, and TNFRII$^{+/+}$ animals treated with secondary SEA.

Corticosterone: Irrespective of genotype, statistical analysis showed that treatment with SEA increased level of CORT ($F_{(1, 40)} = 52.010$, $p < .0001$). See Figure 6.8.

Tumor Necrosis Factor-alpha: Regardless of genotype, there was a significant treatment effect, whereby treatment with SEA increased splenic TNFα ($F_{(1, 48)} = 39.365$, $p < .0001$). See Figure 6.9.

Females

Figures 6.10-6.11 presents mean CORT and splenic TNFα, in female TNFRII$^{+/-}$, TNFRII$^{-/-}$, and TNFRII$^{+/+}$ animals treated with secondary SEA.

Corticosterone: Irrespective of genotype, statistical analysis showed that treatment with SEA increased the level of CORT ($F_{(1, 39)}$ = 48.008, $p < .0001$). See Figure 6.10.

Tumor Necrosis Factor-alpha: Regardless of genotype, there was a significant treatment effect, whereby treatment with SEA increased splenic TNFα ($F_{(1, 39)}$ = 25.341, $p < .0001$). See Figure 6.11.

Discussion

Chapter 4 confirmed that TNFRI is partially responsible for mediating the endocrine response to acute SEA. Therefore, the current study addressed the importance of TNFRI and TNFRII in mediating the endocrine response after secondary exposure to SEA. As shown previously in Experiment 2, the TNFRI$^{-/-}$ male and female animals showed a blunted CORT response after a second injection with SEA. Additionally, as in Experiment 2, in Experiment 4.2 the TNFRII$^{-/-}$ animals did not have a modified CORT response after secondary exposure to SEA. Moreover, while Experiment 2.2 showed that the CORT levels of TNFRII$^{-/-}$ males were exaggerated, regardless of treatment, the present experiment did not replicate those findings. It is possible that this result was due to the animals' pre-exposure to a series of stressful tests, such as the consumption test and the OF/NO tests. Therefore, because the stimulus conditions were not identical between Experiments 2 and 4.2, comparison of CORT levels for the TNFRII$^{-/-}$ animals is not possible.

The effects observed in the TNFRI$^{-/-}$ animals were not due to a modified T cell response, as the levels of TNFα equally increased in response to SEA. Additionally, the attenuated CORT response did not appear to be mediated by changes in CRH expression; however, as stated previously, a longer time point may be necessary to observe any differences in the mRNA expression of CRH. This is supported by Kusnecov et al., (1999), who did not find an increase in CRH in the PVN until 6 hours after treatment with SEB (Kusnecov et al., 1999). This is also corroborated by studies in our laboratory showing that changes in CRH mRNA expression were not observed until 24 hours after treatment with LPS (unpublished report). In addition, the TNFRI$^{-/-}$ males did not show an attenuated level of CRH mRNA expression as compared to the controls, as was observed in Chapter 4. This could be explained by the addition of several stressful tests before sacrifice that could have modulated CRH expression. Other ACTH secretagogues, such as AVP, have been shown to potentiate CRH secretion; therefore, exposure to the stressors throughout the course of the experiment could have modulated CRH expression and AVP (Antoni, 1993).

This present study revealed that secondary treatment with SEA increased the expression of TNFRII in the TNFRI$^{+/-}$ and TNFRI$^{+/+}$ animals but not in the TNFRI$^{-/-}$ subjects. Interestingly, Experiment 2 found no change in TNFRII at two, four, or five hours after acute SEA exposure. This suggests that secondary exposure to SEA increased the expression through TNFRI signaling, in contrast to the results found using acute SEA challenge. It could also be argued that the changes observed in this experiment were a result of the initial injection of SEA, as opposed to the second injection. This scenario remains unlikely, however, due to reports that exposure to SEB did not influence the

expression of TNFRII in the brain at 1, 4, 12, or 24 hours after injection (Bette et al., 2003). A replication of this study with a group of animals sacrificed five days after an acute injection of SEA would help to clarify this problem. Regardless, the attenuated TNFRII expression in the TNFRI$^{-/-}$ animals suggests the possible involvement of TNFRI in altering the expression of TNFRII in response to an immunological stimulus.

Signaling through TNFRI initiates gene transcription via NF-κB which can induce both inflammatory and anti-inflammatory gene expression (Bazzoni and Beutler, 1996; Sedgwick et al., 2000; Kruglov et al., 2008). For example, it has been determined that TNFRI$^{-/-}$ animals exhibit lower expression for various inflammatory genes including interleukin-1 receptor I, silencer of cytokine signaling 3, and multiple chemokines (Quintana et al., 2007). Further supporting our findings, Simen et al., (2006) found a reduction in the mRNA expression of TNFRII in the hippocampus and the frontal cortex in TNFRI$^{-/-}$ animals (Simen et al., 2006). While they did not report a difference in TNFRII in the hypothalamus, we saw differential expression of TNFRII only after secondary challenge with SEA. It is not known whether this decrease in TNFRII may have contributed to the attenuated CORT response observed in the TNFRI$^{-/-}$ animals after re-exposure to SEA. Studies utilizing double knockouts for both receptors are needed to clarify whether TNFRII partially mediates the increase in CORT after secondary treatment with SEA in the absence of TNFRI.

The current results suggest that the initial activation of the HPA axis and increase in hypothalamic TNFRII mRNA by repeated SEA is dependent on TNFRI. The downregulation of TNFRII under these circumstances may have maintained an attenuated CORT response, although futures studies are necessary to clarify this. Moreover, the

precise pathway of the influence of TNFα on these receptors still remains unclear. Future

studies should elucidate the importance of peripheral versus central TNFRI in mediating

these effects, and is discussed further in Chapter 9.

Chapter 7

Role of the Glucocorticoid Response to SEA and LPS Induced Cytokine Regulation

Introduction

Previous experiments showed that SEA increased CORT production. The significance of glucocorticoid elevations has been argued to serve as a negative feedback on immune activity to prevent toxic shock or autoimmune reactivity (Besedovsky and del Rey, 2000b; del Rey and Besedovsky, 2000). Glucocorticoids have many immunosuppressive properties, including inhibition of macrophage function, decreased synthesis of proinflammatory cytokines, and a shift in the T cell response to Th2-like profile (Evans-Storms and Cidlowski, 1995; Elenkov and Chrousos, 2006). It has also been suggested that glucocorticoids play a role in mediating T cell clonal deletion after SAg exposure. For example, it was demonstrated that treatment with the glucocorticoid receptor antagonist RU-486 or adrenalectomy increased mortality following injection with SEA and SEB, while treatment with the synthetic glucocorticoid dexamethasone reversed these effects (Gonzalo et al., 1993). It was also determined that the corticosterone increase after SAg exposure was important for the early clonal deletion of activated Vβ3 or Vβ8 T cells (Gonzalo et al., 1993). Therefore, blockade of the corticosterone response could lead to uncontrolled T cell activation and ultimately septic shock. Due to these findings, it was of interest to determine whether pretreatment with a glucocorticoid inhibitor before the initial injection of SEA would modify the cytokine response to subsequent SEA exposure.

Specifically, the aim of this study was to determine whether inhibiting the synthesis of CORT by Aminoglutethimide (AMINO) or inhibiting the binding of CORT to the glucocorticoid receptors (RU486) would induce mortality and exaggerate sickness behavior in response to acute SEA (or LPS). Due to the importance of the CORT response in mediating T cell clonal deletion, it was also of interest to establish whether pretreatment with a glucocorticoid receptor antagonist before the initial injection of SEA would modify any tolerance in cytokine reactivity to SEA re-exposure. Moreover, because repeated exposure to other antigens, such as LPS, have also been shown to induce tolerance, this study examined the effect of blocking CORT before an initial exposure to LPS upon re-exposure to LPS (Urbach-Ross and Kusnecov, 2007).

Materials and Methods

Animals

See 'General Methods" section

Reagents

Staphyloccal enterotoxin A (SEA), Lipopolysaccharide (LPS), Aminoglutethimide, and RU486 were purchased from Sigma-Aldrich (St. Louis, MO). All injections of SEA or LPS were given intraperitoneally (ip) at doses of 5 µg/mouse in pyrogen-free physiological saline in a volume of 0.2 ml. Aminoglutethimide (50 µg/Kg) was administered subcutaneously (sc) in a volume of 0.1 ml from a stock solution of 12.5 µg/ml diluted in DMSO and mineral oil. Injection of aminoglutethimide was two hours

before the injection of SEA or LPS. Mifepristone (RU-486) (50 mg/Kg sc) was also given two hours prior to the first injection of SEA or LPS and was diluted in DMSO.

Aminoglutethimide Paradigm

Experiment 5.1 Time Course of Aminoglutethimide on the CORT and Cytokine Response to SEA

There is a paucity of information regarding the effect of glucocorticoid inhibition on the cytokine response following SEA challenge. To ensure that the dose of AMINO and time course chosen was sufficient at blocking CORT, an initial time course experiment was conducted to assess the efficacy of AMINO in modulating CORT and cytokine production after acute SEA (N=4-6/group). Animals were sacrificed at two and four hours after treatment with SEA or saline.

Experiment 5.2 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Inhibition Before Primary Exposure to SEA

To examine the effect of CORT inhibition on sickness behavior after exposure to SEA, animals were given one injection of AMINO two hours before given an injection of SEA (N=8/group). Animals were then assessed for sickness behavior and mortality. Due to the fact that blocking the CORT response can produce significant mortality after SAg exposure (Gonzalo et al., 1993), it was expected that if these animals did survive, there would be an exaggerated and prolonged sickness response. In order to assess this, animals were given a palatable novel food substance after pretreatment with AMINO.

Animals were placed into a consumption test at 2 hours, 5 hours, 8 hours, and 24 hours after their last injection. See Table 11.

Experiment 1.3 showed there to be a significant reduction of TNFα following secondary SEA treatment. To understand the impact of glucocorticoids in regulating this tolerance, animals were given a second injection of SEA one week after their initial treatment of AMINO and SEA. Therefore the groups were split up so that animals that were pretreated with AMINO and treated with SEA were split one week later and given a post-treatment of SEA or saline (AMINO-SEA-SEA (N=5) or AMINO-SEA-Sal (N=3)). Animals that were given AMINO and Saline were split one week later and given SEA or saline (AMINO-Sal-SEA (N=5) or AMINO-Sal-Sal (N=3)). The vehicle groups were split similarly so that the groups included Veh-SEA-SEA (N=5), Veh-SEA-Sal (N=3), Veh-Sal-SEA (N=5), and Veh-Sal-Sal (N=3). See Table 11.

Mifepristone (RU-486) Paradigm

Experiment 5.3 RU-486 Dose Response and Time Course: Effect of Plasma ACTH
An initial dose response and time course experiment was conducted to ensure that the dose and time point chosen was sufficient at blocking the glucocorticoid receptor. Animals were given 25 mg/kg (N=6), 50 mg/kg (N=6), or vehicle (N=4) and sacrificed at two or four hours post injection. An increase in plasma ACTH was assessed to ensure sufficient blockade of the glucocorticoid receptor.

Experiment 5.4 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Receptor Inhibition Before Primary Exposure to SEA

In order to assess the effects of blocking the glucocorticoid receptor on sickness behavior after exposure to SEA animals were pretreated with either RU-486 or vehicle. Animals were then treated with SEA or saline two hours later. Subjects were placed in the consumption test at 2, 5, 8, and 24 hours after the last injection. See Table 12.

To understand the impact that blocking the glucocorticoid receptors before an initial injection of SEA will have on re-exposure to SEA, animals were given a second injection of SEA or saline four days after RU-486/SEA treatment. Note that secondary exposure to SEA is closer in time than the aminoglutethimide study (See Table 11 and 12). Therefore the groups were split up so that animals that were pretreated with RU-486 and treated with SEA were split four days later and given a post-treatment of SEA or saline (RU-486-SEA-SEA (N=5) or RU-486-SEA-Sal (N=3)). Animals that were given RU-486 and Saline were split four days later and given SEA or saline (RU-486-Sal-SEA (N=5) or RU-486-Sal-Sal (N=2)). The vehicle groups were split similarly so that the groups included Veh-SEA-SEA (N=5), Veh-SEA-Sal (N=3), Veh-Sal-SEA (N=5), and Veh-Sal-Sal (N=2). See Table 12.

Experiment 5.5 Time Course of Aminoglutethimide on the CORT and Cytokine Response to LPS

To ensure that the dose of AMINO and time course chosen was sufficient at blocking CORT following LPS treatment, an initial time course experiment was conducted to

assess the efficacy of AMINO in modulating cytokine production after acute LPS. Animals were given AMINO + LPS (N=6) or Veh + LPS (N=4) and assessed two hours after the injection of LPS for CORT and splenic proinflammatory cytokine production to ensure that the this dose was also sufficient at blocking CORT and altering cytokine production after treatment with LPS.

Experiment 5.6 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Inhibition Before Primary Exposure to LPS

To examine the effect of CORT inhibition on sickness behavior after exposure to LPS, animals were given one injection of AMINO two hours before given an injection of LPS (N=8/group). Animals were then assessed for sickness behavior and mortality. Due to the fact that blocking the CORT response can produce significant mortality after LPS exposure (Hawes et al., 1992; Beishuizen and Thijs, 2003), it was expected that if these animals did survive, there would be an exaggerated and prolonged sickness response. In order to assess this, animals were given a palatable novel food substance after pretreatment with AMINO. Animals were placed into a consumption test at 2 hours, 5 hours, 8 hours, and 24 hours after their last injection. See Table 13.

Glucocorticoids have long been known to play an important role in mediating endotoxin tolerance (Zuckerman et al., 1991). To understand the impact of blocking the CORT response to secondary insult with LPS, animals were given a second injection of LPS one week after their initial injection. Therefore the groups were split up so that animals that were pretreated with AMINO and treated with LPS were split one week later and given a

post-treatment of LPS or saline (AMINO-LPS-LPS (N=5) or AMINO-LPS-Sal (N=3)).

Animals that were given AMINO and Saline were split one week later and given SEA or

saline (AMINO-Sal-LPS (N=5) or AMINO-Sal-Sal (N=2)). The vehicle groups were split

similarly so that the groups included Veh-LPS-LPS (N=5), Veh-LPS-Sal (N=3), Veh-Sal-

LPS (N=5), and Veh-Sal-Sal (N=2). See Table 13.

Expreriment 5.7 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Receptor

Inhibition Before Primary Exposure to LPS

In order to assess the effects of blocking the glucocorticoid receptor on sickness behavior

after exposure to LPS, animals were pretreated with either RU-486 or vehicle. Animals

were then treated with LPS or saline two hours later. Subjects were placed in the

consumption test at 2, 5, 8, and 24 hours after the last injection (N=6/group). See Table

14.

To understand the impact that blocking the glucocorticoid receptors before an initial

injection of LPS will have on re-exposure to LPS, animals were given a second injection

of LPS or saline four days later. Note that secondary exposure to LPS is closer in time

than the AMINO study (See Table 8). Therefore the groups were split up so that animals

that were pretreated with RU-486 and treated with LPS were split four days later and

given a post-treatment of LPS or saline (RU-486-LPS-LPS (N=4) or RU-486-LPS-Sal

(N=2)). The vehicle groups were split the same so that the groups included Veh-LPS-LPS

(N=4), Veh-LPS-Sal (N=2). See Table 14.

Protein extraction and quantification

See "General Methodology"

Cytokine enzyme linked immunosorbent assay (ELISA)

See General Methods"

Corticosterone Radioimmunoassay (RIA)

See "General Methods"

Adrenocorticotropin Hormone Radioimmunoassay (RIA)

See "General Methods"

Statistical Analysis

See "General Methods"

Results

Experiment 5.1 Time Course of Aminoglutethimide on the CORT and Cytokine Response to SEA

Corticosterone

Figure 7.1 shows that there was a significant reduction in CORT in both SEA and saline treated animals at 2 but not 4 hours following SEA treatment ($F_{(1, 15)}$ = 4.901, p = .0428; $F_{(1, 16)}$ = .403, p = .5347). Therefore, this confirmed that treatment with AMINO was successful at blocking CORT production at the two hour time point.

Hypothalamic Cytokine Protein

Tumor Necrosis Factor-alpha: Statistical analysis revealed that treatment with SEA significantly reduced TNFα mRNA expression in the hypothalamus two hours after treatment ($F_{(1, 15)}$ = 11.573, p = .0039). Pretreatment with AMINO did not alter the decrease in TNFα. There were no changes in TNFα four hours after treatment with SEA in both AMINO and Veh treated animals. See Figure 7.2.

Interleukin-1: The data showed a significant main effect, whereby AMINO significantly blocked the decrease IL-1β seen in the hypothalamus two hours after treatment with SEA ($F_{(1, 15)}$ = 5.014, p = .0407). There were no changes in IL-1β four hours after treatment with SEA. See Figure 7.3.

Splenic Cytokines

Figure 7.4-7.5 summarizes the splenic cytokine concentrations measured in response to treatment with AMINO prior to SEA. Animals were sacrificed at two or four hours following SEA treatment.

Interleukin-2: Regardless of pretreatment with AMINO or Veh, SEA induced a similar increase in IL-2 at two and four hours post exposure ($F_{(1, 16)}$ = 82.451, p < .0001; $F_{(1, 16)}$ = 39.600, p < .0001).

Interferon-gamma: Pretreatment with AMINO or treatment with SEA did not alter splenic IFNγ levels at either time point.

Interleukin-1: Analysis by ANOVA revealed a significant treatment effect, whereby treatment with SEA increased splenic IL-1β at the two hour time point ($F_{(1, 16)}$ = 36.601, p < .0001). Although not significant, there was a trend to suggest that pretreatment with

AMINO increased splenic IL-1β levels ($F_{(1, 16)}$ = 4.219, p = .0567). By four hours there were no elevations of splenic IL-1β.

Interleukin-6: Analysis by ANOVA revealed a significant treatment effect, whereby treatment with SEA increased splenic IL-6 at the two hour time point ($F_{(1, 16)}$ = 25.619, p < .0001). The four hour time point revealed a significant interaction effect in which the AMINO pretreated animals showed elevated levels of splenic IL-6 after treatment with SEA $F_{(1, 16)}$ = 12.790, p < .0025).

Tumor Necrosis Factor-alpha: Irrespective of pretreatment, SEA induced a similar significant increase in splenic TNFα at two and four hours ($F_{(1, 16)}$ = 49.409, p < .0001; $F_{(1,16)}$ = 37.488, p < .0001).

Plasma Interleukin-6: The two hour time point showed a significant interaction effect, whereby pretreatment with AMINO exaggerated IL-6 levels after treatment with SEA ($F_{(1, 16)}$ = 18.738, p = .0005). The four hour time point did not reveal any changes in IL-6 levels. See Figure 7.6.

Experiment 5.2 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Inhibition Before Primary Exposure to SEA

Prosobee Liquid Diet Consumption: Figure 7.7 demonstrates that treatment with SEA, regardless of pretreatment with AMINO, significantly reduced the consumption of Prosobee at 2 and 5 hours post injection ($F_{(1, 28)}$ = 25.803, p <.0001; $F_{(1, 28)}$ = 8.656, p = .0065). There was no effect of SEA on consumption at 8 hours post injection. There was a significant interaction effect at 2 and 5 hours, however this appeared to be due to

AMINO decreasing the consumption of Prosobee in saline treated animals while having no effect on the SEA treated group ($F_{(1, 28)} = .0082$, $p = .0082$; $F_{(1, 28)} = 4.208$, $p = .0497$). This pretreatment effect continued at the 8 hour consumption test, in that both the SEA and Saline animals pretreated with AMINO showed a decrease in consumption ($F_{(1, 28)} = 5.889$, $p = .0219$).

Figures 7.8-7.9 summarizes splenic and plasma cytokine concentrations after secondary SEA challenge following AMINO and SEA pretreatment.

Splenic Cytokines

Interleukin-2: Regardless of pretreatment with AMINO, there was a significant increase in splenic IL-2 after both acute and secondary treatment with SEA ($F_{(1, 24)} = 51.105$, $p < .0001$). It is important to note that treatment with SEA one week before a second injection of SEA did not produce a reduction of IL-2. This is similar to our previous findings (Urbach-Ross et al., 2008) (Experiment 1.1), that secondary exposure to SEA did not produce tolerance to the IL-2 response.

Interferon-gamma: Pretreatment with AMINO did not modify the IFNγ response to secondary SEA. There was a significant post-treatment effect, however, in that there was an increase in the levels of IFNγ after exposure to SEA on the day of the sacrifice ($F_{(1, 24)} = 13.260$, $p = .0013$). An unpaired t-test, however, revealed that there was no increase of IFNγ in animals that received AMINO and saline before exposure to SEA ($t(6) = .144$, $p = .8900$). Therefore, inhibition of CORT one week before exposure to SEA alters the IFNγ response.

Interleukin-1β: Irrespective of pretreatment with AMINO, there was a significant

increase in splenic IL-1β after both acute and secondary treatment with SEA ($F_{(1, 24)}$ =

109.064, $p <$.0001). Interestingly, there was also a significant interaction effect between

treatment and post-treatment. Similar to Experiment 1.3, it was demonstrated that

previous exposure to SEA significantly increased the level of IL-1β after secondary

exposure ($F_{(1, 24)}$ = 4.832, p = .0378). It is interesting to note this effect is still observable

one week after the initial SEA injection.

Interleukin-6: Regardless of pretreatment with AMINO, there was a significant increase

in splenic IL-6 after both acute and secondary treatment with SEA ($F_{(1, 24)}$ = 39.722, p

<.0001).

Tumor Necrosis Factor-alpha: There was no significant effect of pretreatment with

AMINO or treatment with SEA. There was a significant increase in the level of TNFα

after post-treatment with SEA in all of the groups ($F_{(1, 24)}$ = 58.245, $p <$.0001). It is

important to note that a secondary exposure to SEA did not produce tolerance in the

production of TNFα. Although there was no significant interaction effect, it appeared as

though the animals receiving Veh before primary SEA exposure produced a reduction in

TNFα after the second exposure compared to animals that received AMINO prior to the

initial SEA exposure. If the data is expressed as a percent change from the AMINO-Sal-

SEA or Veh-Sal-SEA it becomes apparent that pretreatment with AMINO may have

prevented tolerance to TNFα after secondary exposure to SEA ($t(8)$, = 7.667, $p <$.0001).

Plasma Tumor Necrosis Factor-alpha: There was a significant increase in the level of

plasma TNFα, regardless of AMINO pretreatment or exposure to primary SEA, on the

day of the sacrifice ($F_{(1,24)}$ = 51.103, p <.0001). Although there was not a significant

interaction effect, similarly to the splenic TNFα data, it appeared as though animals

receiving AMINO before their first injection with SEA showed a higher plasma TNFα

response after secondary exposure to SEA compared to animals that received Veh before

two injections of SEA. Once again, this becomes clear if the data is expressed as a

percent change from the AMINO-Sal-SEA or Veh-Sal-SEA ($t(8)$ = 2.546, p = .0344).

Therefore, the initial blockade of CORT before the first injection of SEA allows for a

greater plasma TNFα response after secondary exposure to SEA, and supports the splenic

TNFα results.

Corticosterone: Figure 7.10 shows that there was no significant effect of pretreatment

with AMINO or primary treatment with SEA on CORT production after secondary SEA

exposure. There was a significant increase in the level of CORT after treatment with SEA

on the day of sacrifice, regardless of pretreatment with AMINO or primary exposure to

SEA ($F_{(1, 24)}$ = 28.818, p <.0001). Therefore, it appears that secondary exposure to SEA

one week later does not induce a tolerance to the CORT response.

Experiment 5.3 RU-486 Dose Response and Time Course: Effect of Plasma ACTH

The previous set of experiments involved inhibition of glucocorticoid synthesis by

Aminoglutethimide. In the next series of experiments, we determined whether blocking

the glucocorticoid receptor would affect the behavioral, endocrine, and cytokine response

to SEA. To determine a dose of RU-486 that would be effective in disinhibiting the HPA

axis, a dose response and time course was established.

In response to a stressor the hypothalamus secretes corticotrophin releasing hormone CRH which promotes the release of ACTH from the anterior pituitary. The HPA axis has the ability to self regulate, employing a negative feedback loop to ensure that circulating levels of glucocorticoids do not exceed homeostatic levels. Figure 7.11 shows that both doses chosen significantly increased ACTH at two and four hours after treatment with the drug ($F_{(2, 24)}$ = 7.909, p = .0023). The increase in ACTH in this experiment is a reflection of the glucocorticoid receptors being bound by RU-486, thereby preventing feedback inhibition of the HPA axis. The data here show that both doses successfully blocked the GR at two and four hours. Therefore, the 50 mg/kg dose was chosen for all future experiments.

Experiment 5.4 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Receptor Inhibition Before Primary Exposure to SEA Prosobee Consumption

Figure 7.12 reveals that there were no significant interaction effects between pretreatment with RU-486 and treatment with SEA on liquid diet consumption during the first three consumption tests. Treatment with SEA, regardless of pretreatment with RU-486, significantly decreased consumption at 2, 5, and 8 hours after injection ($F_{(1, 24)}$ = 19.174, p = .0002; $F_{(1, 24)}$ = 12.124, p = .0019, $F_{(1, 24)}$ = 6.916, p = .0147). Twenty four hours after the injection of SEA there was no significant treatment effect, although, there appeared to be a significant interaction effect between pretreatment with RU-486 and treatment with SEA ($F_{(1, 24)}$ = 5.049, p = .0341). Pretreatment with RU-486 appeared to allow the SEA treated animals to recover while the Veh treated animals continued to show a reduction in food intake.

Figure 7.13-7.14 summarizes the mean splenic cytokine concentrations following secondary SEA after RU-486 and SEA pretreatment.

Interleukin-2: There were similar increases in the expression of IL-2 after treatment with SEA compared to saline on the day of sacrifice, regardless of pretreatment with RU-486 or previous exposure to SEA ($F_{(1, 20)} = 438.456$, $p < .0001$). Similar to previous two experiments, prior exposure to SEA did not induce tolerance to the IL-2 response to secondary SEA exposure.

Interferon-gamma: Pretreatment with RU-486 did not affect the production of IFNγ on the day of sacrifice in any of the treatment groups. There was a significant increase in the production of IFNγ after treatment with SEA on the day of sacrifice ($F_{(1, 20)} = 786.177$, $p < .0001$). There was a significant interaction effect between previous treatment with SEA and treatment with SEA on the day of sacrifice, in which secondary exposure to SEA produced an exacerbated IFNγ response ($F_{(1, 20)} = 9.891$, $p = .0051$). This is consistent with the repeated SEA study, demonstrating that secondary exposure to SEA enhances the IFNγ response.

Interleukin-1β: There was no effect of pretreatment with RU-486 or primary treatment with SEA on the production of IL-1β on the day of testing. There was an increase in the production of IL-1β in all animals injected with SEA regardless of pretreatment with RU-486 or primary exposure to SEA ($F_{(1, 20)} = 163.475$, $p < .0001$). In contrast to the previous experiments, previous exposure to SEA did not increase the levels of IL-1β to a secondary exposure.

Interleukin-6: There were similar increases in the production of IL-6 after treatment with SEA on the day of sacrifice, regardless of pretreatment or previous exposure to SEA ($F_{(1, 20)}$ = 53.388, $p < .0001$). There was no effect of pretreatment with RU-486 or previous exposure to SEA on the production of IL-6.

Tumor Necrosis Factor-alpha: Pretreatment with RU-486 did not affect the production of TNFα on the day of sacrifice. Previous exposure to SEA, however, significantly decreased the level of TNFα compared to animals with no previous SEA exposure, but who received secondary SEA. This occurred regardless of pretreatment with RU-486 or Veh ($F_{(1, 20)}$ = 39.096, $p < .0001$). These findings are consistent with the results of prior experiments (Experiment 1.3), demonstrating that a second injection of SEA produces an attenuated TNFα response.

Corticosterone: Figure 7.15 shows that pretreatment with RU-486 did not influence the CORT response to an injection of SEA on the day of sacrifice. Treatment with SEA did increase the production of SEA, however, contrary to the repeated SEA study (Experiment 1.3), secondary exposure to SEA did not attenuate the CORT response ($F_{(1, 20)}$ = 36.564, $p < .0001$).

Experiment 5.5 Time Course of Aminoglutethimide on the CORT and Cytokine Response to LPS

Due to the observation that there was no enhanced mortality in the AMINO + SEA treated animals, the question arose as to whether the immune response to SEA in the context of inhibited CORT production had minimal pathological impact. Therefore, a

second experiment was conducted in which splenic cytokines and CORT were assessed

in AMINO + LPS treated animals compared to Veh + LPS controls to ensure that the

dose and time course employed in this study was sufficient at blocking the CORT

response. Indeed, Figure 7.16 reveals that pretreatment with AMINO decreased CORT

and increased splenic IL-1β, TNFα, and IL-6 compared to Veh + LPS controls (CORT:

$t_{(8)}$ = -3.8843, p = .0049; IL-1β: $t_{(8)}$ = 2.371, p = .0452; TNFα: $t_{(8)}$ = 4.261, p = .0028; IL-

6: $t_{(8)}$ = 7.584, p = .0001). Due to these findings, it is surprising that there was no

enhanced mortality or sickness behavior in the SEA treated animals that were pretreated

with AMINO.

Experiment 5.6 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid

Inhibition Before Primary Exposure to LPS

Prosobee Consumption

Figure 7.17 displays a similar effect to what was observed in the SEA study. It shows that

regardless of treatment with AMINO, LPS decreased consumption at 2 and 5 hours post

treatment ($F_{(1, 25)}$ = 5.128, p = .0026; $F_{(1, 25)}$ = 7.233, p = .0126). While there was no

interaction effect, there seemed to have been a pretreatment effect with AMINO at 2, 5,

and 8 hours. This may have been due to the decrease in the saline treated animals in

consumption similar to what was observed in the SEA experiment ($F_{(1, 25)}$ = 3.553, p

= .0711; $F_{(1, 25)}$ = 10.210, p = .0039; $F_{(1, 25)}$ = 5.976, p = .0219).

Figure 7.18 summarizes the mean splenic cytokine concentrations following secondary

LPS after an initial AMINO exposure and LPS treatment seven days earlier.

Interleukin-1β: Treatment with LPS on the day of sacrifice significantly increased splenic levels of IL-1β ($F_{(1, 22)}$ = 5.463, p = .0289). Although there was no significant interaction effect, it appeared as though treatment with LPS one week before a second injection of LPS attenuated the IL-1β response ($F_{(1, 22)}$ = 5.463, p = .0289). There was no significant effect of pretreatment with AMINO on the IL-1β response to secondary LPS exposure.

Tumor Necrosis Factor-alpha: There was a significant interaction effect between pretreatment with AMINO and post-treatment with LPS on the day of sacrifice. It seems as though pretreatment with AMINO increased the level of TNFα after a secondary injection and after a primary injection of LPS compared to Veh treated animals ($F_{(1, 22)}$ = 5.380, p = .0300).

Interleukin-6: Treatment with LPS on the day of sacrifice significantly increased splenic levels of IL-6 ($F_{(1, 22)}$ = 21.183, p = .0001). There was a trend to suggest that there was a significant interaction effect between treatment and post-treatment on the day of sacrifice. It appears as though there is a reduction in the levels of splenic IL-6 after secondary exposure to LPS ($F_{(1, 22)}$ = 3.901, p = .0609). This tolerance was not altered by pretreatment with AMINO. Therefore, blockade of the CORT response before initial exposure to LPS does not interfere with the induction of tolerance to the production of proinflammatory cytokines in the spleen after a secondary exposure.

Experiment 5.7 Behavioral, Endocrine, and Cytokine Effects of Glucocorticoid Receptor Inhibition Before Primary Exposure to LPS

Prosobee Consumption: Figure 7.19 shows that there was no significant interaction between pretreatment with RU-486 and treatment with LPS in consumption measured 2,

5, and 8 hours after LPS exposure. Overall, there was a significant reduction in food intake at 2, 5, and 8 hours post-injection due to LPS exposure ($F_{(1, 20)}$ = 27.669, p < .0001; $F_{(1, 20)}$ = 51.857, p < .0001; $F_{(1, 20)}$ = 22.611, p < .0001). There was no longer a reduction in food intake due to LPS exposure at 24 hours post injection, however there appeared to be a significant pretreatment effect, with both LPS and saline animals pretreated with RU-486 showing a generally lower level of consumption ($F_{(1, 20)}$ = 5.026, p = .0365).

Figure 7.20-7.21 summarizes the mean splenic cytokine concentrations following secondary LPS after RU-486 and LPS pretreatment.

Interleukin-2: There was no significant treatment effect for the production of IL-2 after secondary LPS exposure.

Interferon-gamma: There were no significant changes in the production of IFNγ after secondary LPS exposure.

Interleukin-1β: There was a significant treatment effect for the production of IL-1β after different regimens of LPS exposure ($F_{(3, 8)}$ = 18.949, p = .0005). Animals treated with RU-486 before the initial injection of LPS did not differ from Veh-LPS in the IL-1β response to a secondary injection of LPS. Post hoc analysis revealed that both groups significantly increased the level of IL-1β compared to their respective controls (p = .0005, p = .0010)

Interleukin-6: There was no significant treatment effect for the production of IL-6 after secondary LPS exposure.

Tumor Necrosis Factor-alpha: There was a significant treatment effect for the production of TNFα after exposure to LPS or saline ($F_{(3, 8)}$ = 4.153, p = .0476). There was no

difference in the production of TNFα between the animals that received RU-486 and LPS before the secondary LPS injection compared to animals that received Veh and LPS. Post hoc analysis, however, revealed that it was only the RU-486 pretreated LPS-LPS group that increased TNFα production in response to a secondary injection compared to controls ($p = .0214$). In keeping with the AMINO and SEA study, this may suggest that antagonizing CORT may alter the tolerance effect to TNFα after secondary exposure to LPS.

Corticosterone: Figure 7.22 demonstrates that secondary treatment with LPS increased plasma level of CORT ($F_{(3, 8)} = 3.670$, $p = .0628$). Pretreatment with RU-486 did not alter the CORT response after secondary exposure to LPS compared to vehicle treated animals.

Discussion

The release of glucocorticoids is a critical adaptive feedback mechanism for controlling excessive inflammation in response to SAgs and LPS. For example, multiple experiments have shown that glucocorticoids regulate peripheral blood mononuclear cells and lymphocytes proliferation following SAg exposure (Gonzalo et al., 1993; Gonzalo et al., 1994; Arai et al., 2007; Fukushima et al., 2007). While glucocorticoids have been shown to be important for regulating proliferation of peripheral blood mononuclear cells and lymphocytes following SEA and regulating the cytokine response following LPS (Evans and Zuckerman, 1991), little is known regarding the role of glucocorticoids in regulating the cytokine response to SAgs. In addition, repeated stimulation with LPS and SEA has been shown to induce a tolerance to the cytokine response, specifically to TNFα

(Heremans et al., 1990; Zuckerman et al., 1991; Urbach-Ross and Kusnecov, 2007; Urbach-Ross et al., 2008). Therefore, this study determined the role of glucocorticoids during TNFα tolerance to SEA and LPS. The current study also assessed the global cytokine response following glucocorticoid inhibition during SEA and LPS induced tolerance. Before answering this question, the current study aimed to determine the efficacy and time course for AMINO and RU-486 pretreatment.

The first experiment in this study demonstrated that treatment with AMINO prior to treatment with SEA moderately altered cytokine production in the brain, spleen, and plasma. In the brain, treatment with SEA decreased TNFα and IL-1β. This was a surprising finding, as previous reports have shown the SAg SEB to have no effect on cytokine production in the brain (Del Rey et al., 2000a). The attenuated TNFα and IL-1β may have been a reflection of increased utilization of these cytokines, or it may have been an indication of glucocorticoid inhibition of cytokine production. Indeed, the decrease in IL-1β was attenuated with pretreatment with AMINO, demonstrating that inhibition of CORT kept the IL-1β levels in the brain close to the controls. As for the periphery, pretreatment with AMINO increased splenic IL-6 at four hours after SEA treatment and two hours in the plasma. There was also a trend to suggest that treatment with AMINO prior to SEA increased splenic IL-1β at the two hour time point as well. This is consistent with the hypothalamic changes in IL-1β suggesting that inhibition of CORT modifies cytokine IL-1β production in both the brain and the periphery. In terms of consumption, treatment with SEA reduced the consumption of Prosobee at two and five hours and thereafter showed recovery. Regardless of treatment with SEA, AMINO itself appeared to decrease food intake in these animals. One hypothesis for this

phenomenon is that AMINO may increase CRH, which has been shown to be anxiogenic and regulate SEA induced anorexia (Koob and Heinrichs, 1999; Kaneta and Kusnecov, 2005).

The mechanism through which SAgs develop T cell unresponsiveness and tolerance has not been fully elucidated. While the role T cell internalization and production of IL-10 are likely candidates, glucocorticoids may also play a role. Due to the ability of glucocorticoids to suppress the production of cytokines such as TNFα, the purpose of the following experiment was to determine the effect of glucocorticoid inhibition during TNFα tolerance to SEA. The results confirmed that although the AMINO was successful in increasing certain cytokines in response to SEA, it did not affect the IL-2, IL-1β, IL-6, or CORT responses to a secondary exposure of SEA. While IFNγ was increased on the day of sacrifice for most of the treatment groups, it appeared that AMINO pretreatment one week before an initial injection of SEA attenuated the IFNγ response. This suggests that the CORT response may prime cells for IFNγ production. For TNFα, it appeared that the animals receiving Veh before primary SEA exposure produced a greater reduction in TNFα after the second exposure than animals that received AMINO. This was supported in the plasma TNFα data, which illustrated that animals receiving AMINO before their first injection of SEA showed a higher TNFα response after secondary exposure to SEA compared to animals that received Veh. Therefore, it seems that treatment with AMINO enhances splenic TNFα as a function of secondary SEA exposure. This suggests that CORT may be partially responsible for the tolerance to TNFα after chronic SAg exposure.

In order to understand the findings from Experiment 5.2, we chose to use a different method for blockade of CORT. The drug mifepristone (RU-486) was chosen to specifically block the glucocorticoid receptor as opposed to preventing the synthesis of CORT actions. Aminoglutethimide is an aromatase and P450 scc (cholesterol 20, 22 lyase) inhibitor and therefore inhibits the synthesis of estrogen and hormones such as corticosterone. The use of RU-486, allowed us to determine whether blocking the glucocorticoid receptor would alter the tolerance to secondary exposure to SEA and LPS. The dose response and time course revealed that the dose chosen for the following experiments (50 mg/kg) successfully blocked the CORT response at both 2 and 4 hours.

Although we expected to see enhancement of anorexia in the consumption studies, the results showed that pretreatment with RU-486 did not alter the anorexic response to SEA at 2, 5, or 8 hours. In fact, the results demonstrated that pretreatment with RU-486 improved recovery from the anorexic effects of SEA 24 hours later. Additionally, while blocking CORT signaling should have enhanced mortality and cytokine production, these animals showed a faster recovery from sickness behavior and no mortality. This lack of mortality was surprising given that previous studies have shown that administration of RU-486 twelve hours before treatment with LPS increased the inflammatory response to LPS (Nadeau and Rivest, 2003). Moreover, the data from the dose response experiment confirmed that the timing and dose of RU-486 successfully blocked the glucocorticoid receptor at the time of SEA and LPS administration.

Regarding the tolerance to SEA, pretreatment with RU-486 did not alter either the production of cytokines or the CORT response to secondary SEA challenge. Although

TNFα was not affected as it was in previous experiments, the spacing of injections was different and cannot be directly compared (Refer to Table 11 and Table 12).

Experiment 5.2 showed that pretreatment with AMINO attenuated TNFα tolerance to SEA. This is consistent with studies showing that LPS induced tolerance to TNFα involves glucocorticoids (Evans and Zuckerman, 1991). Therefore, Experiments 5.6 and 5.7 determined the effect of glucocorticoid inhibition to LPS. To ensure that the dose and time courses used in this study were sufficient to block the CORT response after challenge with LPS, splenic cytokines and CORT were assessed in AMINO and LPS treated animals. Pretreatment with AMINO in these animals decreased CORT and increased splenic IL-1β, TNFα, and IL-6 compared to Veh and LPS treated controls. Due to these findings, it is surprising that neither SEA nor LPS animals that were pretreated with AMINO exhibited enhanced mortality or sickness behavior. Although AMINO pretreatment increased splenic cytokines and decreased CORT, it did not enhance the reduction of consumption of Prosobee after LPS challenge. Treatment with LPS did decrease consumption at two and five hours and showed recovery thereafter. Similar to the results found in Experiment 5.2, AMINO appeared to decrease food intake in these animals, irrespective of their treatment with LPS. This supports the hypothesis that the reduction in CORT may have driven up CRH, thereby inducing anorexia. The vehicle used for AMINO could also have contributed to the reduction in consumption. Mineral oil has been shown to induce inflammation and combined with the reduction in CORT may have resulted in excessive sickness behavior in these animals (Nordan and Potter, 1986; Shacter et al., 1992).

Although mortality and sickness remained unchanged in this experiment, this study established that there was successful inhibition of CORT. Therefore, animals were re-exposed to LPS one week later to see whether the initial inhibition of CORT and increase in cytokine production would interfere with tolerance to a secondary exposure. Intriguingly, it was revealed that treatment with AMINO prior to an acute or a secondary LPS challenge resulted in exaggerated splenic TNFα. This further supports the findings from Experiment 5.2 demonstrating the important role CORT plays in regulating TNFα production. It is also notable that inhibition of CORT one week before an acute exposure to SEA altered splenic TNFα production, however the reasons for this remain unclear.

Pretreatment with RU-486 did not alter the anorexic response to LPS either, with both RU-486 and Veh treated animals showing a similar decrease in consumption at 2, 5 and 8 hours. Although many studies have previously reported enhanced lethality, there are notable differences in the methodology between those experiments and the current study. Previous inquiries have involved administration of RU-486 after immune challenge, and used a significantly higher dose of LPS (Kovacs et al., 2008), or used intravenous SEB instead of SEA (Gonzalo et al., 1993).

In the current study, the timing of the RU-486 injections may have played an important role in the decreased mortality. It has been shown that the rise in glucocorticoids after SAg challenge serves to control the potential lethal effects of the enhanced cytokine response by producing an initial clonal deletion of lymphocytes (Gonzalo et al., 1993). This lymphocyte deletion seems to be sensitive to the time of exposure to glucocorticoids. In another study, splenic lymphocyte proliferation to SEA or SEB was shown to be resistant to the suppressive properties of the synthetic

glucocorticoid dexamethasone (DEX) (Weng et al., 1999). The timing and dose of DEX was important in determining its suppressive effects. This is consistent with Gonzalo et al., (1993) who reported that treatment with DEX was only protective if administered within two hours of treatment with SEB (Gonzalo et al., 1993). Therefore, the timing of AMINO or RU-486 treatment in the current study may have been sufficient to alter cytokines and decrease CORT but ultimately did not alter mortality. In addition, the lower dose of LPS and the use of SEA instead of SEB in the current study provides another probable explanation for the lack of mortality observed.

Interestingly, treatment with RU-486 seemed to enhance consumption in both LPS and saline treated animals 24 hours later. Importantly, treatment with RU-486 increased splenic levels of TNFα after secondary exposure, thereby removing the normal tolerance to TNFα usually observed with repeated SEA. This confirms the findings from Experiment 5.2 and 5.4, where blockade of CORT may have prevented tolerance to TNFα to secondary challenge with SEA. This suggests that inhibition of CORT during the initial exposure to SEA or LPS may induce long lasting effects on TNFα production. It is interesting to note there was not a global change in the other cytokines measured here. This is consistent with reports showing that TNFα is more sensitive to glucocorticoid inhibition compared to IL-1β and suggests differential regulation of cytokines during tolerance (Zuckerman et al., 1991). Given the cytotoxic and neurotoxic potential of TNFα, it is not surprising that TNFα is particularly sensitive to glucocorticoid regulation. Therefore, while the other cytokines measured here escaped glucocorticoid regulation, TNFα appears to be particularly sensitive.

In conclusion, pretreatment with AMINO before SEA or LPS exposure attenuated TNFα tolerance to a secondary challenge four days later. Similarly, pretreatment with RU-486 before LPS challenge abrogated TNFα tolerance to secondary LPS given seven days later. Although pretreatment with RU-486 did not alter TNFα tolerance to secondary SEA as it did in the first experiment, the second exposure was given much later and therefore these two studies cannot be directly compared. Therefore, TNFα tolerance to secondary exposure is removed by inhibiting the synthesis of CORT or blocking the glucocorticoid receptor before the initial immune challenge. Consequently, CORT may be partially responsible for the reduction in TNFα observed after repeated treatment with SEA and LPS.

Chapter 8

The Effect of Chronic Restraint Stress on the Endocrine and Cytokine

Response to Acute and Repeated SEA

Introduction

In a typical stress reaction, the adrenal gland releases epinephrine and norepinephrine to elicit a fight or flight response. These catecholamines can increase heart rate, increase catalysis of glycogen to glucose in the liver, and suppress appetite. Additionally, a stress response involves activation of the hypothalamic-pituitary-adrenal (HPA) axis (McEwen, 2000). However, the severity and duration of these responses depends on the specific paradigm employed in the study. An acute stressor can include a single session of a brief tail shock, restraint, cold swim, intruder stress, or sleep deprivation. Chronic stress involves prolonged exposure to the stressor of choice, although exposure to a homotypic stressor may result in habituation of the stress response. Therefore, to counteract this phenomenon, a heterotypic stressor is often employed.

Restraint is a popular model of acute and chronic stress induction and depending on the protocol used, produces neurobiological adaptation, measured in the form of a habituated response (Magarinos & McEwen 1995). However, it was found that when mice were exposed to 2 hours of daily restraint for 14 days, they spent less time in the open arms of the elevated plus maze, demonstrating that this regimen was sufficient to induce anxiogenic like behavior (Kim and Han, 2006). This study also found increased depressive like behavior, as measured by an increase in immobility time in the forced swim test. This is consistent with findings that repeated restraint sensitizes animals to

show higher CORT response to a novel mild stressor compared to non-stressed animals (Chotiwat and Harris, 2006).

Various acute stressors have been shown to alter the immune response. Inescapable tail shock has been shown to improve healing after subcutaneous challenge with Escherichia coli, while restraint has been shown to enhance T cell reactivity as measured by improved skin delayed-type hypersensitivity response (DTH) (Dhabhar and McEwen, 1996, 1997; Deak et al., 1999). Stress has been shown to increase cytokine production, lymphocyte proliferation, and macrophage function in vitro (Lysle et al., 1990; Lyte et al., 1990; Wood et al., 1993; Chancellor-Freeland et al., 1995). With respect to the immune response to LPS, prior exposure to acute stress has been shown to exaggerate levels of IL-1β and TNFα in the plasma and IL-1β in the brain, as well as sensitize the CORT (Carswell et al., 1975) and ACTH response (Johnson et al., 2002b; Johnson et al., 2002a). Overall, there is considerable evidence that acute stressors can enhance various parameters of immune function.

While acute stress seems to enhance immune function, chronic stress appears to be immunosuppressive (Dhabhar and McEwen, 1996, 1997; Deak et al., 1999). For example, repeated stress has been shown to impair wound healing and suppress the DTH response (Dhabhar and McEwen, 1997; Padgett et al., 1998). This is consistent with reports that chronic stress induces an anti-inflammatory bias, which results in immunodeficiency. Chronically stressed mice have exhibited an increase in bacterial burden after infection with Listeria monocytogenes and Escherichia coli, attributable to reduced proliferation of T cells (Zhang et al., 1998; Kiank et al., 2006). Chronic stress has also been shown to cause an increase in apoptosis of lymphocytes, a reduction in the

number of thymocytes, a decrease in phagocytic activity, and an impairment in the production of TNFα, IL-6, IFNγ, and IL-10, which demonstrates an anti-inflammatory cytokine bias (Yin et al., 2000; Kiank et al., 2006). Conversely, it has been argued that chronic stress can induce susceptibility to infection by inducing glucocorticoid resistance, thereby allowing excessive production of proinflammatory cytokines (Quan et al., 2001; Quan et al., 2003). Moreover, multiple studies have noted a chronic stress induced increase in cytokine production after treatment with LPS (Quan et al., 2001; Johnson et al., 2002a; Stark et al., 2002; Avitsur et al., 2003; Johnson et al., 2003; Quan et al., 2003; Avitsur et al., 2005). At present, it is not known whether chronic restraint can affect the cytokine and HPA-activating effects of bacterial SAgs. The difference in the severity and duration of the stressor as well as the diversity of stressors used makes it difficult to hypothesize whether exposure to restraint stress will enhance or suppress the CORT response and cytokine production after challenge with SEA. However, because SEA relies on the induction of activated T cells and restraint stress has been shown to promote lymphocyte apoptosis, we hypothesize that chronically restrained stressed animals will show a blunted CORT and an anti-inflammatory bias following SEA challenge.

Systemic infection models using LPS mainly characterize the effects of stress on macrophage mediated inflammation. The current study aimed to extend those findings to a T cell mediated immune response by utilizing the SAg SEA. The stressor used in this study was chronic restraint stress since considerable information exits on its immunologic effects. Therefore, the current study sought to address the endocrine and immunological effects of chronic restraint after acute and repeated SEA administration. Additionally, this

study examined stress and SEA induced changes in hypothalamic CRH, TNFRI, and TNFRII mRNA expression.

Material and Methods

Animals

See "General Methods".

Reagents

Staphyloccal enterotoxin A was purchased from Sigma-Aldrich (St. Louis, MO). All injections of SEA were given intraperitoneally (IP) at a dose of 5 µg/mouse in pyrogen-free physiological saline in a volume of 0.2 ml.

Restraint Protocol

Mice were placed into 50 ml conical tubes, containing 80 air holes for ventilation. Each restraint session lasted 90 minutes and was repeated for ten days. Control animals remained in their home cage.

Experiment 6.1: Effects of Acute and Repeated Restraint Stress on Brain c-Fos Immunoreactivity

To ensure that the animals did not completely habituate to the restraint a set of animals were sacrificed immediately after removal from the last restraint session and compared to animals exposed to restraint for the first time (acute restraint). Immunohistochemistry

was performed for the detection of the immediate early gene c-Fos in the PVN as an assessment for neuronal activation (N = 6/group).

To date, there have been no studies assessing the impact of chronic stress on the immune response to bacterial superantigens. In Experiment 6.2, SEA was administered 24 hours after the last restraint session. Since we had previously shown that SEA produces unique patterns of cytokine production, while retaining HPA axis reactivity after multiple injections of SEA (Urbach-Ross et al., 2008), two treatment regimens with SEA were tested following restraint stress exposure in Experiment 6.3. The two treatment regiments included an acute injection of SEA 6 days after restraint as well as a second injection of SEA on day 6 after the initial treatment at 24 hours.

Experiment 6.2: Acute SEA Administration Protocol

Subjects were given the first injection of SEA or Saline 24 hours from the last restraint session and sacrificed two hours later. The groups included Restraint + SEA (N = 6/group), Restraint + saline (N = 6/group), Home cage + SEA (N = 6/group), and Home cage + saline (N = 6/group).

Experiment 6.3: Repeated SEA Administration Protocol

Studies from our laboratory have found that when secondary exposure to SEA is given five days after the initial exposure there is an attenuated splenic TNFα and CORT response compared to acute exposure while the splenic IL-2 response remained unchanged (See Chapter 3) (Urbach-Ross et al., 2008). Therefore, the next study

determined whether chronic restraint altered the secondary response to SEA or to acute exposure to SEA given 6 days after the final restraint session. Specifically, twenty four hours after the last restraint session animals were given either SEA or saline (immunological and endocrine results of this treatment were measured in Experiment 6.2). Mice were then returned to their home cage after which they were given either SEA or saline five days later and sacrificed two hours after the injection. The groups included Restraint plus repeated SEA (i.e. two injections of SEA; designated Restraint + SEA-SEA, N = 8/group), Restraint plus acute SEA (an injection of saline and then SEA on the day of sacrifice; designated Restraint + Sal-SEA, N = 4/group), saline controls (restraint plus two injections of saline; Restraint + Sal-Sal, N = 4/group), and a control to ensure there was no residual effect from the initial injection of SEA (Restraint plus an initial injection of SEA and a secondary injection of saline on the day of sacrifice; Restraint + SEA-Sal, N = 4/group). Home cage controls were included for all groups with the Home cage + SEA-SEA having 8/group while all of the other groups had 4/group.

Protein extraction and quantification
See "General Methods".

Cytokine enzyme linked immunosorbent assay (ELISA)
See "General Methods".

Corticosterone Radioimmunoassay (RIA)
See "General Methods".

Statistical Analysis

See "General Methods".

Results

Experiment 6.1 Repeated Restraint c-fos

A two way ANOVA revealed that exposure to repeated restraint stress produced a habituated but still significant increase in c-Fos activation in the PVN ($F_{(2, 15)}$ = 75.931, p < .0001) (See Figure 8.1). Therefore, the paradigm used in this study continued to be perceived as stressful to these animals on the tenth day of exposure.

Experiment 6.2 Repeated Restraint + Acute SEA

Figure 8.2 summarizes mean splenic cytokine concentrations in response to acute SEA following repeated restraint.

Tumor Necrosis Factor-alpha: As was expected, there was a significant increase in splenic TNFα after treatment with SEA ($F_{(1, 20)}$ = 195.155, p < .0001). Repeated restraint, however, did not alter the levels of TNFα in the spleen.

Interleukin-1β: Acute exposure to SEA increased the levels of IL-1β in the spleen ($F_{(1, 20)}$ = 148.889, p < .0001). Although not significant, there is a trend to suggest that repeated restraint increased splenic levels of IL-1β in both SEA and saline animals ($F_{(1, 20)}$ = 3.392, p = .0804). This is consistent with reports that physiological stressors can increase central IL-1β (Nguyen et al., 1998; Nair and Bonneau, 2006; Urbach-Ross and Kusnecov, 2007).

Interleukin-2: There was a significant interaction effect between restraint and exposure to SEA, in which exposure to restraint blocked the increase in IL-2 normally observed after treatment with SEA ($F_{(1, 20)} = 11.259$, $p = .0031$).

Interferon-gamma: There were no significant changes of splenic IFNγ after restraint or treatment with acute SEA.

Interleukin-10: An acute injection of SEA failed to elicit an IL-10 response in both restrained and control animals. This is consistent with data from our laboratory, showing that acute exposure to SEA does not increase splenic levels of IL-10 (See Chapter 3). Interestingly, exposure to restraint stress increased levels of IL-10, regardless of treatment with SEA or saline ($F_{(1, 19)} = 10.922$, $p = .0037$).

Corticosterone: Figure 8.3 reveals that there was no significant effect of restraint on the CORT response after treatment with SEA. Exposure to SEA significantly increased levels of CORT regardless of treatment with restraint ($F_{(1, 20)} = 98.355$, $p < .0001$).

Hypothalamic mRNA: Analysis by ANOVA revealed there to be no changes in the mRNA expression of CRH, TNFRI, and TNFRII after restraint or treatment with SEA. See Figure 8.4.

Experiment 6.3 Repeated Restraint + Repeated SEA

Figure 8.5 summarizes mean splenic cytokine concentrations in response to acute and secondary SEA following repeated restraint.

Tumor Necrosis Factor-alpha: There was no significant effect of restraint on the splenic TNFα response. There was a significant treatment effect ($F_{(3, 31)} = 41.969$, $p < .0001$), in that both a single treatment of SEA and repeated exposure to SEA increased the levels of TNFα in the spleen relative to the saline controls (Sal-SEA, $p < .0001$; SEA-SEA, $p < .0001$). Post hoc analysis replicated previous findings from our laboratory (Chapter 3 and 7) that secondary exposure to SEA attenuated the splenic TNFα response when compared to acute exposure ($p = .0013$). Post hoc analysis also revealed that splenic TNFα responses were enhanced to acute SEA challenge given six days after the last restraint session ($p = .029$).

Interleukin-1β: There was no significant effect of restraint on the production of IL-1β, however there was a significant treatment effect ($F_{(3, 31)} = 23.205$, $p < .0001$). Post hoc analysis revealed that both a single injection of SEA as well as two injections increased IL-1β compared to the saline controls (Sal-SEA, $p < .0001$; SEA-SEA, $p < .0001$).

Interleukin-2: Analysis by ANOVA revealed there to be a significant increase in the IL-2 response after both acute and repeated exposure to SEA ($F_{(3, 31)} = 30.175$, $p < .0001$). This is consistent with the repeated SEA study (Chapter 3) in that both acute and secondary exposure to SEA continues to elicit and IL-2 response. Fascinatingly, post hoc analysis revealed that splenic IL-2 was enhanced to a primary exposure to SEA given six days after the last restraint session ($p = .0067$). These changes were not observed when the animals were given the second of two SEA injections (first at 24 hrs, second on the 6th day after last restraint).

Interferon-gamma: There were no significant changes in the IFNγ levels. There was a trend, however, that suggested that restraint itself increased levels of splenic IFNγ ($F_{(1, 31)}$ = 4.079, p = .0521). This study did replicate the findings from Experiment 1.3 (Chapter 3) in that two injections of SEA five days apart drove up the IFNγ response compared to a single exposure (p = .0231).

Interleukin-10: The treatment given to the animals significantly altered the IL-10 response in the spleen ($F_{(3, 30)}$ = 10.524, p < .0001). Post hoc analysis revealed that a single exposure to SEA did not increase IL-10, similarly to what was observed in the previous experiment. Secondary exposure, however, did increase splenic IL-10 compared to the control (p = .0028). Interestingly, the level of IL-10 was also increased in animals that received an initial injection of SEA followed by an injection of saline (p < .0001). There was also a trend to suggest that repeated restraint stress itself increased splenic IL-10 levels, specifically in the SEA-SEA and SEA-Sal animals ($F_{(1, 30)}$ = 4.106, p = .0517).

Corticosterone: Figure 8.6 shows that exposure to restraint did not alter the CORT response after treatment with either acute or repeated SEA. There was an increase in the CORT response after both acute and repeated SEA, which was not altered by restraint ($F_{(3, 31)}$ = 13.200, p < .0001). Interestingly, a second injection of SEA continued to drive up the CORT response, demonstrating that a second injection of SEA continued to be involved in the activation of the HPA axis, regardless of the attenuation in TNFα. These observations were observed previously in our laboratory.

Hypothalamic mRNA: Analysis by ANOVA revealed there to be no changes in the mRNA expression of CRH, TNFRI, and TNFRII after restraint or treatment with SEA. See Figure 8.7.

Discussion

Repeated restraint has been widely used to assess the influence of chronic stress on both innate and adaptive immunity. To examine the effects of chronic stress on a T cell mediated immune response, we exposed animals to chronic restraint followed by acute or repeated SEA. This study explored whether chronic restraint stress altered either the HPA axis activation or the cytokine response to SEA.

Exposure to restraint stress has been shown to increase lymphocyte apoptosis by signaling through the Fas-mediated pathway (Yin et al., 2000). It was therefore hypothesized that treatment with repeated restraint would suppress the CORT response and pro-inflammatory cytokine profile after challenge with T cell mediated immune stimulus such as SEA. This study determined that repeated restraint did not interfere with the upregulation of TNFα, IFNγ, or CORT after treatment with acute SEA. Although there were no changes in these cytokines, the data revealed that mice exposed to chronic restraint showed an increase in IL-10 and a trend to suggest an increase in IL-1β as well. In fitting with our hypothesis, there was an attenuated IL-2 response after challenge with acute SEA in the restraint stressed animals.

The increase in IL-10 and the attenuated IL-2 response is consistent with reports that chronic stress is immunosuppressive. A recent study confirmed that restraint stress

enhanced resistance to lethal doses of E *coli* and LPS, which was associated with a reduction in pro-inflammatory cytokines (Wang et al., 2008). The Wang et al., (2008) study found no resistance against the lethality of S *aureus* and therefore they did not measure the differences in cytokine levels in the animals after restraint. Our study, however, confirmed that restraint stress attenuated splenic IL-2 after primary SEA challenge but left the TNFα levels unchanged. Given that TNFα has been shown to be the primary mediator of septic shock (Cauwels and Brouckaert, 2007), out data suggests that the intact sensitivity to lethal doses of S *aureus* in the Wang (2008) study could have been mediated through maintained elevation of TNFα.

The decrease in the proinflammatory cytokine IL-2 was accompanied by an overall increase in the anti-inflammatory cytokine IL-10 in all restraint stress treated animals. This increase in IL-10 was corroborated by Kiank et al., (2006), who noted an enhanced IL-10 inducibility after chronic restraint stress (Kiank et al., 2006). Indeed, an inverse relationship between levels of IL-10 and IL-2 has previously been reported (Sundstedt et al., 1997). Hence, the enhanced splenic IL-10 after restraint stress may have been responsible for driving down the IL-2 levels after challenge with SEA. Surprisingly, there were no changes in CORT levels in the restrained treated animals, although the maintained increase in TNFα may have sustained the CORT response. This is consistent with previous studies from our laboratory (Chapter 3 and Chapter 6) in which TNFα continued to drive the CORT response under conditions in which there was elevated IL-10 production. Although glucocorticoids have traditionally been associated with stress induced immunosuppression, the CORT response in these studies remained unchanged. Hence, another mechanism for the immunosuppression is likely to be operating. Yin et al.,

(2000) showed that adrenectomized animals showed no difference in the reduction of lymphocytes after restraint stress as compared to sham treated animals (Yin et al., 2000). Furthermore, they found that the immunosuppression was mediated through endogenous opiates, which increased Fas expression on lymphocytes and ultimately induced apoptosis. Therefore, opiate induced immunosuppression and other mechanisms may be involved in mediating the dampened IL-2 and heightened IL-10 observed here.

The second experiment confirmed the increase in IL-10 after repeated restraint stress and found a trend for an increase in IFNγ. Contrary to the results from Experiment 6.2, splenic IL-2 and TNFα responses were enhanced to a primary exposure to SEA given six days after the last restraint session. These changes were not observed when the animals were given the second of two SEA injections (first at 24 hrs, second on the 6th day after last restraint). Moreover, plasma CORT to primary or secondary SEA remained intact in stressed animals, which suggests that the T cell cytokine response to SEA is refractory to the effects of chronic restraint stress when SEA challenge is distal to the termination of chronic stress. This is consistent with the notion that exposure to psychogenic and immunogenic stressors can enhance responsiveness to the same or a different stressor. A study by Hayley et al., (1999) revealed that secondary administration of TNFα provoked a time-dependent desensitization or sensitization to both sickness behavior and CORT, with the length in the interval increasing sensitization (Hayley et al., 1999). The same might be true for repeated restraint stress, in which a psychogenic stressor may desensitize or sensitize immune reactivity in a biphasic manner. An increase in the co-expression of AVP with CRH in the median eminence has been suggested to be important to mediate the sensitization to the enhanced responsiveness to a secondary

stressor (Tilders and Schmidt, 1999). Therefore, future studies should determine whether restraint stress induced changes in AVP are mediating the cytokine enhancement following SEA challenge.

In conclusion, chronic restraint stress abrogated IL-2 levels in response to acute SEA given early after the end of chronic stress, and did not interfere with the CORT response. Alternatively, when acute SEA exposure occurred six days after the last restraint session there was enhanced IL-2 and TNFα, while the CORT response remained unchanged. Finally, if SEA was given both 1 and 6 days after restraint, no changes in the cytokine or CORT response to the second injection were observed. These data suggest that the T cell cytokine response to SEA is attenuated following chronic restraint when SEA challenge is close to termination of the chronic stress and enhanced when the exposure is more distal to termination of chronic stress.

Chapter 9

Conclusions and Future Directions

Previous studies have confirmed that a single systemic challenge with SEA produces significant endocrine and behavioral effects. These effects include neuronal activation of limbic brain regions, activation of the HPA axis, enhanced neophobia to both appetitive and non-appetitive stimuli, and heightened anxiety (Gonzalo et al., 1993; Kawashima et al., 2002; Kawashima and Kusnecov, 2002; Rossi-George et al., 2004; Rossi-George et al., 2005). Studies from our laboratory have shown TNFα to be the primary mediator of these behavioral and endocrine effects of SEA (Rossi-George et al., 2005). The purpose of studied described in Chapter 3 was to extend those previous findings and elucidate the *in vivo* repercussions of repeated challenge with SEA. From this study it was determined that the magnitude of the CORT response differed depending on the interval between SEA challenges. Nevertheless, repeated treatment with SEA continued to induce a CORT response that was dissociated from the behavioral effects. Notably, secondary exposure to SEA resulted in a decrease in TNFα production, while IL-1β, IFNγ, and IL-2 all remained elevated. This was an important finding as the CORT response was also reduced after secondary exposure. Therefore, the CORT response appeared to be independent from regulation by cytokines such as IL-1β that have traditionally been known to increase CORT. More importantly, a hypothesized role for TNFα in mediating the endocrine response to repeated SEA emerged.

While TNFα appears to be important for mediating the effects of acute SEA, the mechanism through which that is achieved had not been determined. Therefore, Chapters 4 through 6 examined the role of TNFRI and TNFRII in mediating the effects of acute

SEA immediately after exposure, several days after exposure, and following secondary exposure to SEA.

Chapter 4 noted the importance of TNFRI but not TNFRII in mediating the anorexia and CORT response immediately following acute SEA in males and females. Interestingly, both anorexia and the CORT response were reinstated in the TNFRI$^{-/-}$ animals by four hours after SEA. Therefore, there may have been compensatory mechanisms, such as the upregulation of IL-2, which may have contributed to the delayed CORT response (Karanth et al., 1994; Pauli et al., 1998; Zalcman et al., 1998; Zalcman, 2001). The use of IL-2$^{-/-}$ animals may help to clarify the role of IL-2. Moreover, immuno-neutralization of IL-2 in TNFRI$^{-/-}$ animals may help to elucidate whether this cytokine is providing a compensatory role in the absence of TNFRI. Another possible mechanism for the delayed CORT response could be signaling by TNFRII. Although TNFRII$^{-/-}$ animals did not show a change in the CORT response following SEA challenge and there were no changes in the mRNA expression of TNFRII in TNFRI$^{-/-}$ animals, the absence of TNFRI may have been enough to enhance signaling through TNFRII. Future studies should utilize double knockout animals to assess the role of both receptors in mediating the effects of acute SEA.

To our knowledge, the long term behavioral consequences of SEA exposure had not been assessed previously. Therefore Chapter 5 explored the repercussions of SEA challenge four days following exposure in the OF/NO test. This study assessed TNFRI$^{+/-}$, TNFRI$^{-/-}$, TNFRI$^{+/+}$ and TNFRII$^{+/-}$,TNFRII$^{-/-}$, and TNFRII$^{+/+}$ males and females. Intriguingly, challenge with SEA four days prior to the OF/NO test provoked an increased in neophobic behavior in all but the male TNFRII cohort. This was interesting

as it showed that treatment with SEA continued to induce anxiogenic like behavior several days after exposure, regardless of signaling through TNFRI or TNFRII. These results suggest further investigation into the long term consequences of exposure to superantigens is needed, addressing other anxiety behavioral tests such as the elevated plus maze and the light dark box. Moreover, other deficits in behavior, such as learning and memory, will also be important to assess. Finally, the use of TNFα or TNFRI and TNFRII double knockout animals may shed some light to the role TNFα may play in mediating the enhanced anxiety.

As was mentioned previously, the immediate CORT response following SEA treatment was mediated through TNFRI signaling. It was also determined that the CORT response following secondary SEA challenge was also mediated through TNFRI signaling. Interestingly, TNFRII mRNA expression was also attenuated in the TNFRI$^{-/-}$ animals in response to secondary SEA. Therefore, TNFRI appears to mediate both the CORT response and hypothalamic TNFRII mRNA expression following repeated SEA exposure. The downregulation of TNFRII under these circumstances may maintain an attenuated CORT response at the four hour time point. Therefore future studies are necessary to determine the CORT response four hours after repeated exposure. Furthermore, it is not possible to determine whether the effects observed in Experiments 4 and 6 were due to the absence of central or peripheral TNFα receptors. Neutralization of central TNFRI and TNFRII will help determine whether central or peripheral receptors or the neutralization of both are necessary for the attenuated CORT response following acute and repeated SEA. If this particular experiment does show that central TNFα receptors are necessary to mediate the effects of SEA, it will be important to determine

the location of the receptors that are specifically responsible for mediating these effects. It has been demonstrated that TNFα administration produced strong c-Fos activation in the PVN and bilateral lesions to the PVN produces blunted ACTH in response to TNFα (Kovacs and Elenkov, 1995; Rossi-George et al., 2005). Moreover, TNFRI expression has been shown to be constitutively expressed in the PVN (Nadeau and Rivest, 1999). Therefore, direct central inhibition of TNF receptors in the PVN may block the effects of SEA. If it is determined that central TNF receptors are responsible for mediating the effects of SEA, the mechanism through which that is achieved will be important to determine. Pan et al. (2007) has determined that there is receptor-mediated transport of peripheral TNFα into the brain parenchyma where it may bind central TNF receptors (Pan et al., 2007). Once TNFα is in the brain, the question remains as to whether it is directly mediating the effects of SEA or eliciting a response by increasing the production of other cytokines such as IL-1.

It is also possible that central receptors are not playing a role in mediating these effects. TNFα has been shown to increase BBB permeability and induce mediators such as prostaglandins, nitric oxide, and other cytokines in the brain (Pober and Cotran, 1990; Megyeri et al., 1992). Nadeau & Rivest (1999) demonstrated that there is a large increase in the expression of TNFα receptors (specifically TNFRI) in the subventricular organs and barrier associated cells after systemic LPS or TNFα, and that these cells may mediate the actions of circulating TNFα after an immunogenic stressor (Nadeau and Rivest, 1999). Ultimately, TNFα may not require its central receptors but may exert its behavioral and neuroendocrine effects to SEA indirectly, via signaling through receptors at the BBB interface.

One way to examine the mechanism of this peripheral signaling is through assessing the down stream consequences of transcription factors such as NF-κB. When TNFα binds to TNFRI, adapter proteins such as TRADD and TRAF are recruited, which results in the phosphorylation of Inhibitor of κB (I-κB) and the release of NF-κB from the NF-κB- I-κB complex. Once NF-κB has been released, it translocates to the nucleus where it regulates many inflammatory genes (Hanada and Yoshimura, 2002). It is through the regulation of these inflammatory genes that TNFα may exert its neuroendocrine effects following SEA. For example, it has been suggested that TNFα can stimulate the release of prostaglandins by incαreasing cyclo-oxygenase-2 (COX-II) via NF-κB signaling at the site of the PVN (Nadeau and Rivest, 1999). Prostaglandins have been shown to induce ACTH release either directly or by stimulating the release of CRH and AVP (Bugajski et al., 2004). It has not yet been determined whether this release of prostaglandins following TNFα is responsible for increased glucocorticoids. However, IL-1β has been shown to signal the release of CRH from the median eminence by stimulating endothelial cells to release prostaglandins (O'Connor et al., 2000). NF-κB activation may also increase TNFα or other cytokines in the brain, which may stimulate the HPA axis. Therefore NF-κB activation and the resulting increase in inflammatory mediators following TNFRI signaling at the site of the BBB remains a likely candidate for mediating the increase in CORT following SEA exposure. (See the figure at the end of the chapter)

While TNFα has been shown to be important in mediating the endocrine effects of both acute and repeated SEA, Chapter 7 revealed the importance of glucocorticoids in mediating TNFα. The increase in glucocorticoids after SAg challenge has been suggested

to play a role in mediating tolerance to SAg and endotoxin. Chapter 7 confirmed that glucocorticoids are partially responsible for the reduction in TNFα following secondary SEA or LPS challenge. Interestingly, other cytokines such as IL-2 and IL-1β were not altered by the inhibition of CORT during the initial exposure. The reason for this remains unclear, but it could be related to the cytotoxic and neurotoxic potential of TNFα overproduction, which makes it particularly important to keep TNFα levels tightly regulated.

While the mechanism through which glucocorticoids mediate tolerance remains unclear, it has been repeatedly demonstrated that a normal glucocorticoid response is necessary for the induction of tolerance (Chautard et al., 1999). In addition, Chapter 7 confirms other studies that demonstrate that treatment with RU-486 deactivates tolerance to TNFα after repeated stimulation with LPS (Fantuzzi et al., 1995). This is consistent with findings that tolerance to TNFα after repeated stimulation with LPS is glucocorticoid dependent (Evans and Zuckerman, 1991). These findings support the idea of glucocorticoid negative feedback. Glucocorticoids can dampen the inflammatory response by decreasing cytokine mRNA stability or by inhibiting NF-κB (O'Connor et al., 2000). Interestingly, the decrease in TNFα mRNA after endotoxin tolerance is associated with a decrease in NF-κB (Ziegler-Heitbrock et al., 1994). Therefore, TNFα mRNA may be particularly sensitive to glucocorticoid downregulation of NF-κB. Another mechanism through which glucocorticoid tolerance occurs could be through the upregulation of IL-10. Indeed, treatment with glucocorticoids has been shown to increase both IL-10 mRNA and circulating IL-10 (Ramirez et al., 1996; Tabardel et al., 1996; van der Poll et al.,

1996). Future studies should address the involvement of NF-κB and IL-10 in mediating glucocorticoid dependent TNFα tolerance to both SEA and LPS.

Finally, while we showed that CORT inhibition modifies TNFα following secondary SEA challenge, Chapter 8 determined the consequence of glucocorticoid disruption through repeated restraint on the effects of acute and repeated challenge with SEA. We showed that chronic restraint induced an overall upregulation of IL-10 and an attenuated IL-2 response after acute SEA challenge. Therefore, while there was no change in CORT or TNFα production, it appeared that chronic restraint produced immunosuppression and an anti-inflammatory bias. Intriguingly, when the initial SEA challenge was given 6 days after the final restraint session there was a continued enhancement of IL-10, but instead of an attenuated IL-2 response, both IL-2 and TNFα were enhanced. Importantly, the restraint stress did not interfere with SAg tolerance, as none of the cytokines were altered when animals were given the second of two SEA injections. These data suggest that the T cell cytokine response to SEA is refractory to the effects of chronic restraint stress when SEA challenge is distal to termination of chronic stress. Future studies should assess the involvement of AVP in mediating the exaggerated IL-2 and TNFα response.

Hypothetical Mechanisms of TNFα Mediated HPA Axis Activation Following SEA Exposure
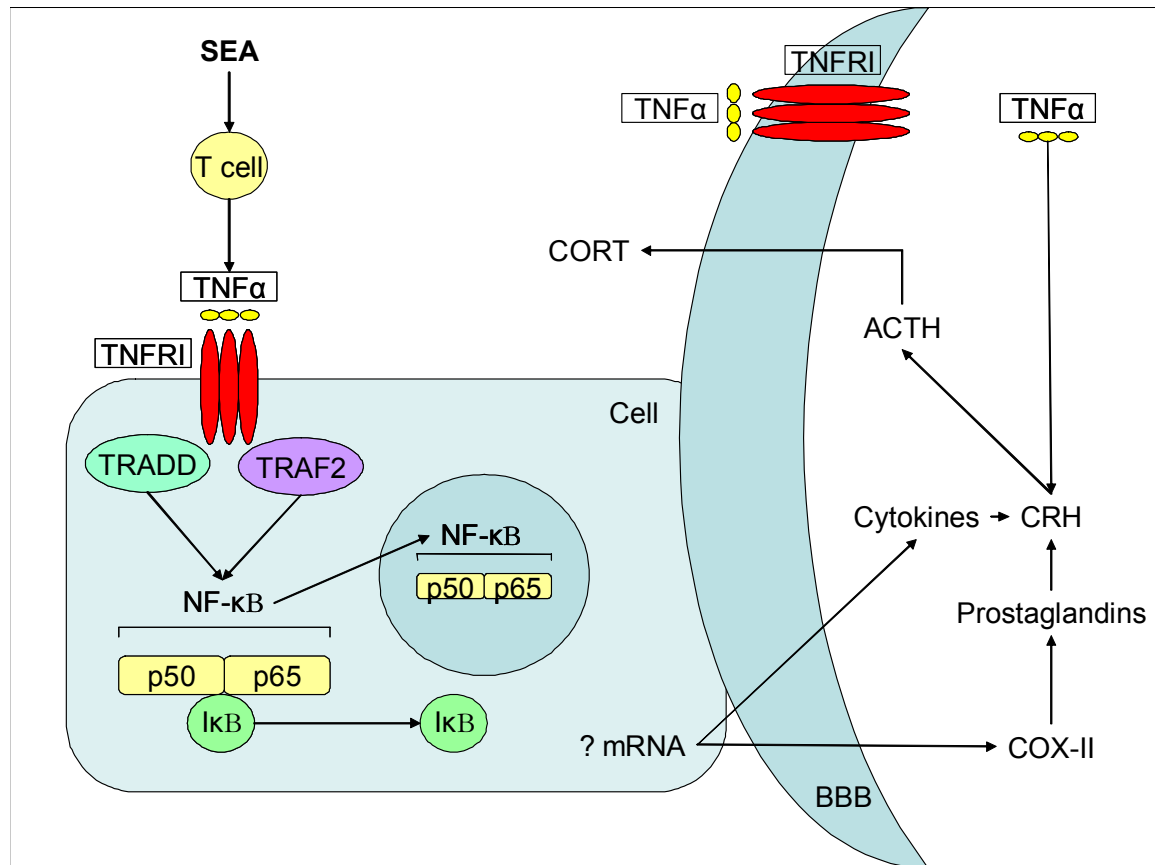
SEA, Staphylococcal Enterotoxin A; **TNFα**, Tumor necrosis factor-alpha; **TNFRI**, tumor necrosis factor-alpha receptor I; **TRADD**, TNF receptor associated death domain; **TRAF2**, TNF receptor associated factor 2; **NF-κB**, Nuclear factor kappa B; **IκB**, Inhibitor of κB; **BBB**, Blood brain barrier; **COX-II**, cyclo-oxygenase-2; **CRH**, Corticotropin releasing hormone; **ACTH**, Adrenocorticotropic hormone; **CORT**, Corticosterone

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Table 1

Biological Properties of Superantigens

T cell stimulation	•Antigen processing independent •T cell dependent •10^4 more T cell stimulation than conventional antigens
Cytokine production	↑ IL-2 ↑ IFNγ ↑ TNFα
HPA axis activation	↑ CRH ↑ ACTH ↑ CORT
Behavior not affected	·Mobility ·Malaise
Behavior affected	↑ Gustatory neophobia ↑ Neophobia to novel object ↑ Anxiety

Table 2

Genotyping PCR primers (all sequences 5' to 3')

	Gene Amplified	**Sequence**
TNFRI$^{-/-}$ genotyping	Tnfr1 "E444"	TGT GAA AAG GGC ACC TTT ACG GC
	Tnfr1 "p55R-636"	GGC TGC AGT CCA CGC ACT GG
	Tnfr1 "HSV-TK"	ATT CGC CAA TGA CAA GAC GCT GG
TNFRII$^{-/-}$ genotyping	Tnfrsfb "A"	CCT CTC ATG CTG TCC CGG AAT
	Tnfrsfb "+"	AGC TCC AGG CAC AAG GGC GGG
	Tnfrsfb "Neo 490V"	CGG TTC TTT TTG TCA AGA C
	Tnfrsfb " NEO NB3"	ATC CTC GCC GTC GGG CAT GC

TNFRI$^{-/-}$ - http://jaxmice.jax.org/strain/002818.html
TNFRII$^{-/-}$ -http://jaxmice.jax.org/strain/002620.html

Table 3

Real Time PCR Primers (all sequences 5' to 3')

Gene Amplified	Forward Primer	Reverse Primer
CRH	GTT GAA TTT CTT GCA ACC GGA G	GAC TTC TGT TGA GGT TCC CCAG
TNFRI	CAA CGT CCT GAC AAT GCA GAC C	ACG CAT GAA CTC CTT CCA AGC G
TNFRII	AAA GAG ATG CCA AGG TGC CTC AT	GAG CTG CTG CTG CTG GAA CTG
GAPDH	AAC TCC CTC AAG ATT GTC AGC AA	GGC TAA GCA GTT GGT GGT GC

Table 4

Summary of Experimental Design for Experiment 1.1

	D0	D1	D3
Acute SEA	Sal		SEA
Acute SEA		Sal	SEA
SEAx2 (2d) (2 days apart)		SEA	SEA
SEAx2 (3d) (3 days apart)	SEA		SEA
Control 1		Sal	Sal
Control 2	Sal		Sal

Table 5

Summary of Experimental Design for Experiment 1.2

	D0	**D2**	**D5**
control	Sal	Sal	Sal
Acute SEA	Sal	Sal	**SEA**
SEAx2 (3d)	Sal	**SEA**	**SEA**
SEAx2 (5d)	**SEA**	Sal	**SEA**
SEAx3	**SEA**	**SEA**	**SEA**

Table 6

Summary of Experimental Design for Experiment 1.3

	D0	**D2**	**D5**	**D10**
Acute SEA	Sal	Sal	Sal	**SEA**
SEA 5d	Sal	Sal	**SEA**	**SEA**
SEAx3a	Sal	**SEA**	**SEA**	**SEA**
SEAx3b	**SEA**	Sal	**SEA**	**SEA**
SEAx4	**SEA**	**SEA**	**SEA**	**SEA**
Acute control	Sal	Sal	Sal	Sal
Control 5d	Sal	Sal	**SEA**	Sal
Controlx3a	Sal	**SEA**	**SEA**	Sal
Controlx3b	**SEA**	Sal	**SEA**	Sal
Controlx4	**SEA**	**SEA**	**SEA**	Sal

Table 7

Summary of Behavior in the OF/NO Apparatus for Experiment 3.1: Males

Percent time in periphery ➢Genotype ➢Treatment ➢Genotype X Treatment	$F_{(2, 68)}$ = .472, p = .6261 $F_{(1, 68)}$ = .019, p = .8911 $F_{(2, 68)}$ = 1.931, p = .1529
Percent time in middle ➢Genotype ➢Treatment ➢Genotype X Treatment	$F_{(2, 68)}$ = .316, p = .7298 $F_{(1, 68)}$ = .1.283E-4, p = .9910 $F_{(2, 68)}$ = .888, p = .4161
Number of entries to center ➢Genotype ➢Treatment ➢Genotype X Treatment	$F_{(2, 68)}$ = .964, p = .3864 $F_{(1, 68)}$ = .2.601, p = .1114 $F_{(2, 68)}$ = .812, p = .4482
Number of contact with NO ➢Genotype ➢Treatment ➢Genotype X Treatment	$F_{(2, 68)}$ = 1.747, p = .1820 $F_{(1, 68)}$ = .246, p = .6218 $F_{(2, 68)}$ = 1.147, p = .3238
Number of rears in periphery ➢Genotype ➢Treatment ➢Genotype X Treatment	$F_{(2, 68)}$ = 2.698, p = .0746 $F_{(1, 68)}$ = 2.169, p = .1454 $F_{(2, 68)}$ = 1.919, p = .1546
Number of rears on NO ➢Genotype ➢Treatment ➢Genotype X Treatment	$F_{(2, 68)}$ = .685, p = .0746 $F_{(1, 68)}$ = .442, p = .1454 $F_{(2, 68)}$ = .682, p = .1546

Table 8

Summary of Behavior in the OF/NO Apparatus for Experiment 3.1: Females

Percent time in periphery	
➢Genotype	$F_{(2, 73)}$ = 1.113, p = .3340
➢Treatment	$F_{(1, 73)}$ = 2.132, p = .1486
➢Genotype X Treatment	$F_{(2, 73)}$ = .245, p = .7837
Percent time in middle	
➢Genotype	$F_{(2, 73)}$ = .012, p = .9885
➢Treatment	$F_{(1, 73)}$ = .282, p = .5971
➢Genotype X Treatment	$F_{(2, 73)}$ = .373, p = .6896
Number of contact with NO	
➢Genotype	$F_{(2, 73)}$ = 1.709, p = .1882
➢Treatment	$F_{(1, 73)}$ = .458, p = .5009
➢Genotype X Treatment	$F_{(2, 73)}$ = 1.159, p = .3195
Latency to contact NO	
➢Genotype	$F_{(2, 73)}$ = .275, p = .7600
➢Treatment	$F_{(1, 73)}$ = .733, p = .3948
➢Genotype X Treatment	$F_{(2, 73)}$ = 1.121, p = .3315
Number of rears in periphery	
➢Genotype	$F_{(2, 73)}$ = .724, p = .4881
➢Treatment	$F_{(1, 73)}$ = 2.383, p = .1270
➢Genotype X Treatment	$F_{(2, 73)}$ = .149, p = .8621
Number of rears on NO	
➢Genotype	$F_{(2, 73)}$ = 1.397, p = .2539
➢Treatment	$F_{(1, 73)}$ = .877, p = .3520
➢Genotype X Treatment	$F_{(2, 73)}$ = .422, p = .6575

Table 9

Summary of Behavior in the OF/NO Apparatus for Experiment 3.2: Males

Percent time in periphery	
➤Genotype	$F_{(2, 48)} = .189, p = .8286$
➤Treatment	$F_{(1, 48)} = 1.907, p = .1737$
➤Genotype X Treatment	$F_{(2, 48)} = .036, p = .9651$
Percent time in middle	
➤Genotype	$F_{(2, 48)} = 1.278, p = .2878$
➤Treatment	$F_{(1, 48)} = .656, p = .4221$
➤Genotype X Treatment	$F_{(2, 48)} = .358, p = .7006$
Percent time in center	
➤Genotype	$F_{(2, 48)} = .470, p = .6282$
➤Treatment	$F_{(1, 48)} = 2.045, p = .1592$
➤Genotype X Treatment	$F_{(2, 48)} = .028, p = .9719$
Latency to enter center	
➤Genotype	$F_{(2, 48)} = 1.436, p = .2480$
➤Treatment	$F_{(1, 48)} = .946, p = .3356$
➤Genotype X Treatment	$F_{(2, 48)} = .565, p = .5719$
Number of entries to center	
➤Genotype	$F_{(2, 48)} = 1.172, p = .3183$
➤Treatment	$F_{(1, 48)} = 2.014, p = .1624$
➤Genotype X Treatment	$F_{(2, 48)} = .694, p = .5045$
Number of contacts with NO	
➤Genotype	$F_{(2, 48)} = .324, p = .7245$
➤Treatment	$F_{(1, 48)} = .387, p = .5371$
➤Genotype X Treatment	$F_{(2, 48)} = .250, p = .7797$
Latency to contact NO	
➤Genotype	$F_{(2, 48)} = .281, p = .7564$
➤Treatment	$F_{(1, 48)} = 1.237, p = .2715$
➤Genotype X Treatment	$F_{(2, 48)} = 1.236, p = .2997$
Number of rears on NO	
➤Genotype	$F_{(2, 48)} = .263, p = .7700$
➤Treatment	$F_{(1, 48)} = .452, p = .5045$
➤Genotype X Treatment	$F_{(2, 48)} = .101, p = .9042$

Table 10

Summary of Behavior in the OF/NO Apparatus for Experiment 3.2: Females

Percent time in periphery	
➢Genotype	$F_{(2, 47)} = .750$, $p = .4479$
➢Treatment	$F_{(1, 47)} = 1.844$, $p = .1810$
➢Genotype X Treatment	$F_{(2, 47)} = 2.148$, $p = .1281$
Percent time in middle	
➢Genotype	$F_{(2, 47)} = 2.967$, $p = .0612$
➢Treatment	$F_{(1, 47)} = .018$, $p = .8946$
➢Genotype X Treatment	$F_{(2, 47)} = .865$, $p = .4278$
Latency to enter center	
➢Genotype	$F_{(2, 47)} = 2.125$, $p = .1308$
➢Treatment	$F_{(1, 47)} = 2.230$, $p = .1420$
➢Genotype X Treatment	$F_{(2, 47)} = .738$, $p = .4836$
Number of contacts with NO	
➢Genotype	$F_{(2, 47)} = .666$, $p = .5186$
➢Treatment	$F_{(1, 47)} = .125$, $p = .7252$
➢Genotype X Treatment	$F_{(2, 47)} = 2.781$, $p = .0722$
Latency to contact NO	
➢Genotype	$F_{(2, 47)} = .713$, $p = .4955$
➢Treatment	$F_{(1, 47)} = 2.144$, $p = .1498$
➢Genotype X Treatment	$F_{(2, 47)} = 1.225$, $p = .3030$
Number of rears in periphery	
➢Genotype	$F_{(2, 47)} = .760$, $p = .4736$
➢Treatment	$F_{(1, 47)} = 3.436$, $p = .0701$
➢Genotype X Treatment	$F_{(2, 47)} = .533$, $p = .5902$

Table 11
Summary of Experimental Design for Experiment 5.2

Pre-treatment	Treatment	Post-treatment
Day 0	Day 0	Day 7

Amino
- SEA
  - SEA
  - Sal
- Sal
  - SEA
  - Sal

Veh
- SEA
  - SEA
  - Sal
- Sal
  - SEA
  - Sal

• 2Hr Sac
• Splenic cytokines
• CORT

↑
Consumption Test

• 2Hr
• 5 Hr
• 8 Hr
• 24 Hr
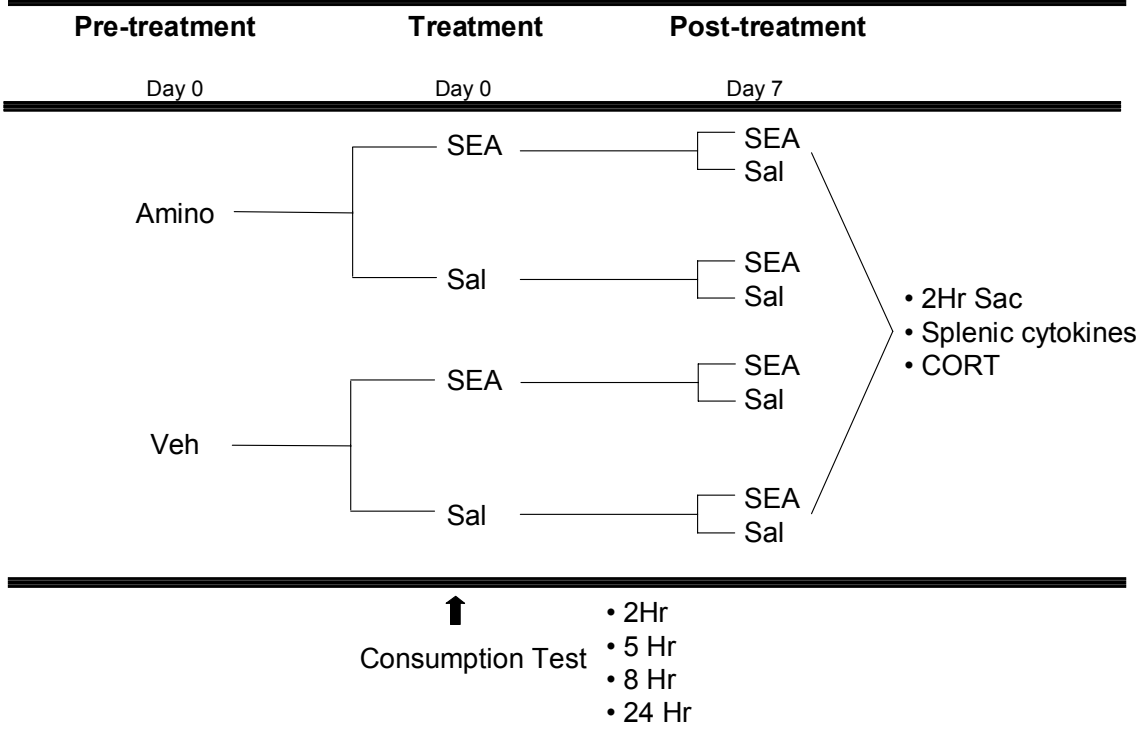
Table 12
Summary of Experimental Design for Experiment 5.4

Pre-treatment	Treatment	Post-treatment
Day 0	Day 0	Day 4

RU-486

    SEA   SEA / Sal

    Sal   SEA / Sal

Veh

    SEA   SEA / Sal

    Sal   SEA / Sal

• 2Hr Sac
• Splenic cytokines
• CORT

↑
Consumption Test

• 2Hr
• 5 Hr
• 8 Hr
• 24 Hr
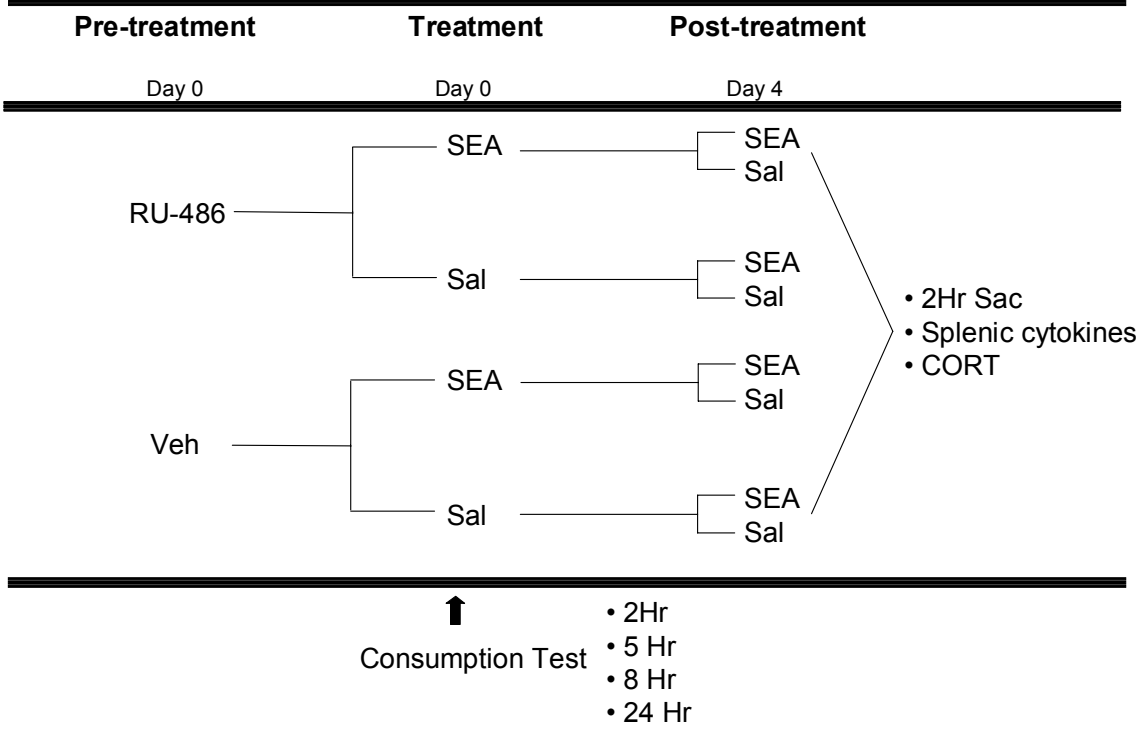
Table 13
Summary of Experimental Design for Experiment 5.6

Pre-treatment	Treatment	Post-treatment
Day 0	Day 0	Day 7

Amino
- LPS
  - LPS
  - Sal
- Sal
  - LPS
  - Sal

Veh
- LPS
  - LPS
  - Sal
- Sal
  - LPS
  - Sal

- 2Hr Sac
- Splenic cytokines
- CORT

↑
Consumption Test
- 2Hr
- 5 Hr
- 8 Hr
- 24 Hr
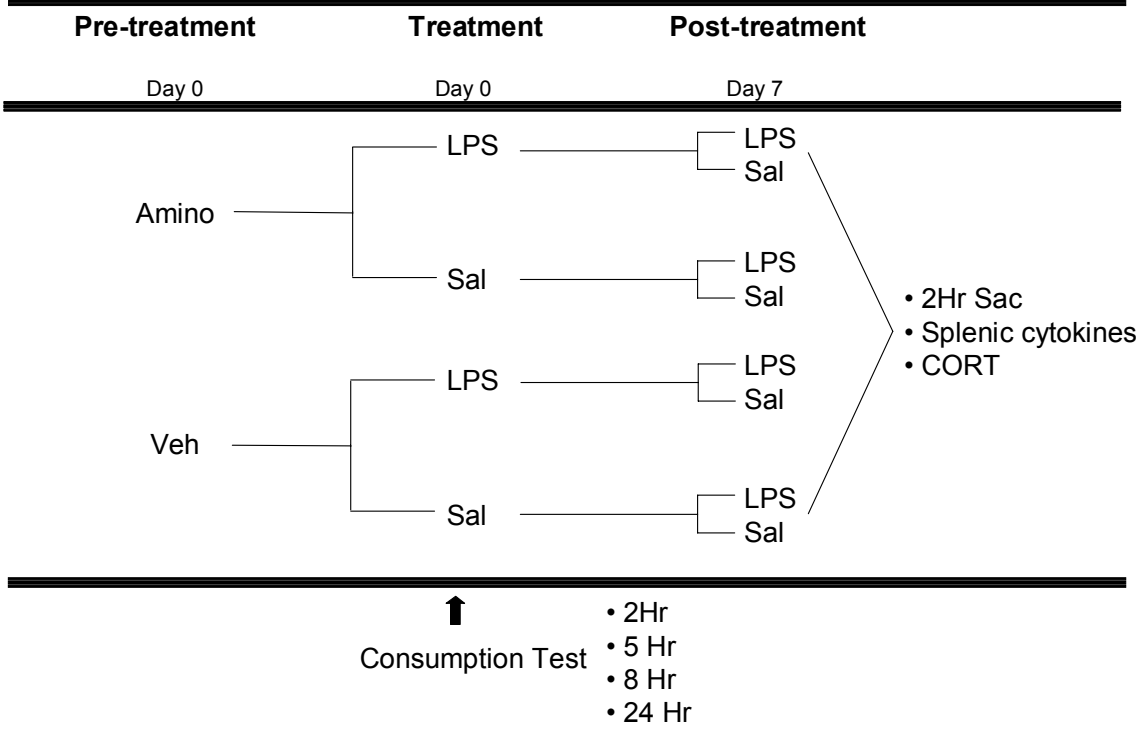
Table 14
Summary of Experimental Design for Experiment 5.7

Pre-treatment	Treatment	Post-treatment
Day 0	Day 0	Day 4

RU-486   LPS ──────┐ LPS
                         └ Sal

              Sal   • 2Hr Sac
                                       • Splenic cytokines
                                       • CORT

Veh   LPS ──────┐ LPS
                         └ Sal

              Sal

⬆
Consumption Test
• 2Hr
• 5 Hr
• 8 Hr
• 24 Hr
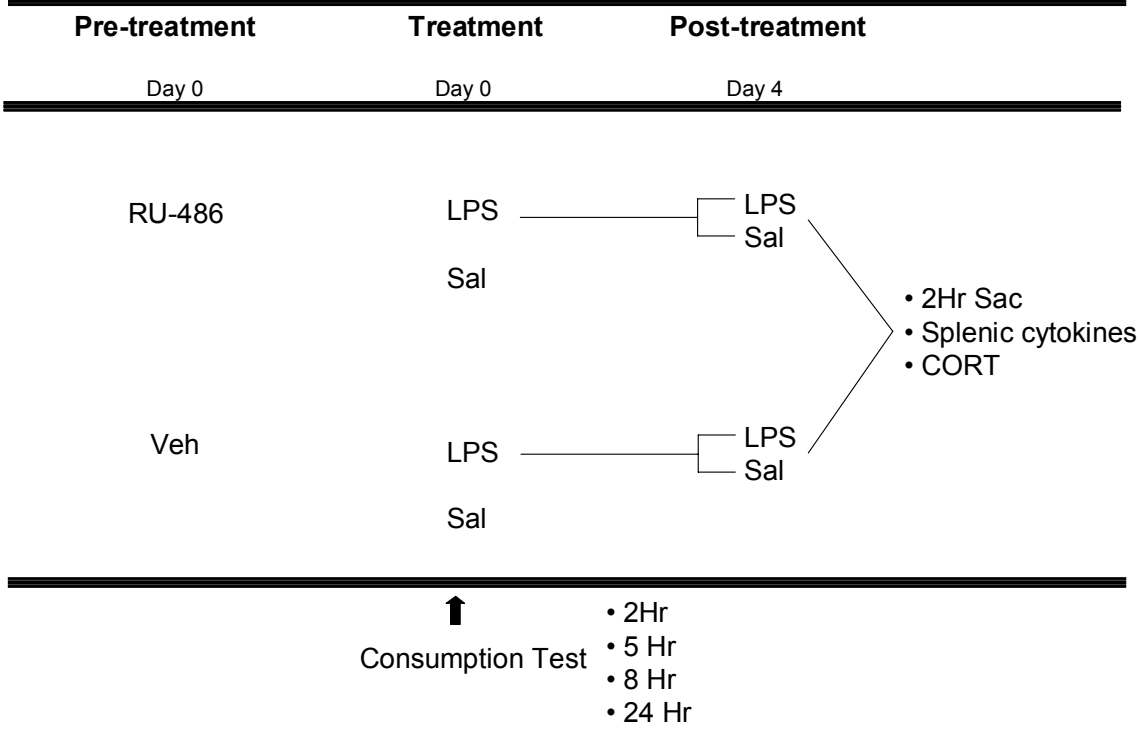
Acute and Repeated SEA: Splenic Cytokines
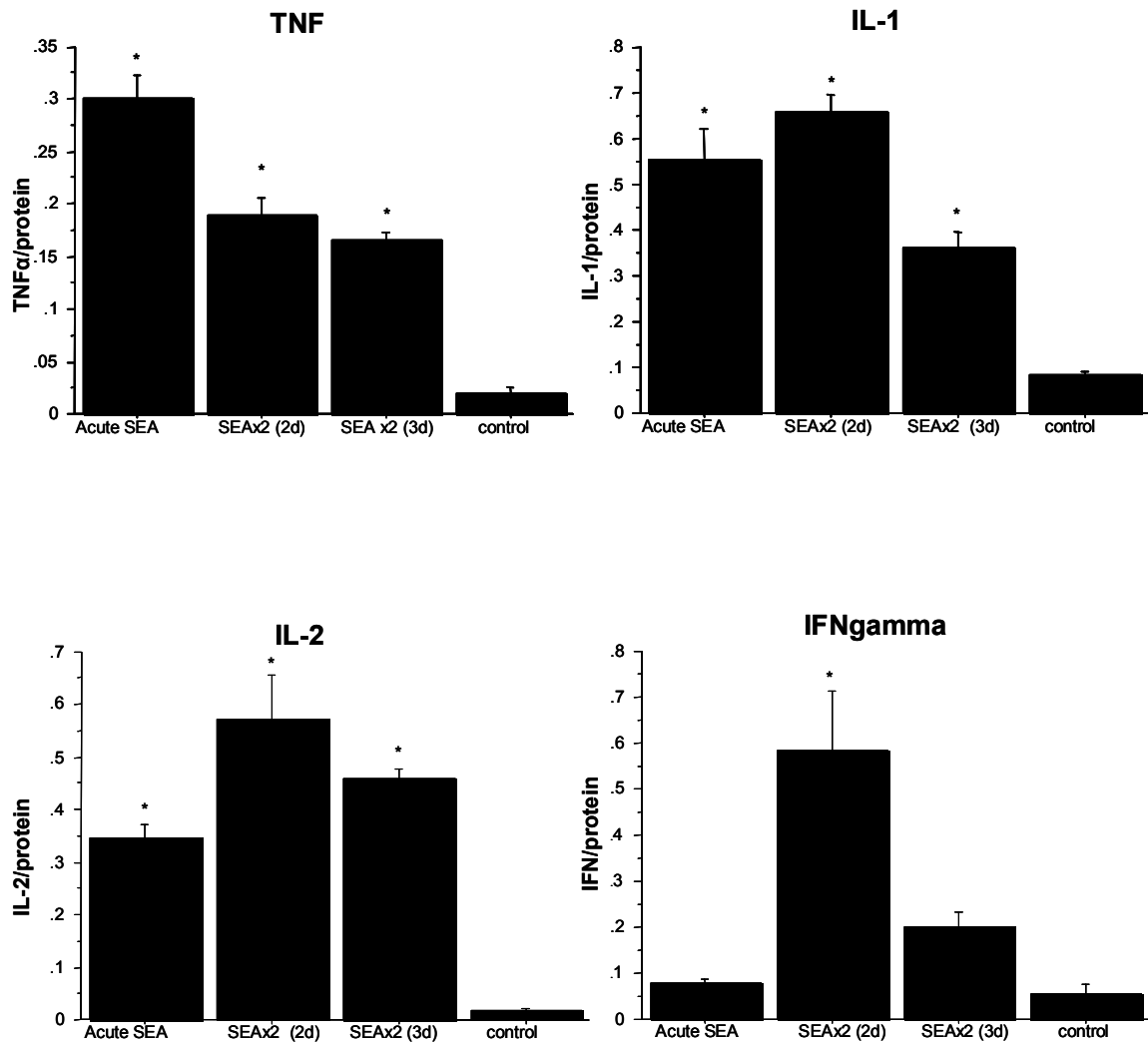
Figure 3.1. Mean splenic TNFα, IL-1β, IL-2 and IFNγ concentrations (+/-SE) after acute SEA, two injections of SEA spaced two days apart (SEAx2 (2d)) or three days apart (SEAx2 (3d)) and the control. Data is expressed as pg cytokine/μg protein. N = 5-11/group; $p<.05$ compared to control. Error bars represent standard error of the mean.

Acute and Repeated SEA: Corticosterone
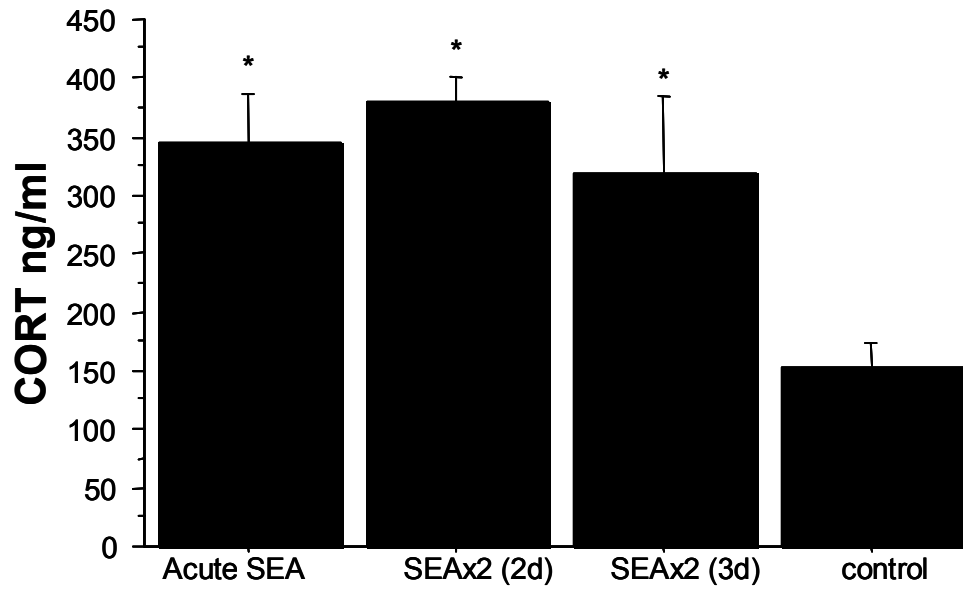

Figure 3.2. Mean plasma corticosterone levels after acute SEA, two injections of SEA spaced two days apart (SEAx2 (2d)), three days apart (SEAx2 (3d)), and the control. N = 5-11/group; *p*<.05 compared to control. Error bars represent standard error of the mean.

Acute and Repeated SEA: Total Consumed
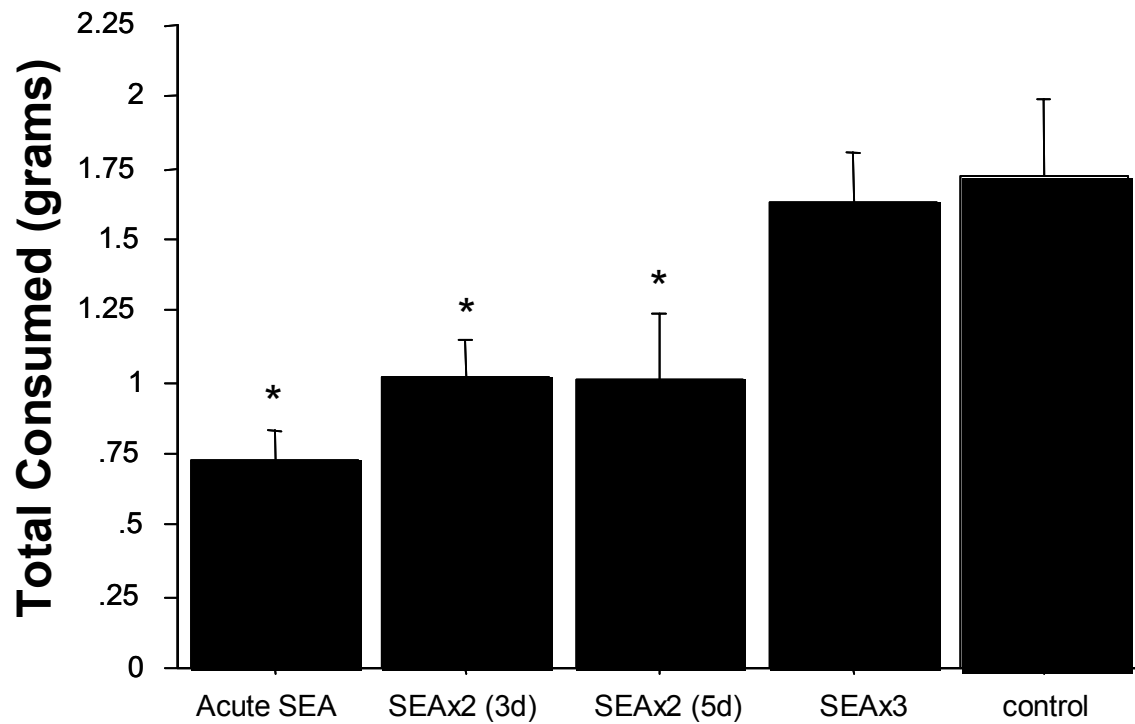
Figure 3.3. Consumption of a novel liquid diet (Prosobee) after acute SEA, two injections of SEA spaced three (SEAx2 (3d)), five days apart (SEAx2 (5d)), three injections of SEA (SEAx3), and the control. N = 8/group; $p<.05$ compared to control. Error bars represent standard error of the mean.

Acute and Repeated SEA: Corticosterone
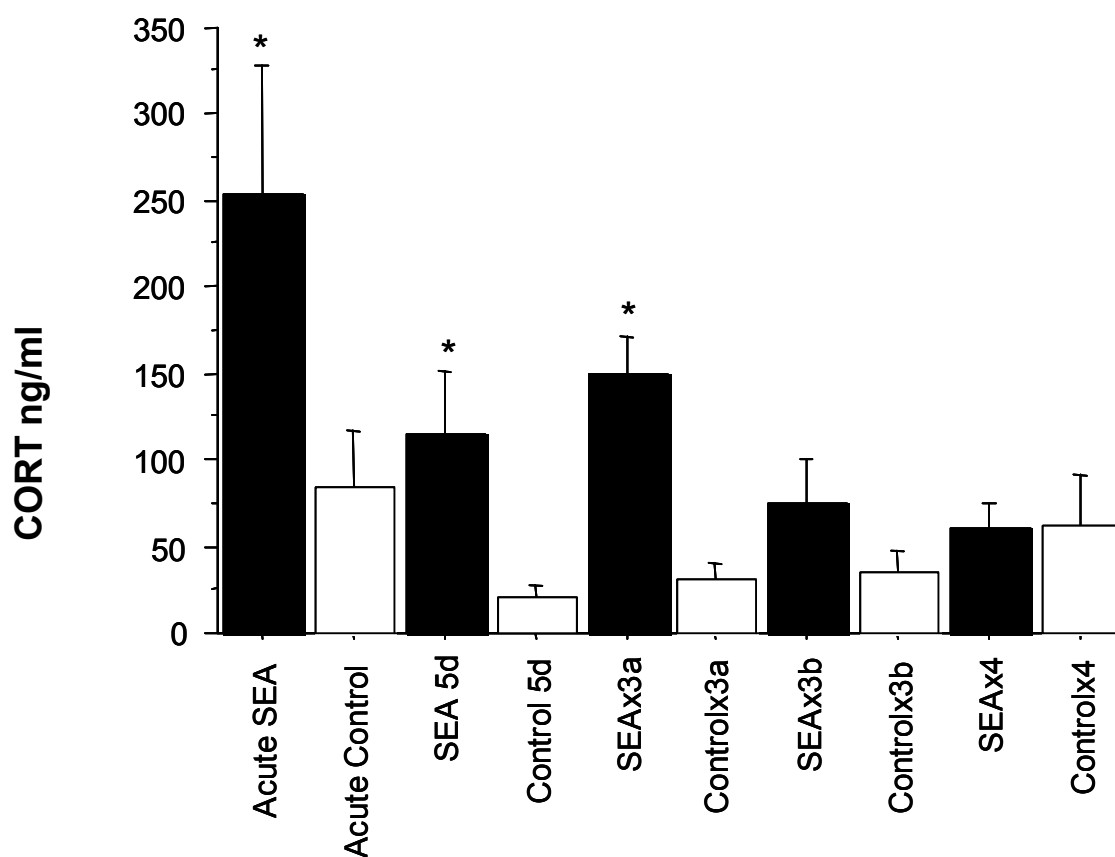
Figure 3.4. Mean plasma corticosterone levels after acute SEA, two injections of SEA spaced five days apart (SEA 5d), three injections of SEA given three and then five days apart (SEAx3a), three injections of SEA given every five days (SEAx3b), and four injections of SEA (SEAx4). See Table 6 for control group designation. N = 4/group; $p<.05$ compared to corresponding control. Error bars represent standard error of the mean.

Acute and Repeated SEA: Splenic Cytokines
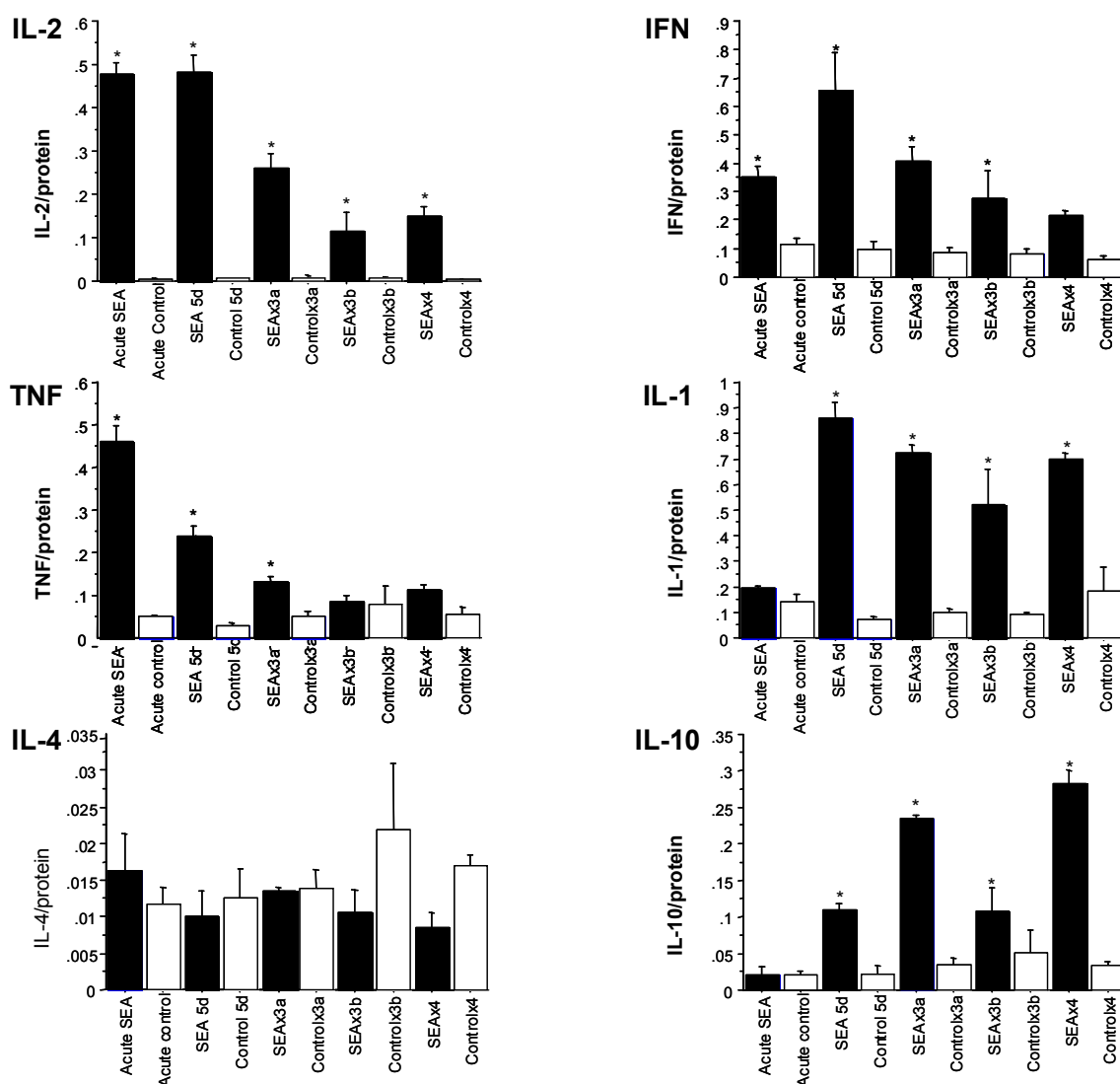
Figure 3.5. Mean splenic TNFα, IL-1β, IL-2 , IFNγ, IL-10 and IL-4 concentrations after acute SEA, two injections of SEA spaced five days apart (SEA 5d), three injections of SEA given three and then five days apart (SEAx3a), three injections of SEA given every five days (SEAx3b), and four injections of SEA (SEAx4). See Table 6 for control group designation. Data is expressed as pg cytokine/μg protein. N = 4/group; *p*<.05 compared to corresponding control. Error bars represent standard error of the mean.

Acute and Repeated SEA: Plasma Cytokines
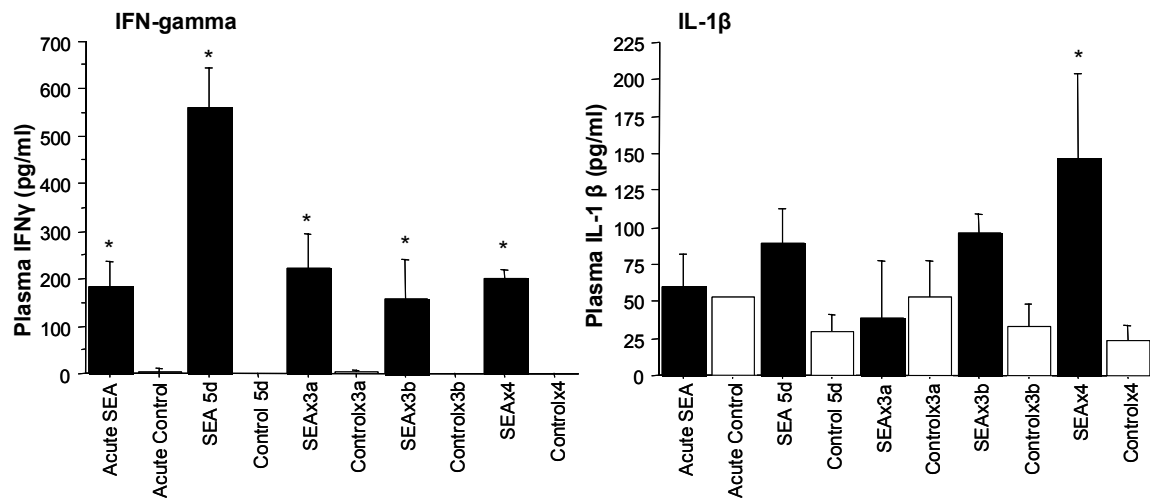
Figure 3.6. Mean plasma IFNγ and IL-1β after acute SEA, two injections of SEA spaced five days apart (SEA 5d), three injections of SEA given three and then five days apart (SEAx3a), three injections of SEA given every five days (SEAx3b), and four injections of SEA (SEAx4). See Table 6 for control group designation. N = 4/group; $p<.05$ compared to corresponding control. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males: Corticosterone
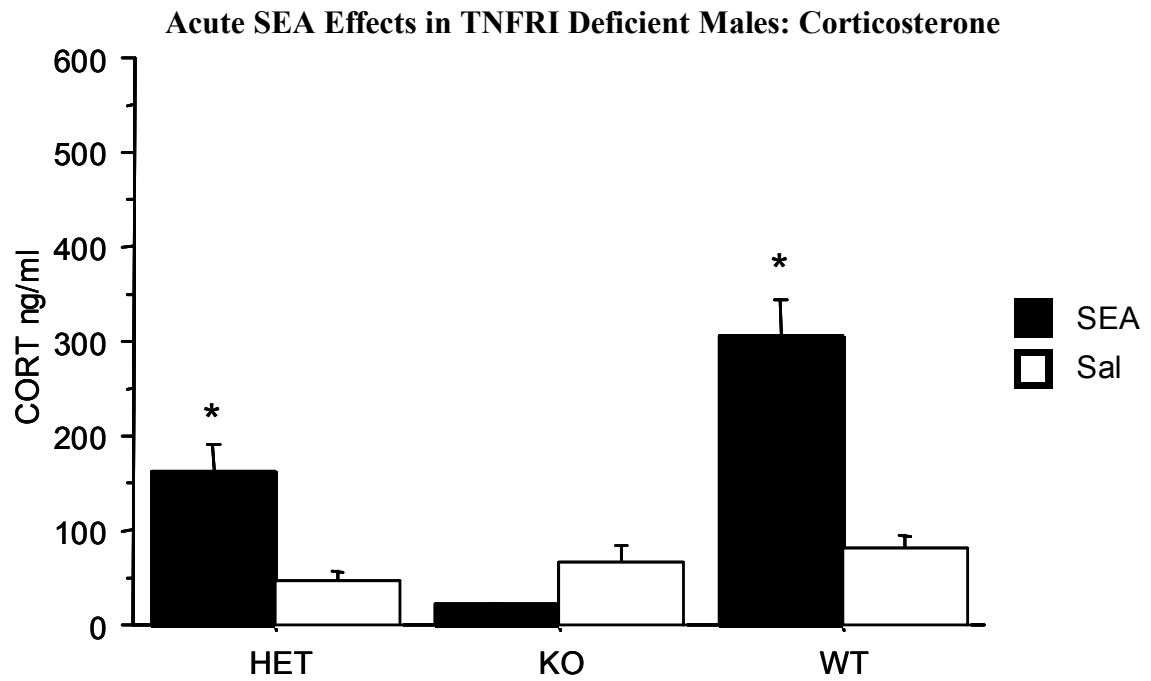
Figure 4.1. Mean plasma corticosterone in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. N = 10/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males: Splenic TNFα
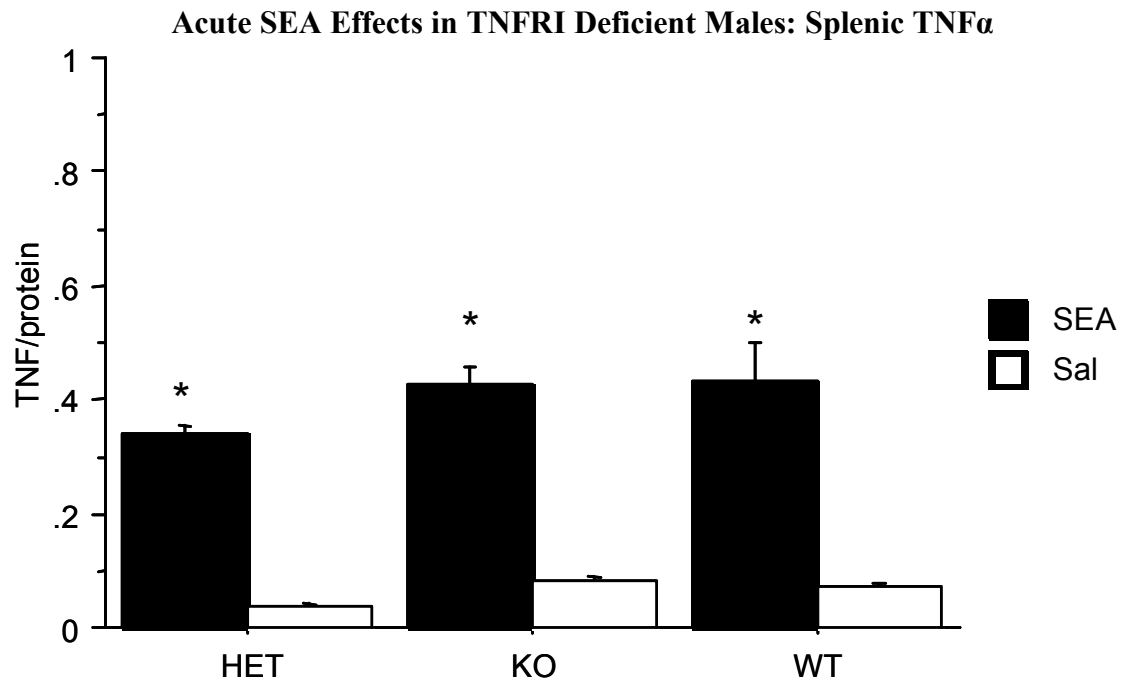
Figure 4.2. Mean spenic TNFα in male TNFRI^{+/-} (HET), TNFRI^{-/-} (KO), and TNFRI^{+/+} (WT) animals after treatment with SEA or Saline. Data is expressed as pg cytokine/μg protein. N = 10/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males: Total Consumed
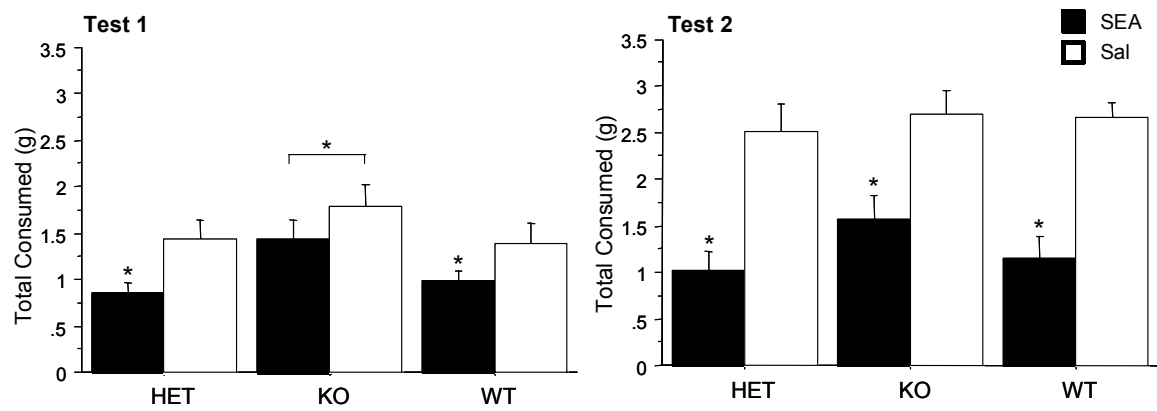
Figure 4.3. Mean consumption in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. N = 10-14/group; $p<.05$ compared to corresponding saline controls or compared to WT animals. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males: Hypothalamic CRH mRNA
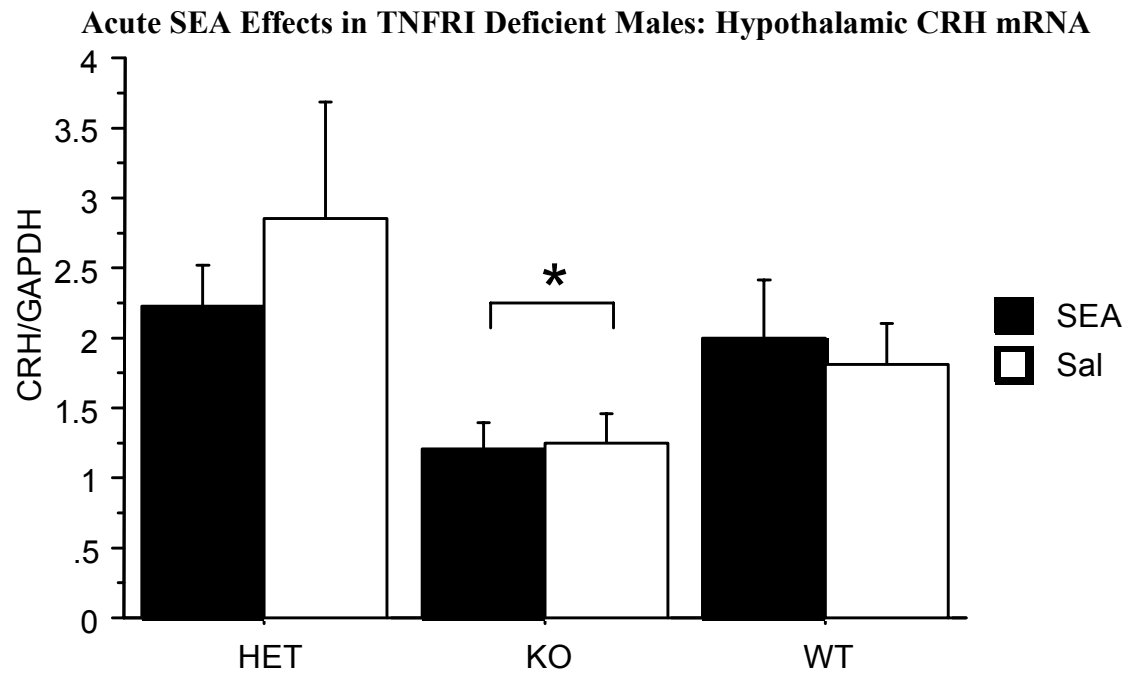
Figure 4.4: Mean CRH mRNA in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. N = 10/group; $p<.05$ compared to WT controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males: Hypothalamic TNFRII mRNA
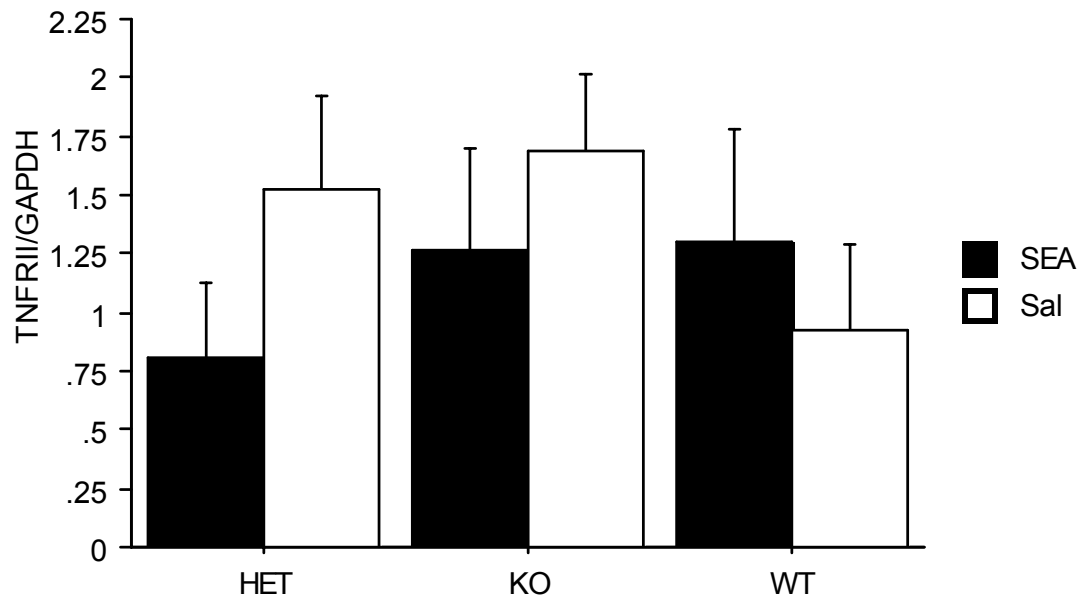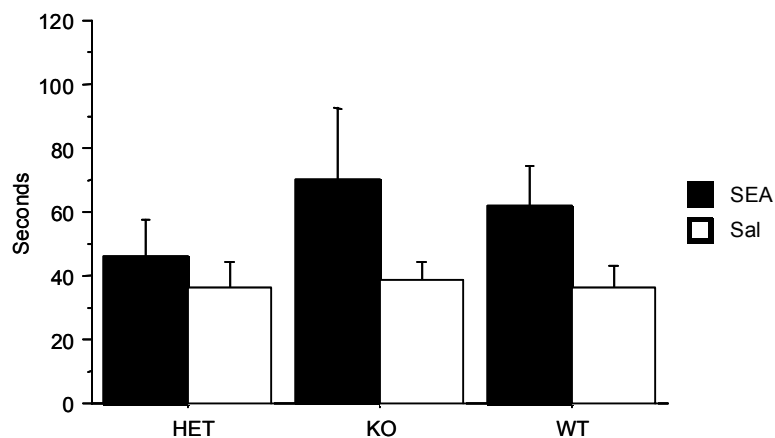

Figure 4.5: Mean TNFRII mRNA in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. N = 10/group. Error bars represent standard error of the mean.

Acute SEA Effects in Wildtype Males: Hypothalamic TNFRI mRNA
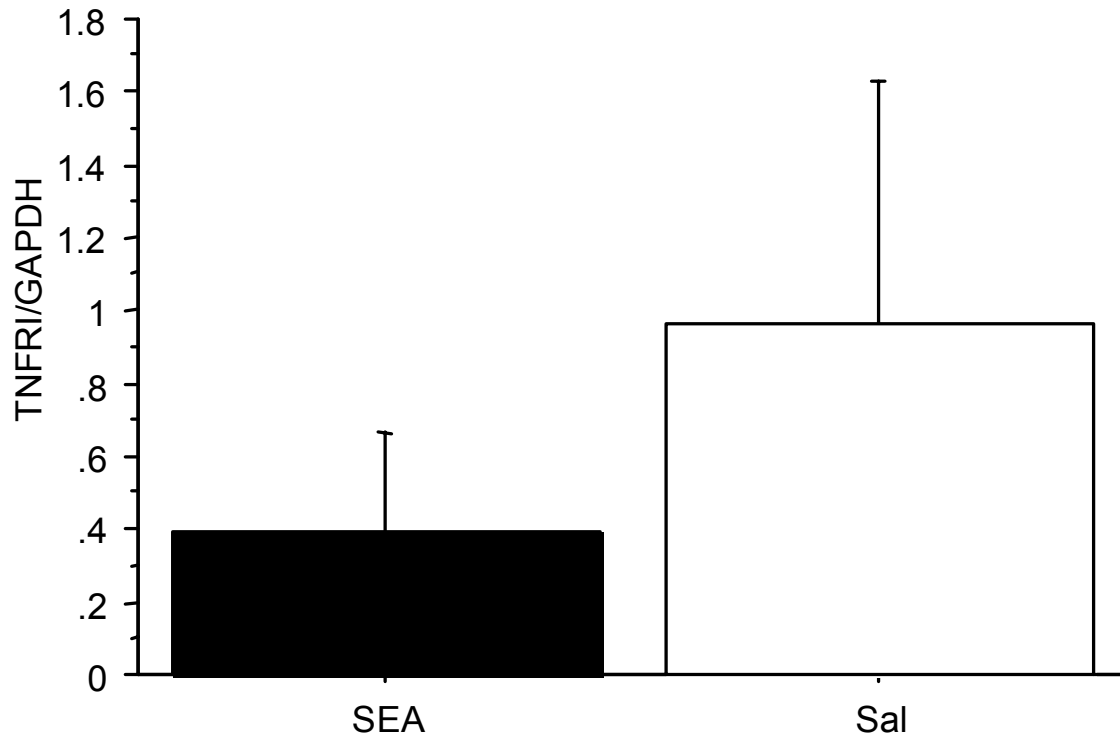
Figure 4.6. Mean TNFRI mRNA in male TNFRI$^{+/+}$ animals treated with SEA or Saline. N = 10/group. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Females: Corticosterone
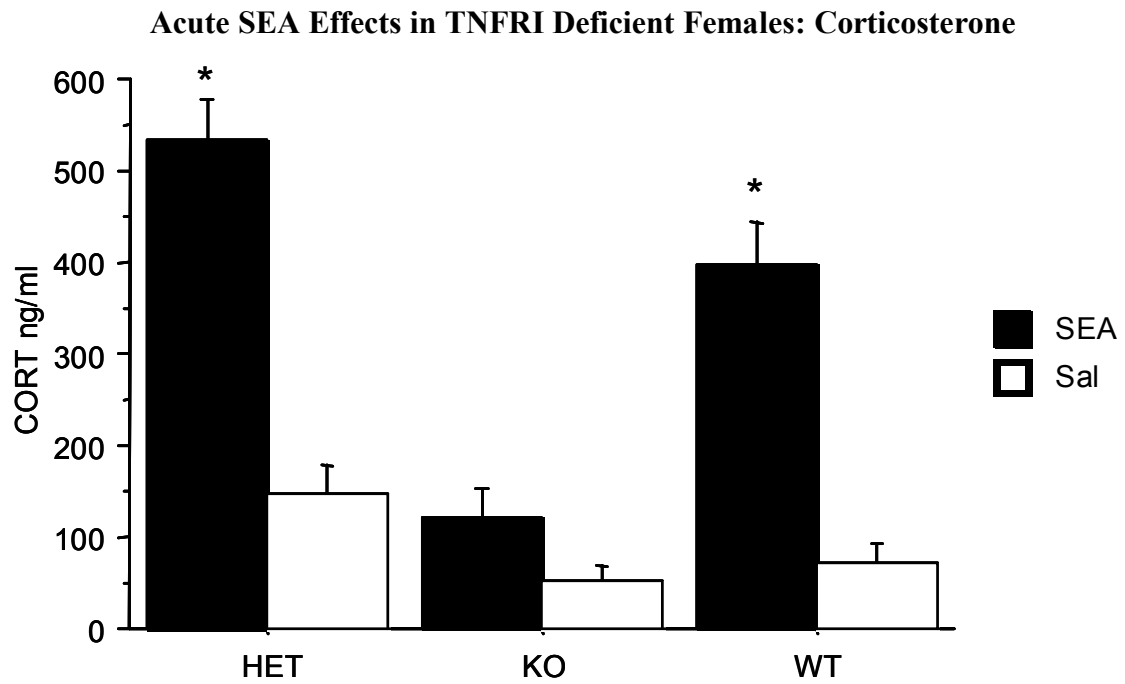
Figure 4.7. Mean plasma CORT in female TNFRI $^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. N = 7-8/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Females: Splenic TNFα
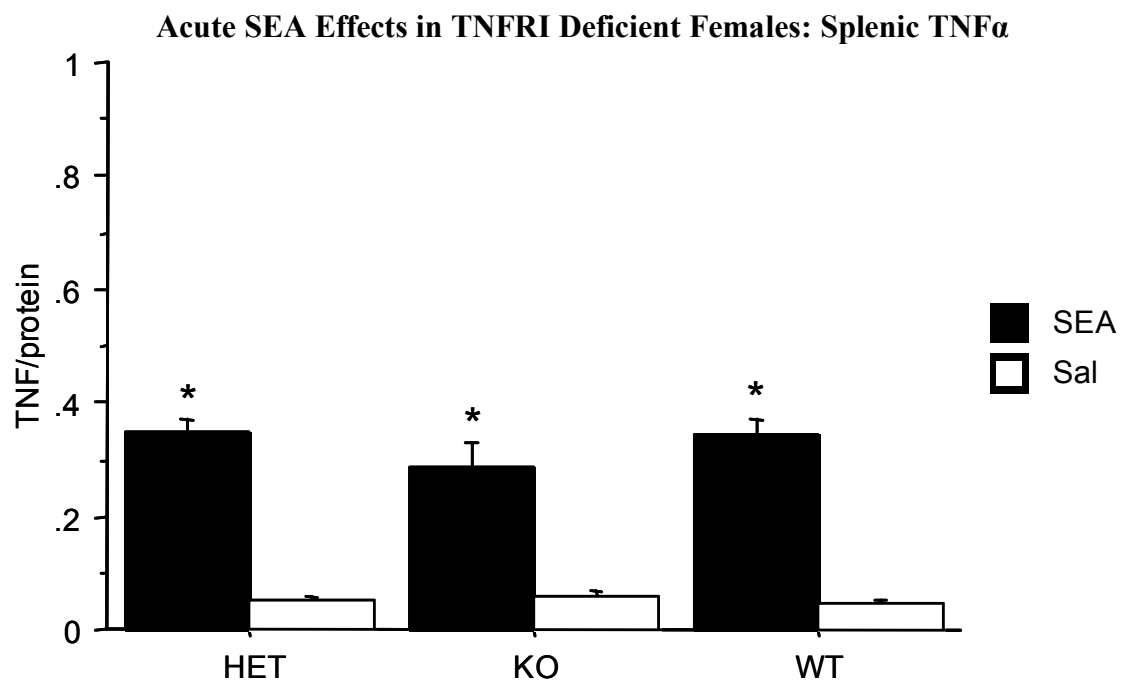
Figure 4.8. Mean splenic TNFα in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. Data is expressed as pg cytokine/μg protein. N = 7-8/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.
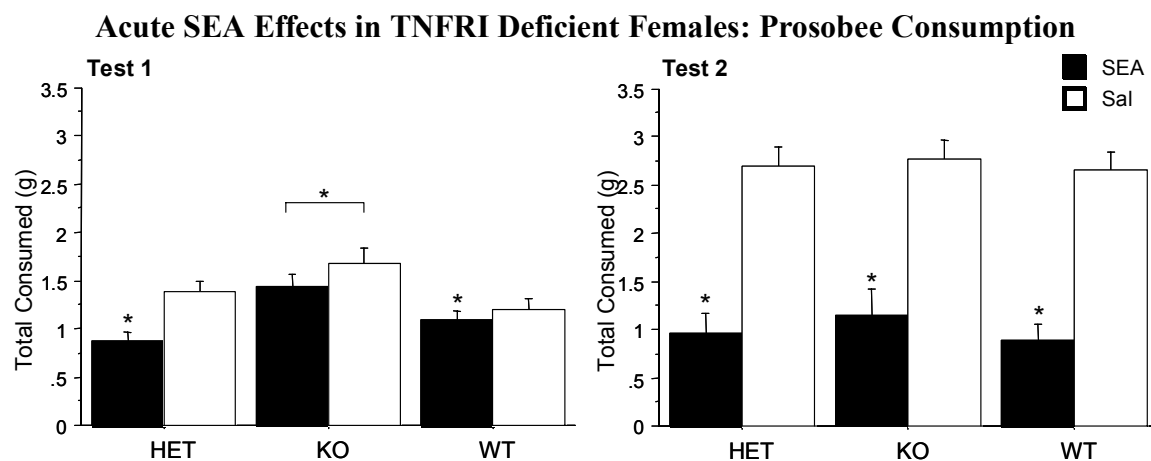
Figure 4.9. Mean consumption in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or Saline. N = 10-15/group; $p<.05$ compared to corresponding saline controls or WT controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRII Deficient Males: Corticosterone
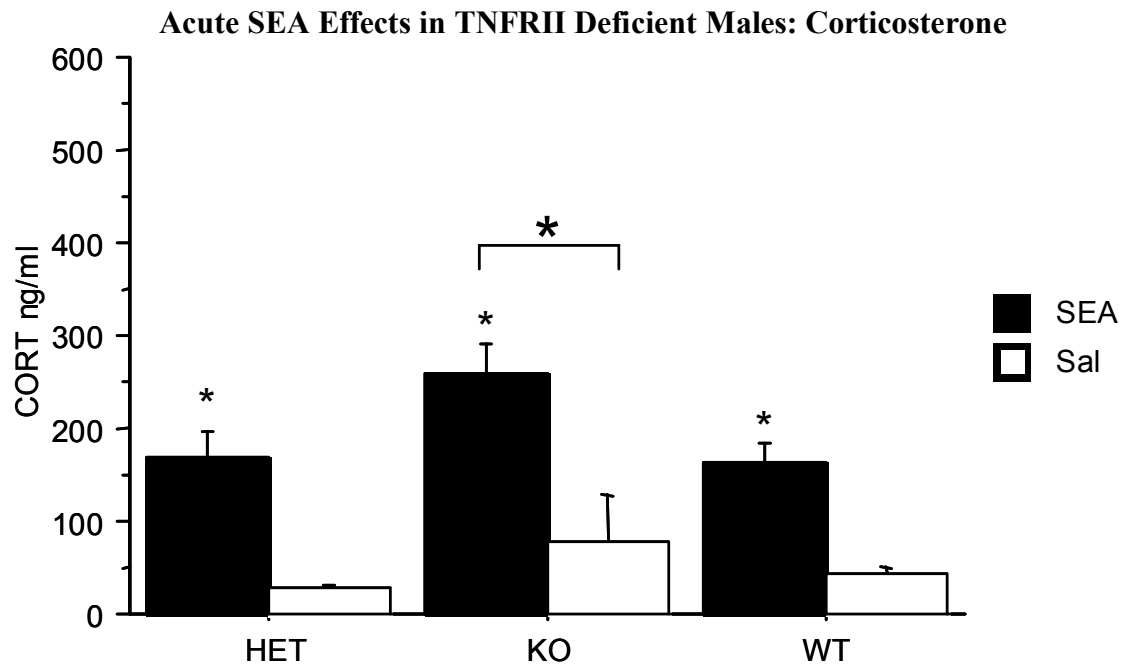
Figure 4.10. Mean plasma CORT in male TNFRII^{+/-} (HET), TNFRII^{-/-} (KO), and TNFRII^{+/+} (WT) animals treated with SEA and saline. N = 8-9/group; *p*<.05 compared to corresponding saline controls or WT controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRII Deficient Males: Splenic TNFα
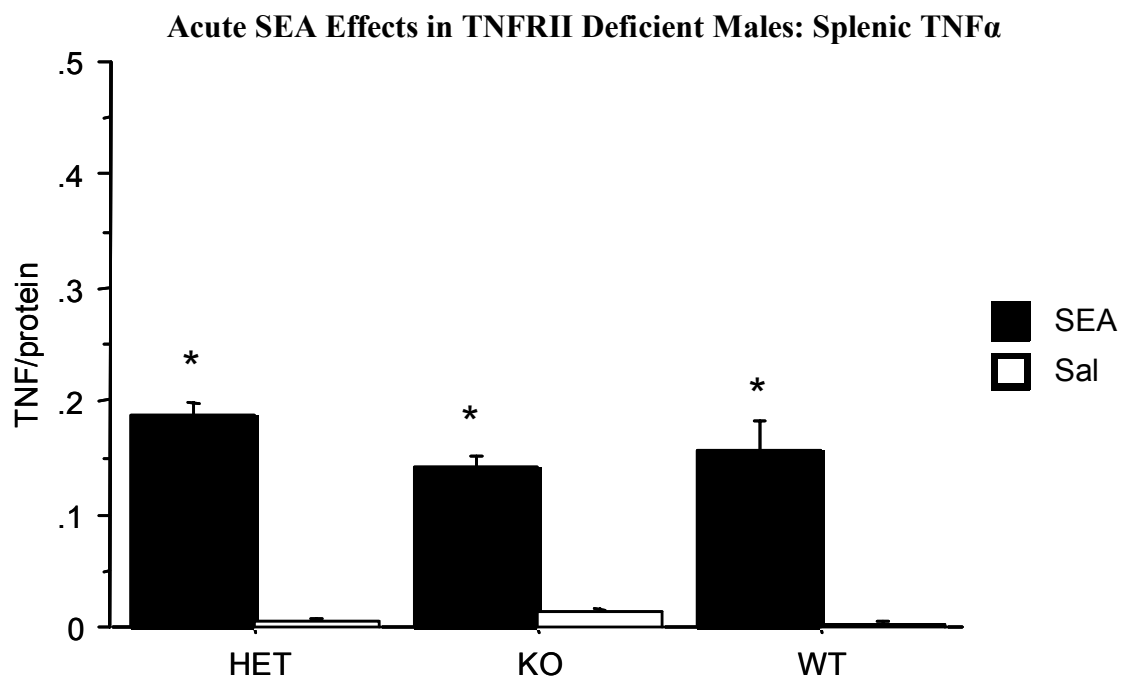
Figure 4.11. Mean splenic TNFα in TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA and saline. Data is expressed as pg cytokine/µg protein. N = 8-9/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRII Deficient Males: Prosobee Consumption
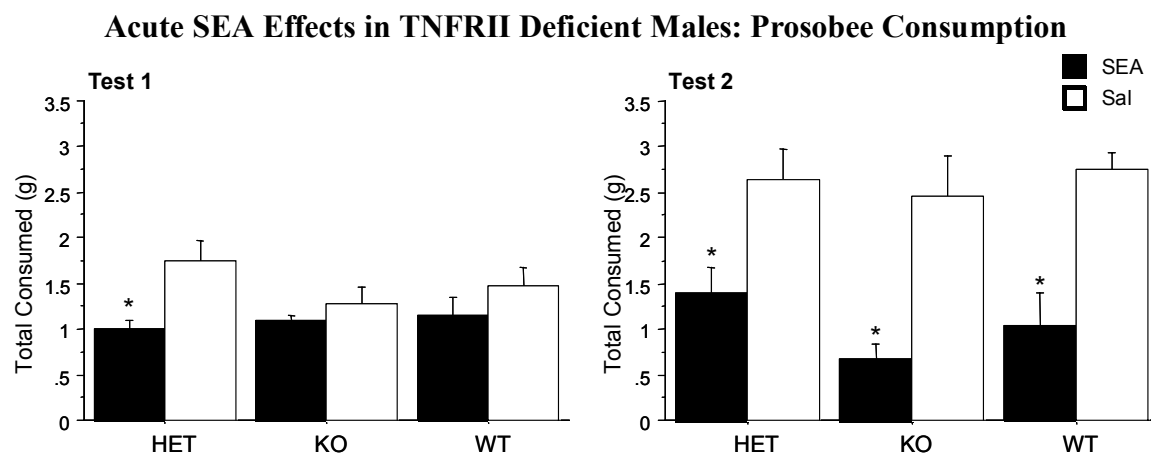
Figure 4.12. Mean consumption in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA and saline. N = 6-12/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRII Deficient Females: Corticosterone
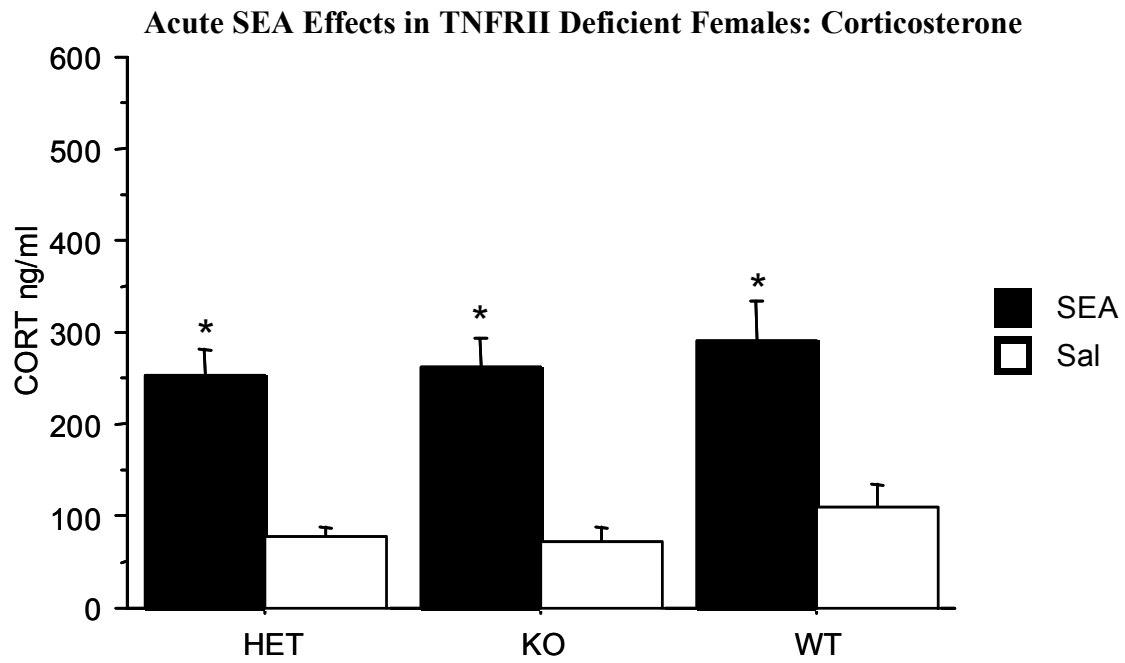
Figure 4.13. Mean plasma CORT in female TNFRII^{+/-} (HET), TNFRII^{-/-} (KO), and TNFRII^{+/+} (WT) animals treated with SEA or saline. N = 7-9/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRII Deficient Females: Splenic TNFα
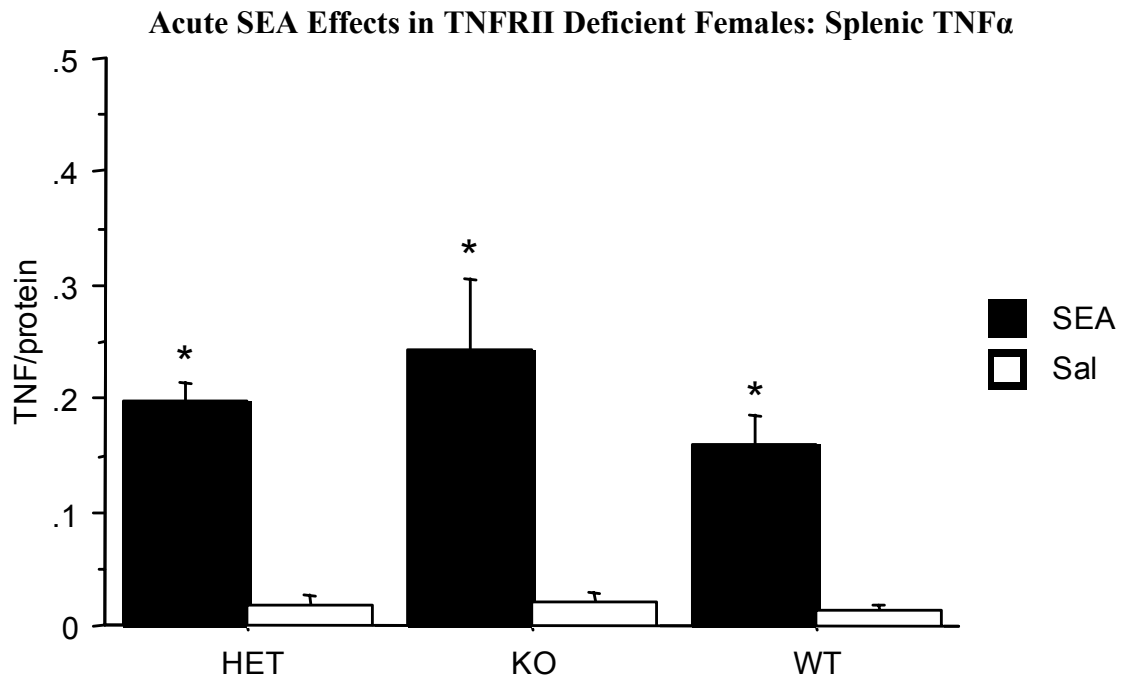
Figure 4.14. Mean splenic TNFα in female TNFRII^{+/-} (HET), TNFRII^{-/-} (KO), and TNFRII^{+/+} (WT) animals treated with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 7-9/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRII Deficient Females: Prosobee Consumption
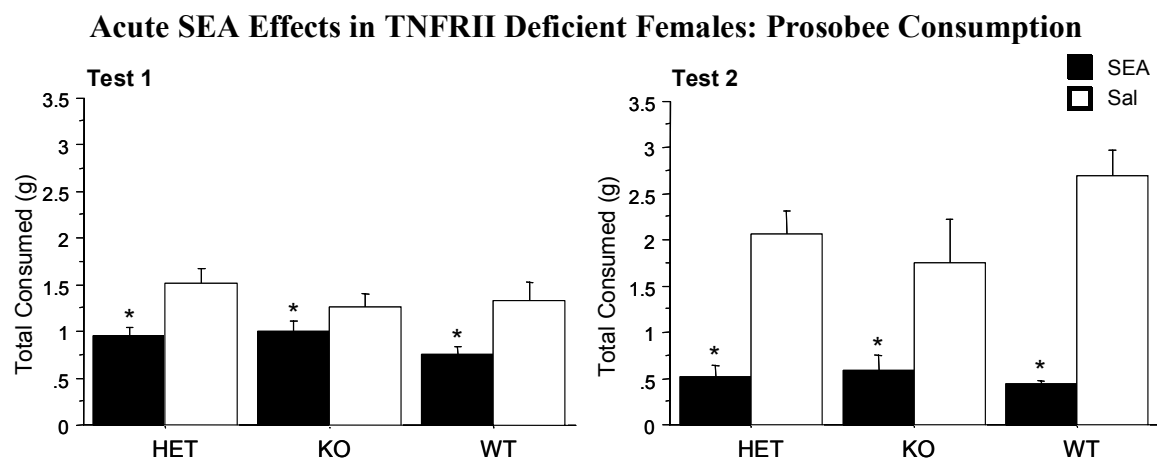
Figure 4.15. Mean total consumption in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 6-12/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males:
Corticosterone at 4 Hours Post-Injection
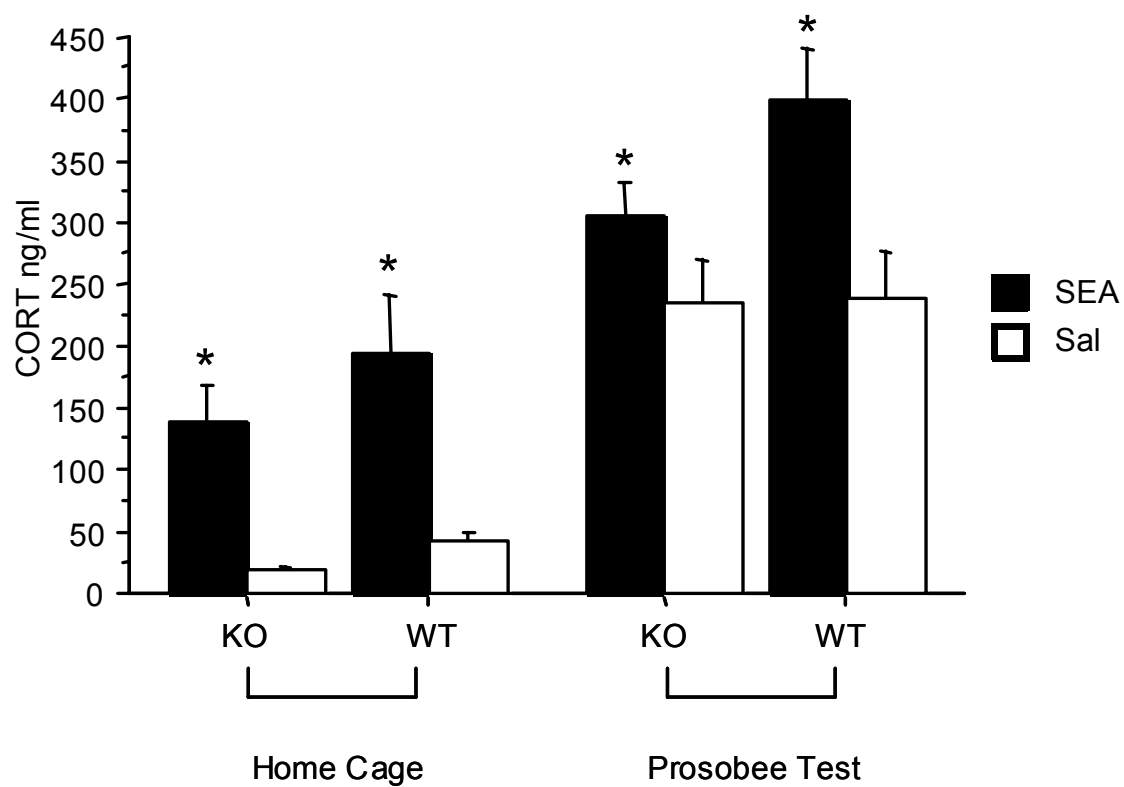
Figure 4.16. Mean plasma CORT in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 5-7/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.
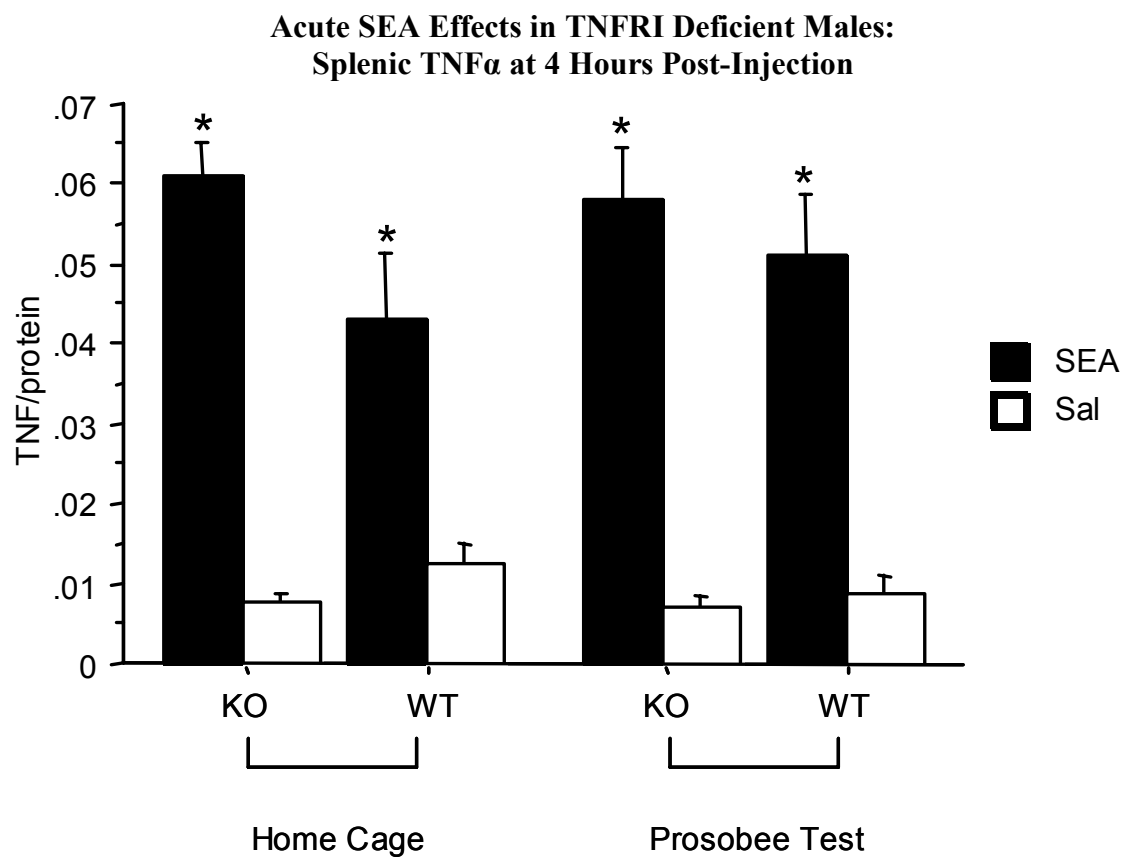
Figure 4.17. Mean splenic TNFα in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. Data is expressed as pg cytokine/µg protein. N = 5-7/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean

Acute SEA Effects in TNFRI Deficient Males:
Splenic IL-2 at 4 Hours Post-Injection
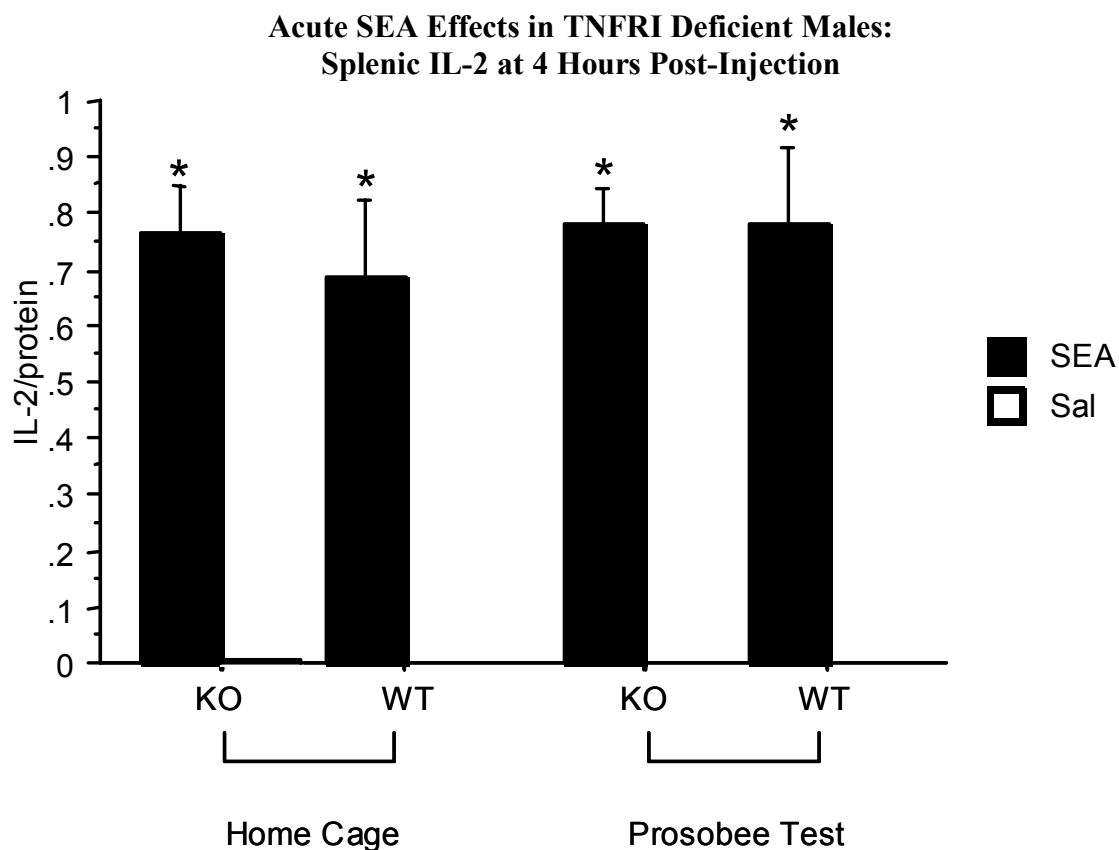
Figure 4.18. Mean splenic TNFα in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 5-7/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean

Acute SEA Effects in TNFRI Deficient Males:
Hypothalamic mRNA at 4 Hours Post-Injection

CRH
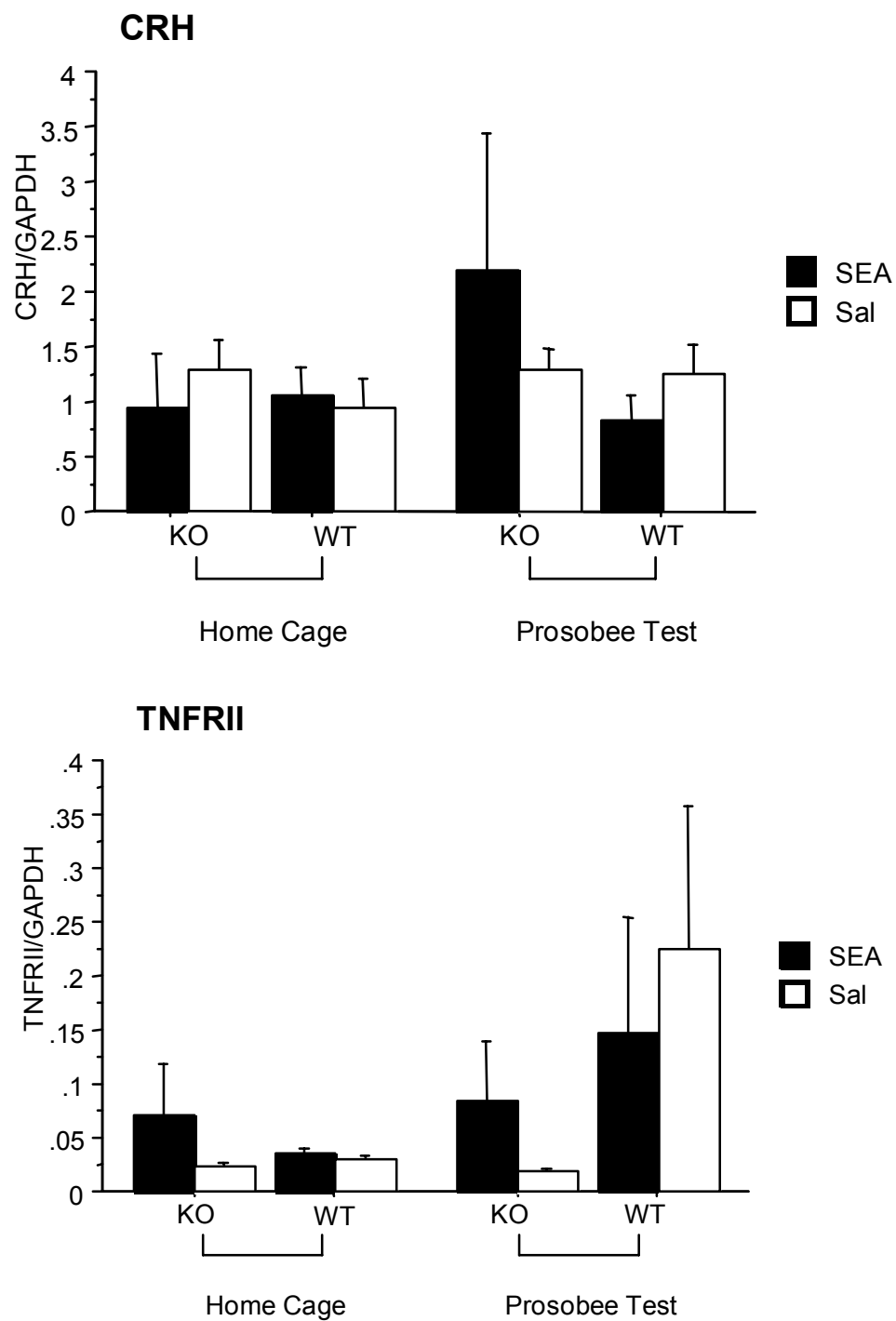
TNFRII

Figure 4.19. Mean hypothalamic CRH and TNFRII mRNA in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 5-7/group. Error bars represent standard error of the mean.

Acute SEA Effects in Wildtype Males:
Hypothalamic TNFRI mRNA at 4 Hours Post-Injection
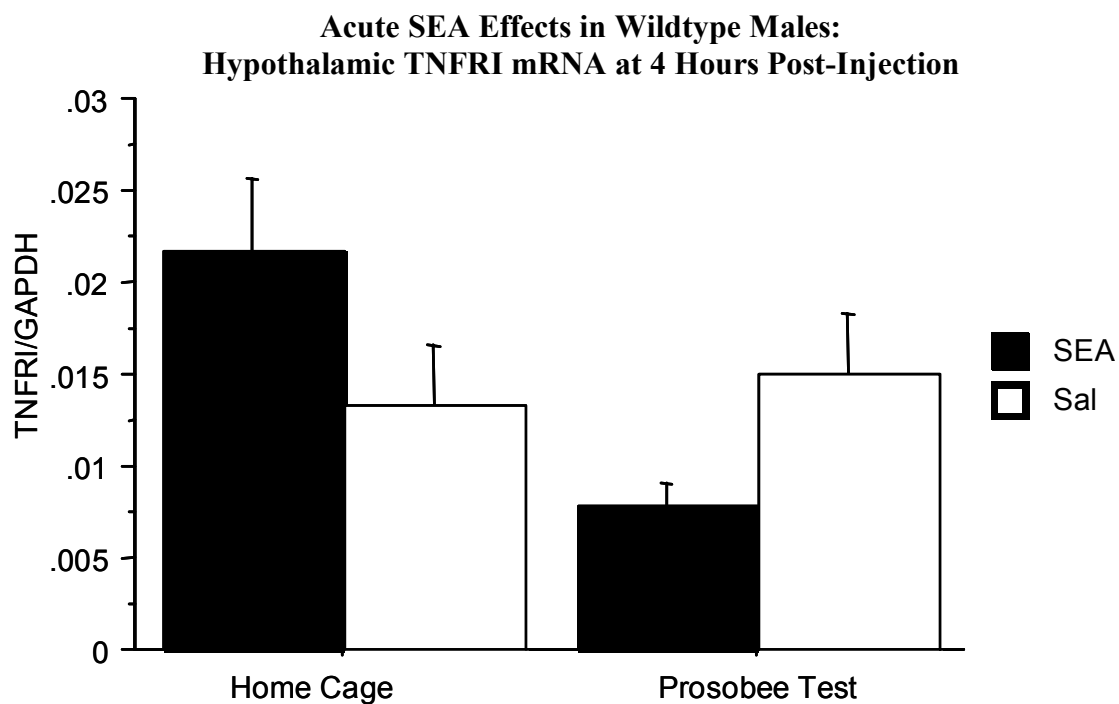
Figure 4.20. Mean hypothalamic TNFRI mRNA in male TNFRI^{+/+} animals treated with SEA or saline. N = 5-7/group. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males:
Corticosterone at 4 and 5 hours post-injection during Prosobee Testing
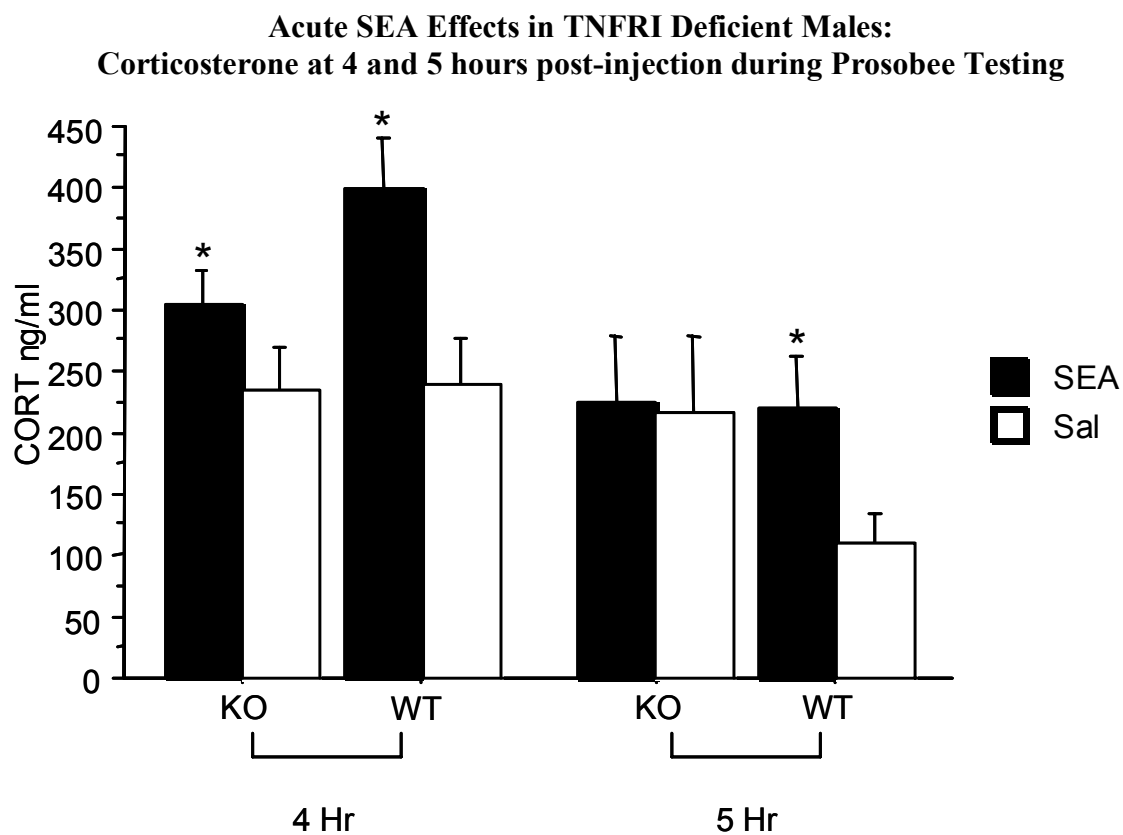
Figure 4.21. Mean plasma CORT in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 5-7/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

**Acute SEA Effects in TNFRI Deficient Males:
Splenic TNFα at 4 and 5 hours post-injection during Prosobee Testing**
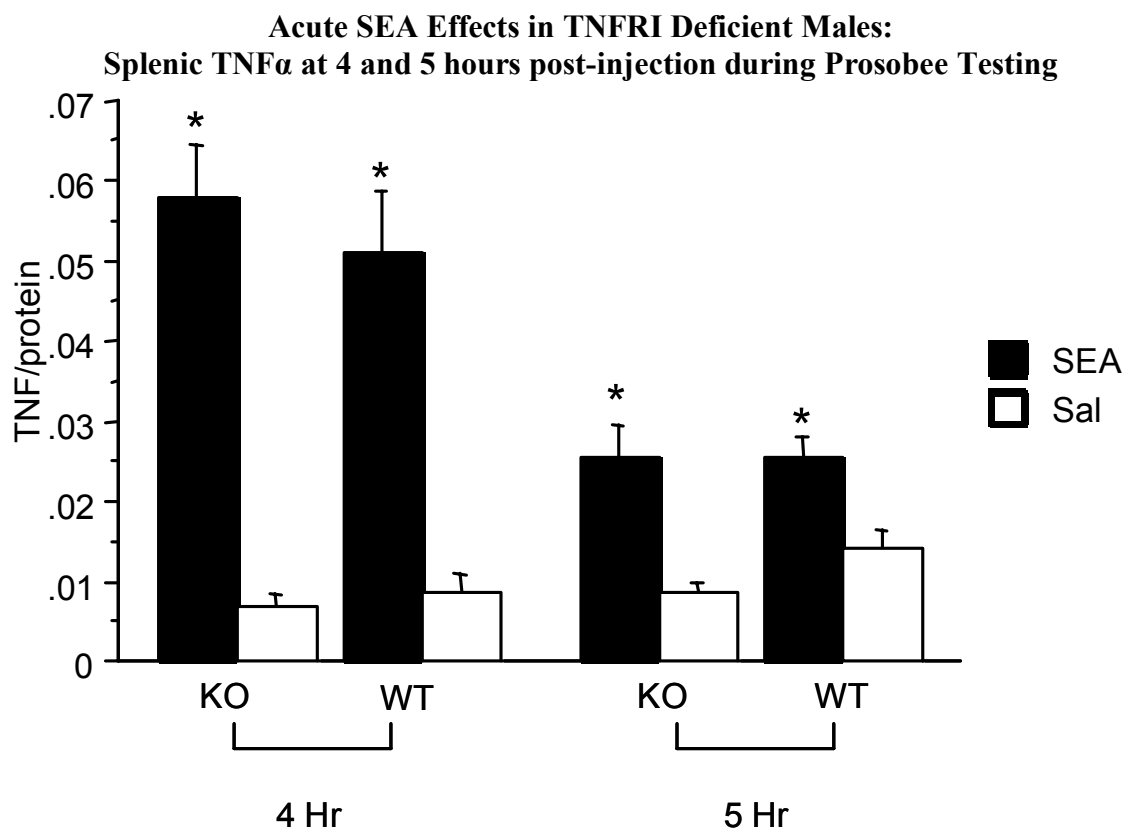
Figure 4.22. Mean splenic TNFα in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. Data is expressed as pg cytokine/µg protein. N = 5-7/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.
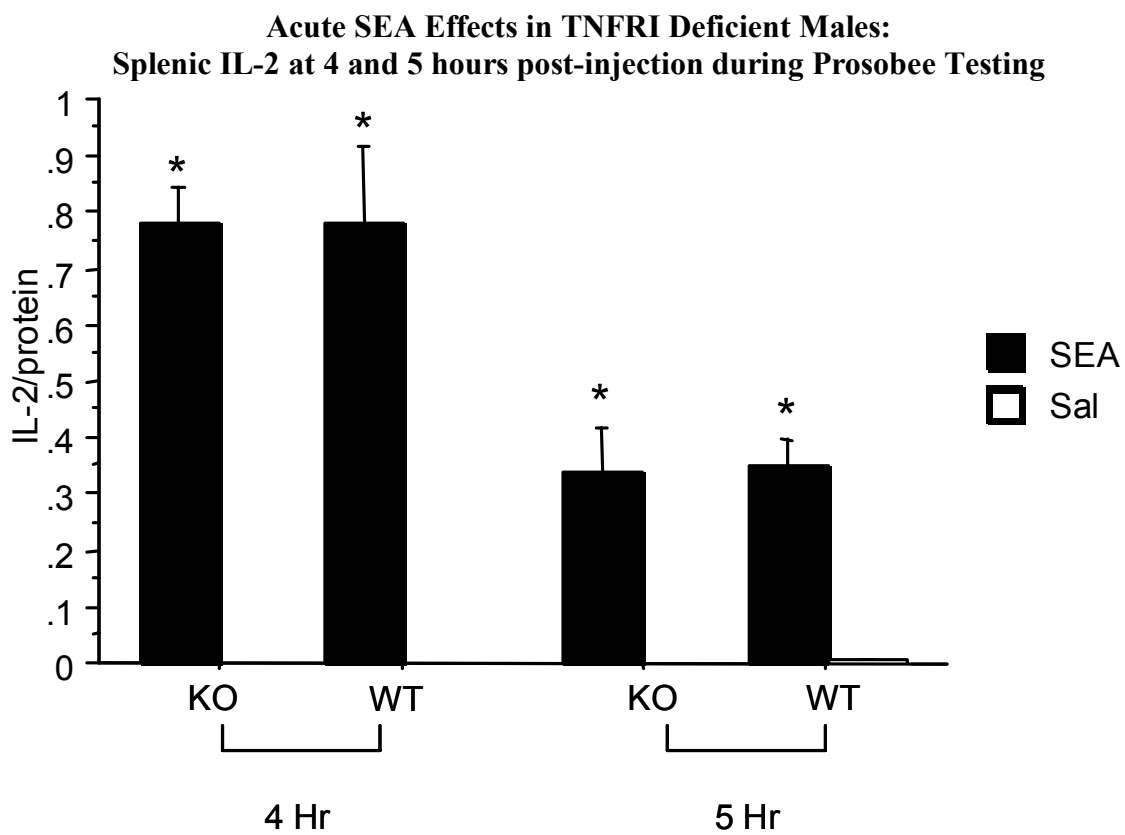
Figure 4.23. Mean splenic TNFα in male TNFRI^{-/-} (KO) and TNFRI^{+/+} (WT) animals treated with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 5-7/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

Acute SEA Effects in TNFRI Deficient Males:
Hypothalamic mRNA at 4 and 5 hours post-injection during Prosobee Testing
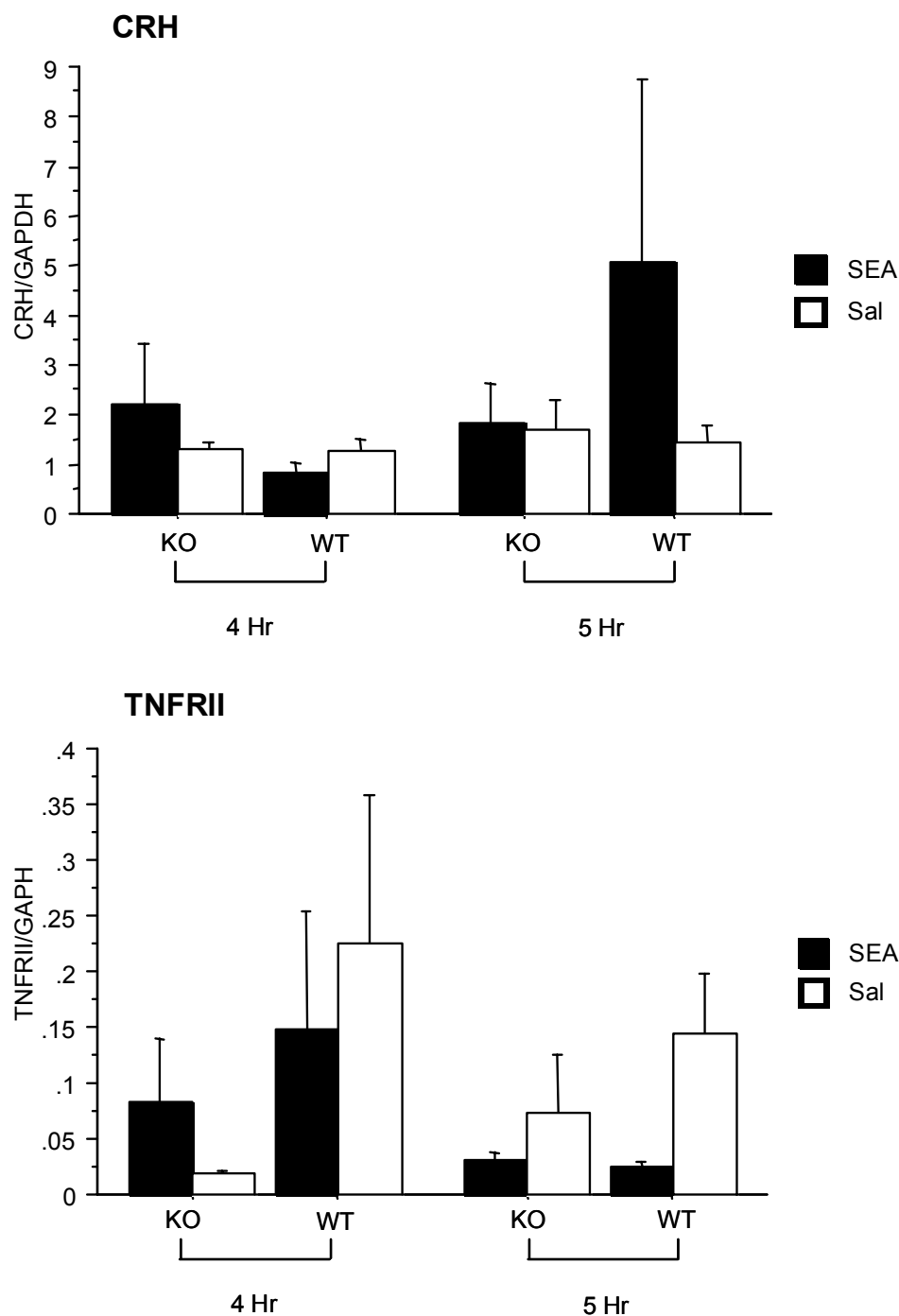
Figure 4.24. Mean hypothalamic CRH and TNFRII mRNA in male TNFRI$^{-/-}$ (KO) and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 5-7/group. Error bars represent standard error of the mean.

Acute SEA Effects in Wildtype Males:
Hypothalamic TNFRI mRNA at 4 and 5 hours
post-injection during prosobee testing
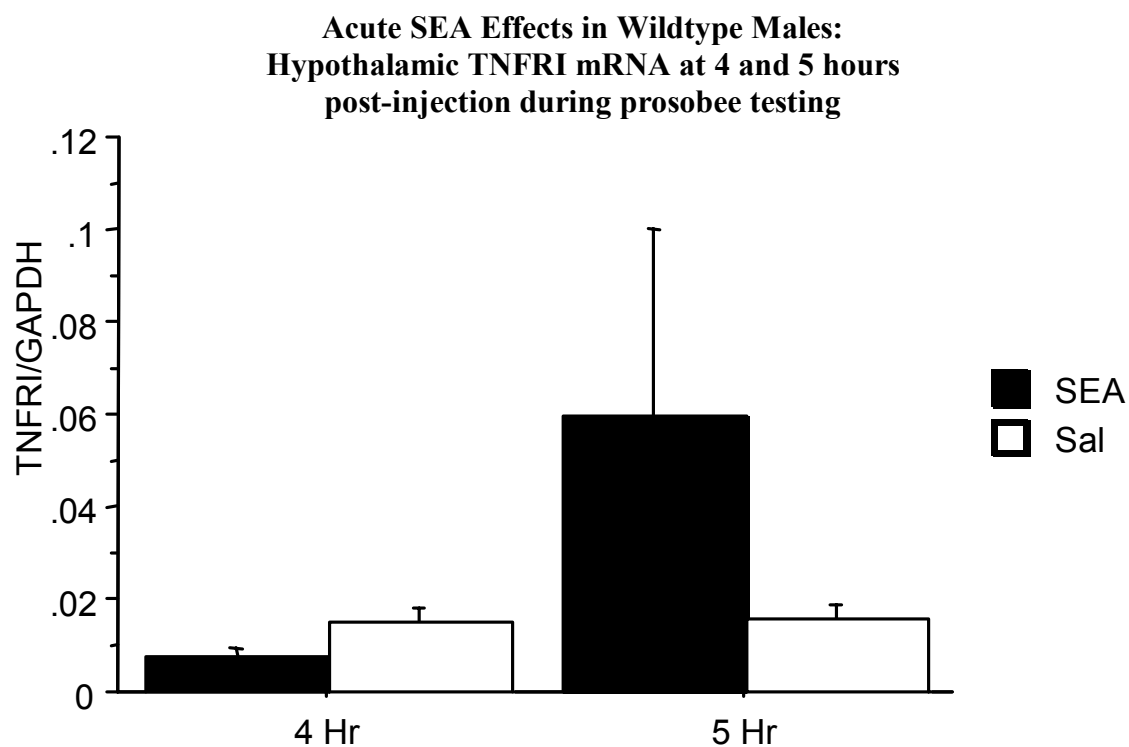
Figure 4.25. Mean hypothalamic TNFRI mRNA in TNFRI^{+/+} animals treated with SEA or saline. N = 5-7/group. Error bars represent standard error of the mean.

Figure 5.1

SEA Effects in TNFRI Deficient Males:
Locomotor Activity in the OF/NO Apparatus
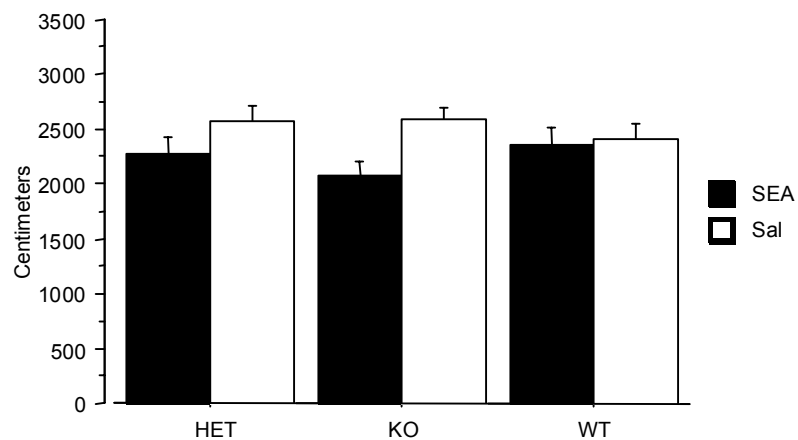
a. Total distance

b. Total number of entries
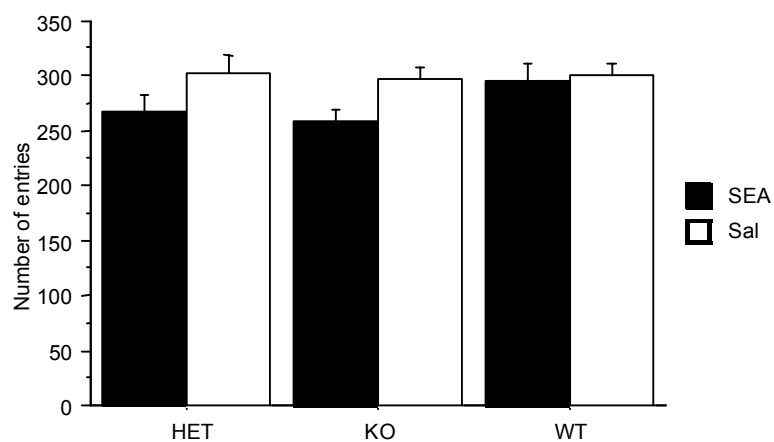
c. Total number of entries
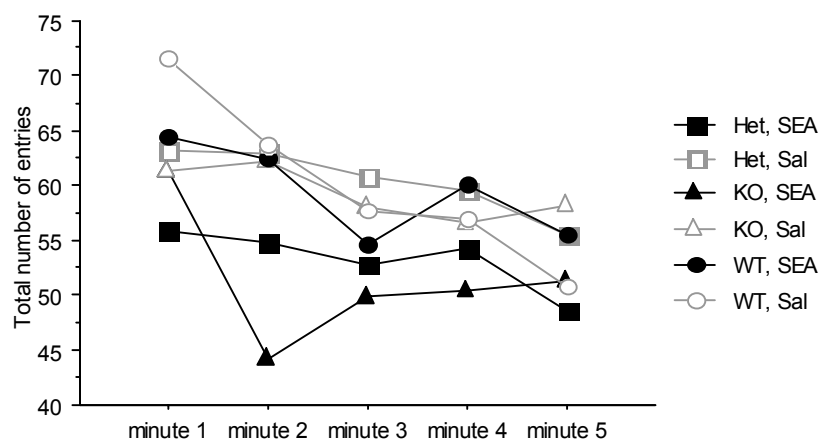
Figure 5.1. a. Mean total distance traveled in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; 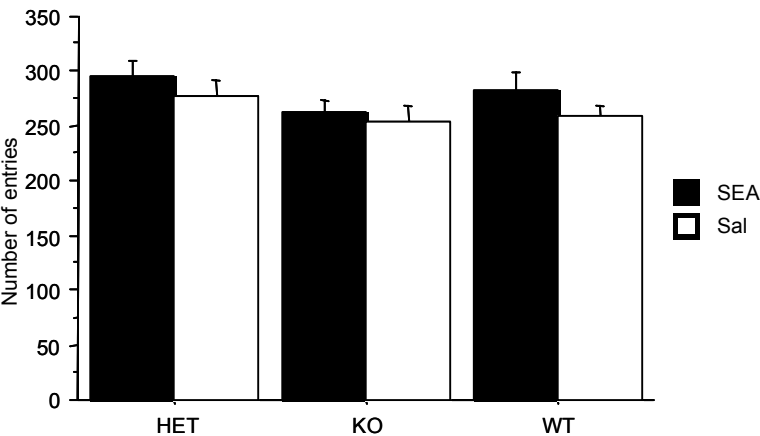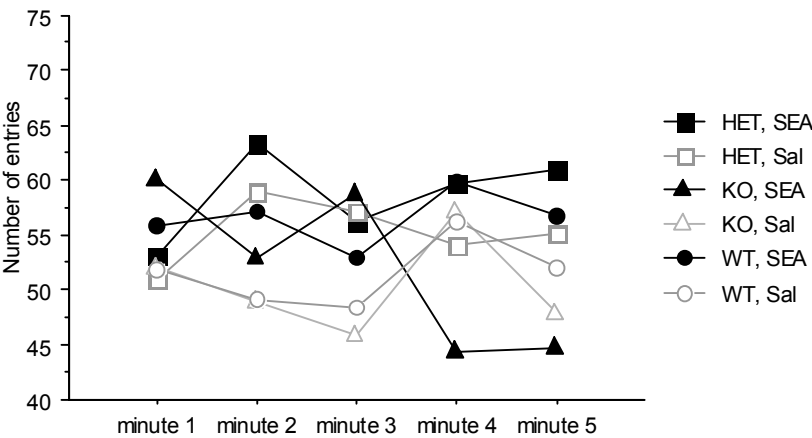$p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean. c. Mean total number of entries expressed over the five minute interval in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals. N = 10-14/group; $p<.05$.

SEA Effects in TNFRI Deficient Males:
Measures of Exploratory Behavior in the OF/NO Apparatus

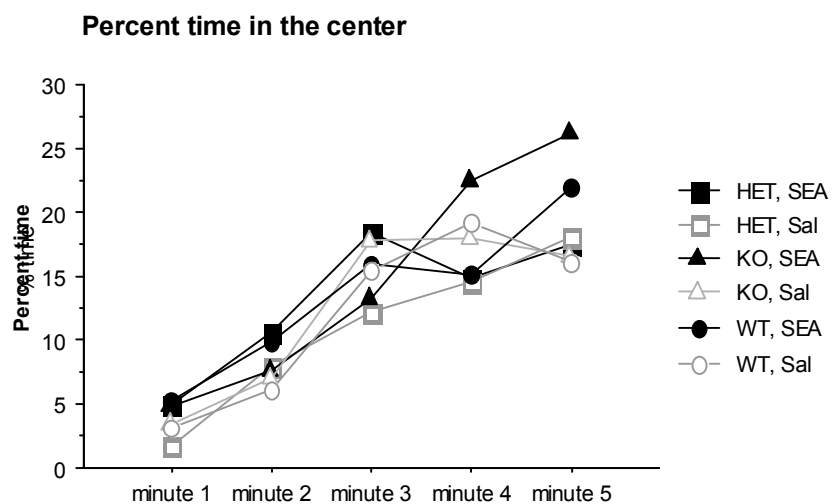### a. Latency to enter center

b. Latency to contact NO
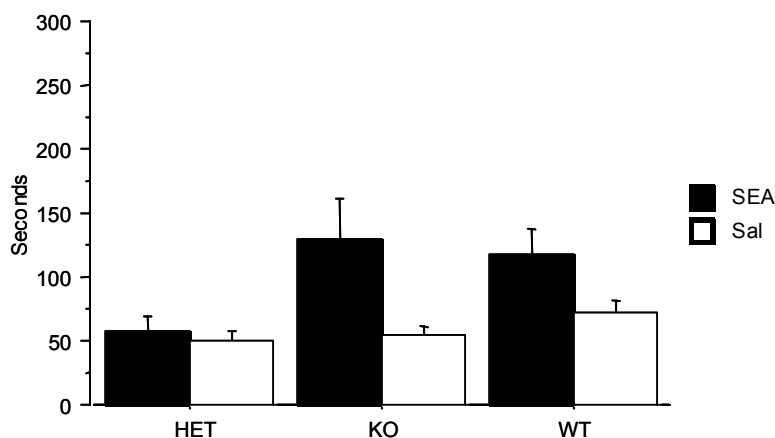
Figure 5.2. a. Mean total latency to enter the center in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean. b. Mean latency to contact the NO in male male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean.

SEA Effects in TNFRI Deficient Males:
Measures of Exploratory Behavior in the OF/NO Apparatus

a. Percent time in center
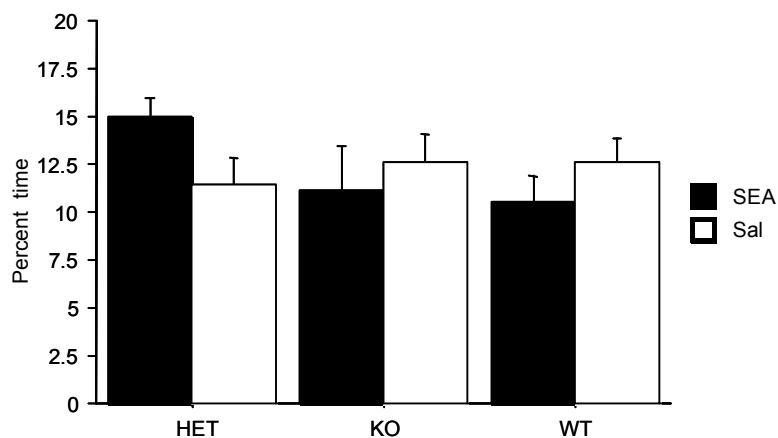
b. Percent time in center
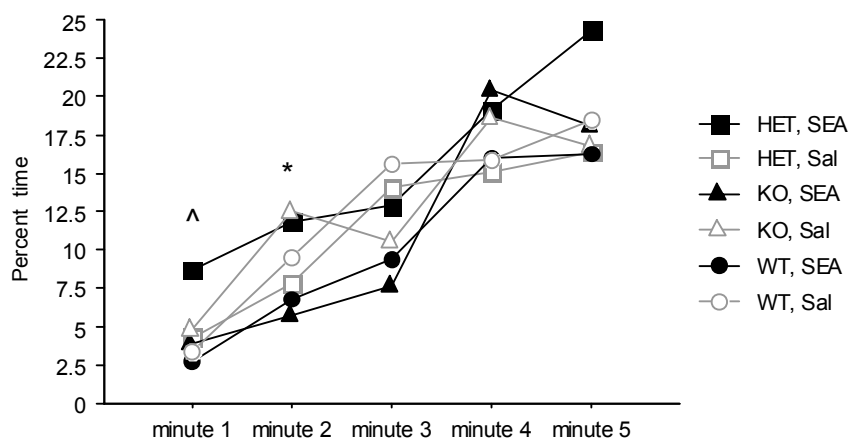
Figure 5.3. a. Mean total percent time spent in the center in male TNFRI^{+/-} (HET), TNFRI^{-/-} (KO), and TNFRI^{+/+} (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total percent time in the center expressed as a repeated measure ANOVA in male TNFRI^{+/-} (HET), TNFRI^{-/-} (KO), and TNFRI^{+/+} (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$.; ^ = p=.0675 compared to corresponding saline controls.

SEA Effects in TNFRI Deficient Males:
Measures of Exploratory Behavior in the OF/NO Apparatus

a. Total number of entries to center
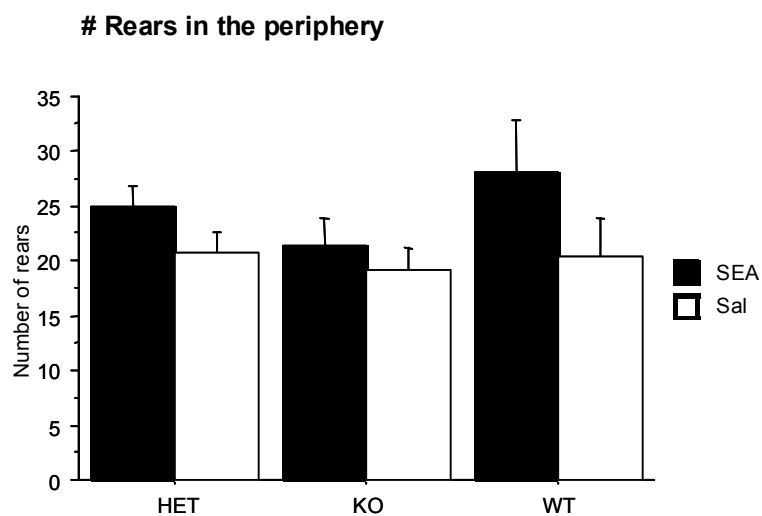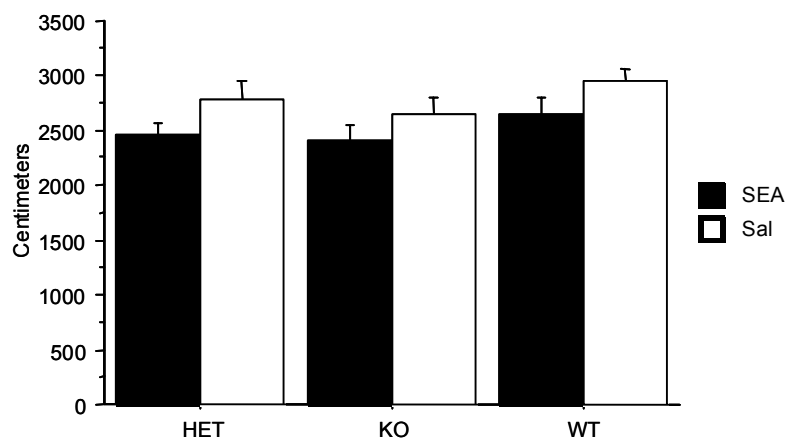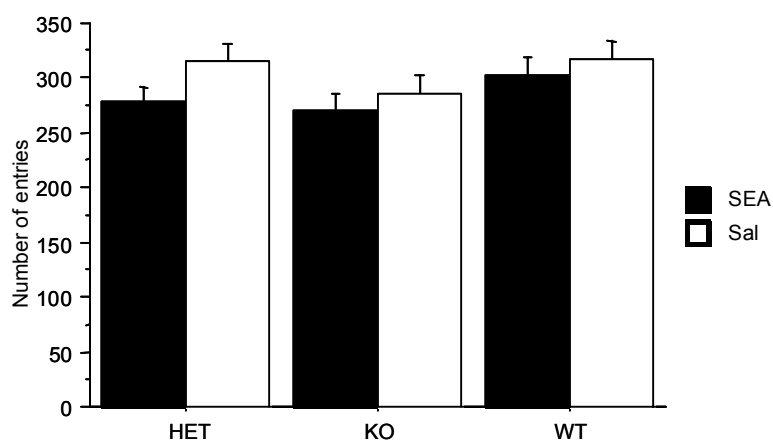

b. Total number of entries to center
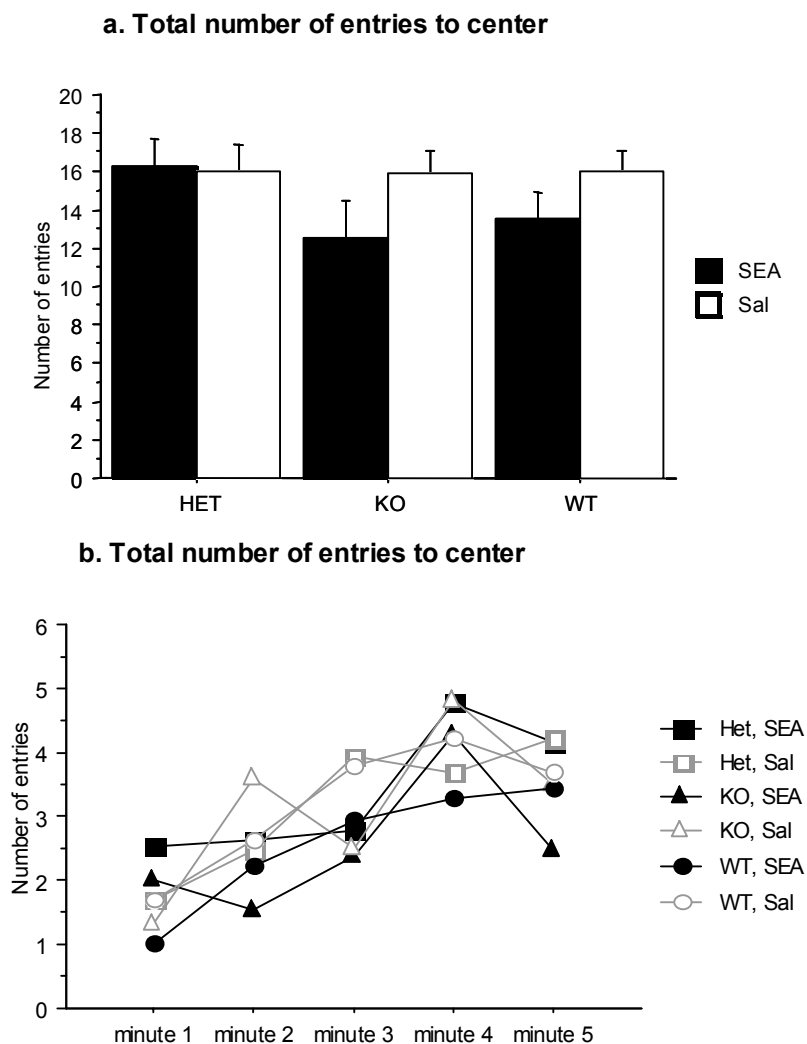
Figure 5.4. Mean total number of entries to the center in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries to the center expressed as a repeated measure ANOVA in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean.

Figure 5.5

**SEA Effects in TNFRI Deficient Females:
Locomotor Activity in the OF/NO Apparatus**
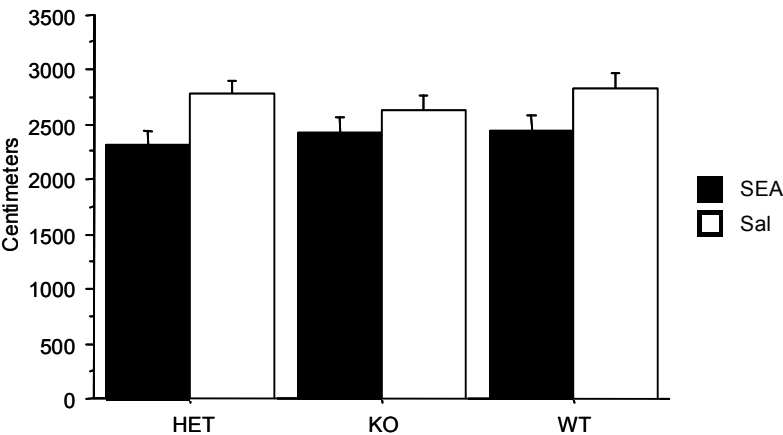
a. Total distance

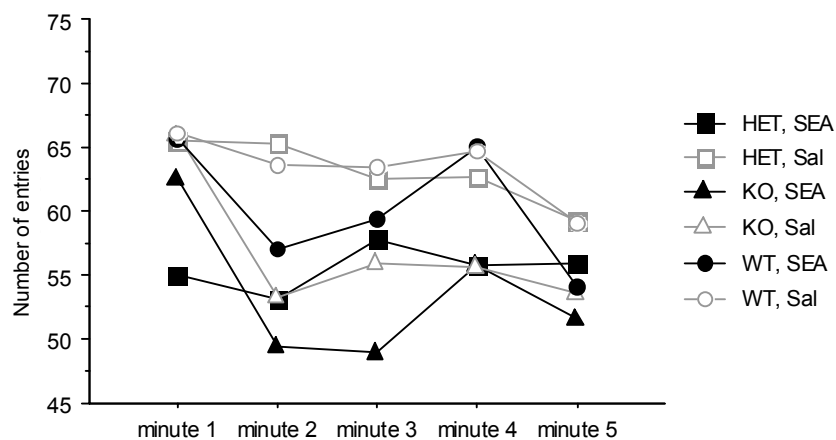**b. Total number of entries**
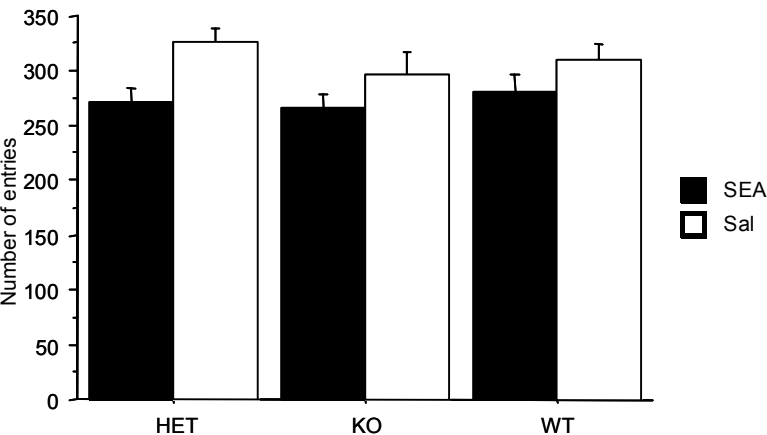
c. Total number of entries
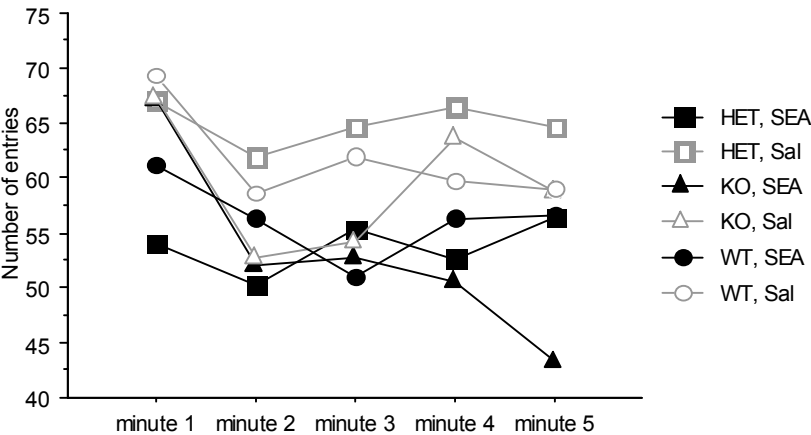
Figure 5.5. a. Mean total distance traveled in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-15/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-15/group; $p<.05$. Error bars represent standard error of the mean. c. Mean total number of total entries over the five minute interval in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals. N = 10-15/group; $p<.05$. Error bars represent standard error of the mean.

**SEA Effects in TNFRI Deficient Females:
Measures of Exploratory Behavior in the OF/NO Apparatus**

a. Total number of entries to center

b. Total number of entries to center
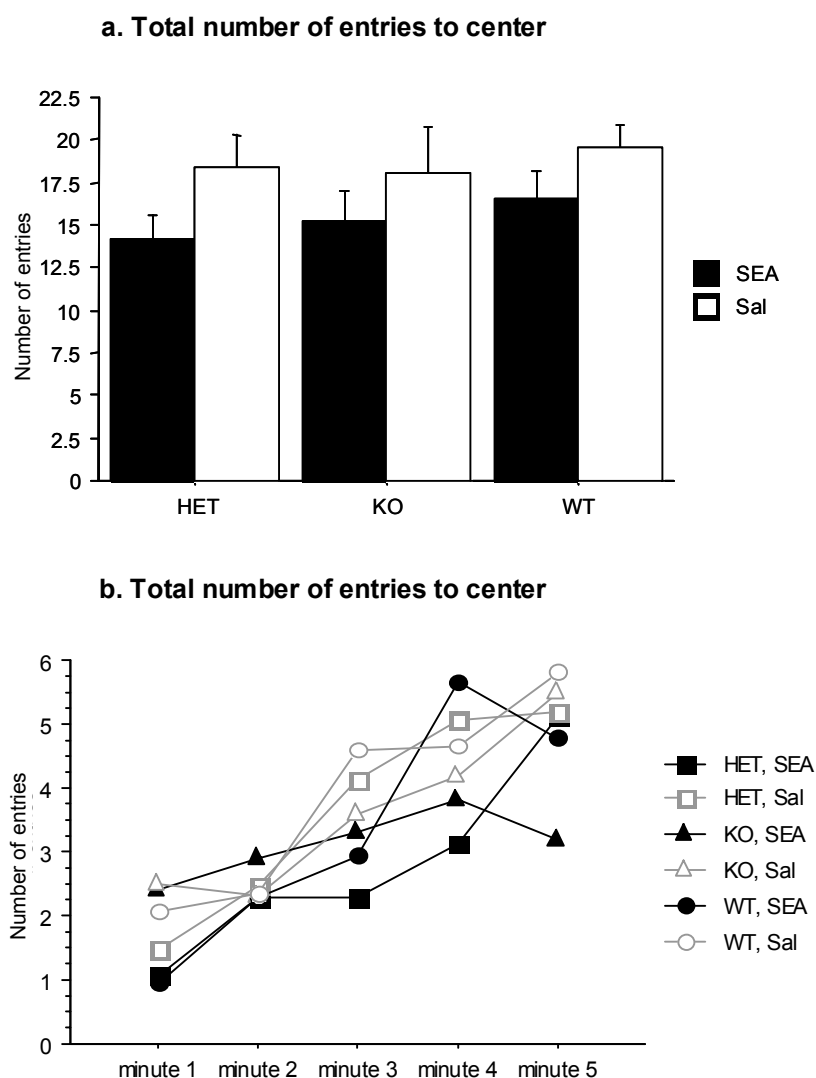
Figure 5.6. a. Mean total number of entries to the center in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-15/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries to the center in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline over the five minute interval. N = 10-15/group; $p<.05$.

SEA Effects in TNFRI Deficient Females:
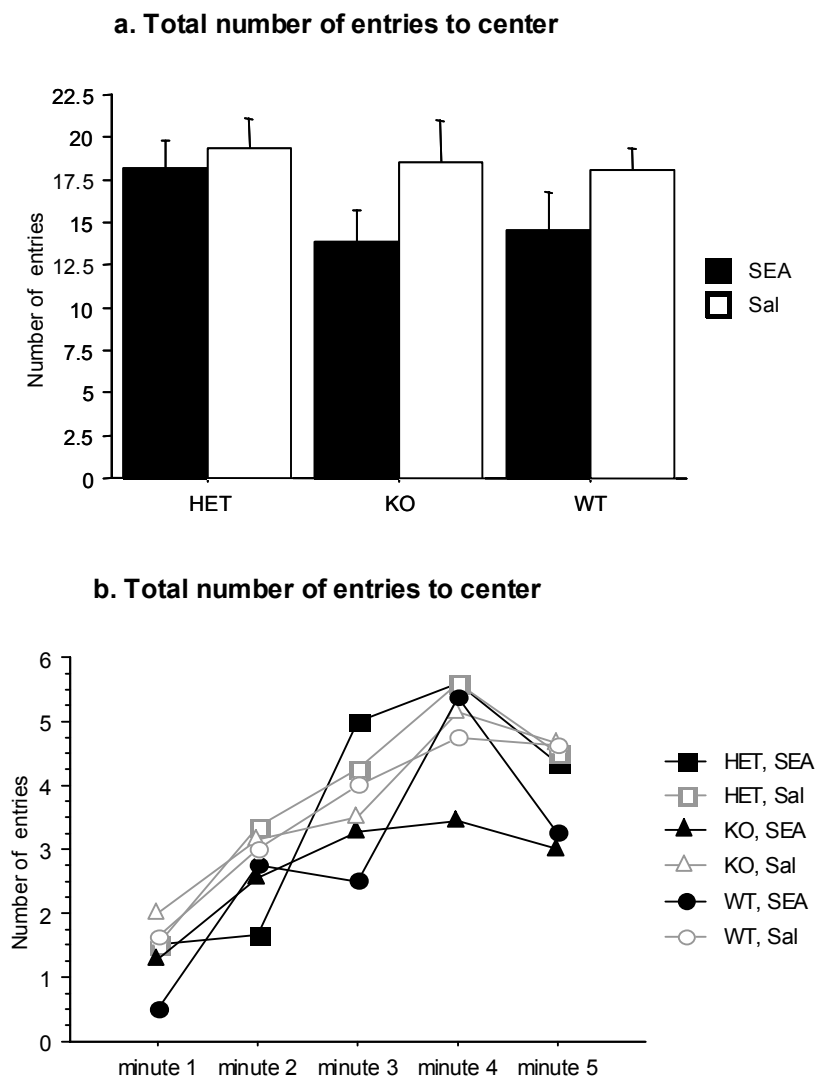**Measures of Exploratory Behavior in the OF/NO Apparatus**
a. Percent time in center
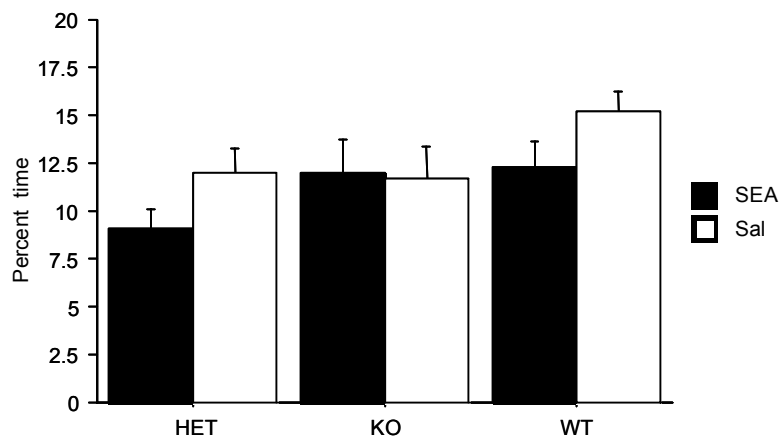
b. Percent time in center
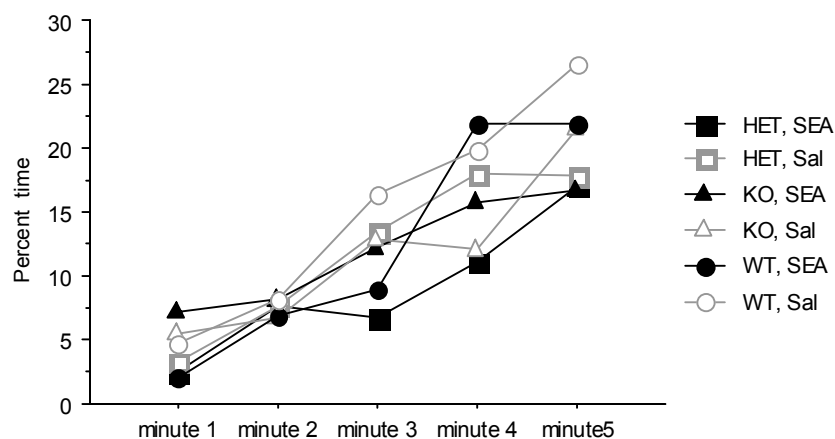
Figure 5.7. a. Mean percent time spent in the center in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-15/group; $p<.05$. Error bars represent standard error of the mean. b. Mean percent time spent in the center in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals over the five minute interval. N = 10-15/group; $p<.05$.

SEA Effects in TNFRI Deficient Females:
**Measures of Exploratory Behavior in the OF/NO Apparatus**

Latency to center
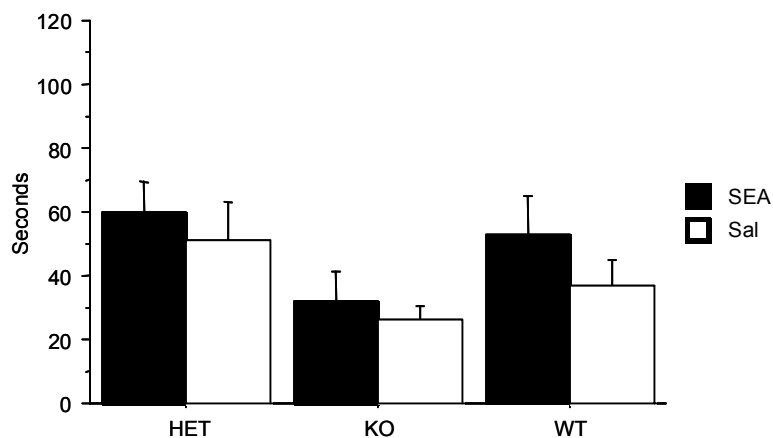
Figure 5.8. Mean latency to enter the center in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-15/group; *p*<.05. Error bars represent standard error of the mean.
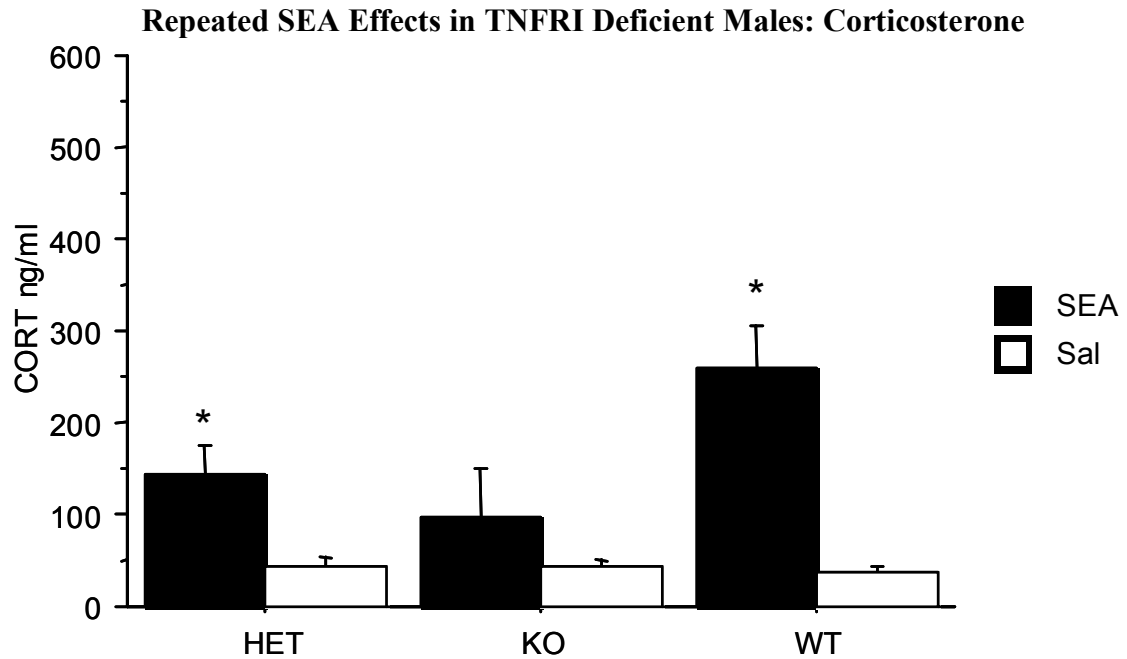
Figure 5.9

SEA Effects in TNFRII Deficient Males:
Locomotor Activity in the OF/NO apparatus
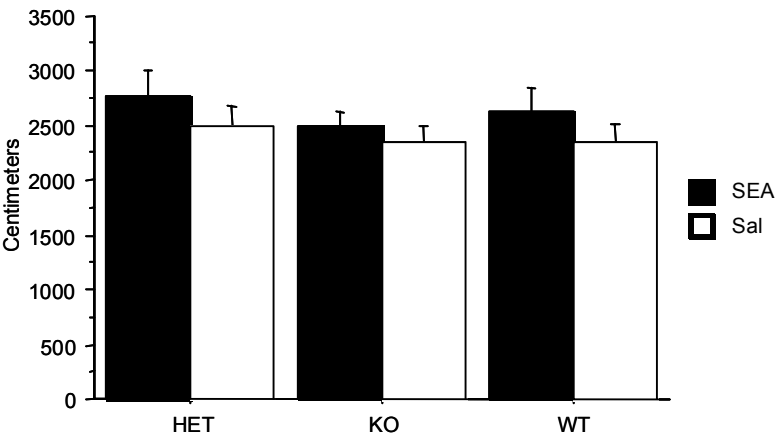
a. Total distance

b. Total number of entries

c. Total number of entries

Figure 5.9. a. Mean total distance traveled in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean. c. Mean total number of entries in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline over the five minute interval. N = 10-14/group; $p<.05$.

SEA Effects in TNFRII Deficient Males:
Measures of Exploratory Behavior in the OF/NO apparatus

Percent time in the center

Figure 5.10. Mean percent time spent in the center in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline over a five minute interval. N = 10-14/group; $p<.05$.

SEA Effects in TNFRII Deficient Males:
Measures of Exploratory Behavior in the OF/NO apparatus

 # Rears in the periphery

Figure 5.11. Mean number of rears in the periphery in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 10-14/group; $p<.05$. Error bars represent standard error of the mean.

Figure 5.12

SEA Effects in TNFRII Deficient Females:
Locomotor Activity in the OF/NO Apparatus

a. Total distance

b. Total number of entries

c. Total number of entries

Figure 5.12. a. Mean total distance traveled in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 6-12/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 6-12/group; $p<.05$. Error bars represent standard error of the mean. c. Mean total number of entries in the female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline over the five minute interval. N = 6-12/group; $p<.05$.

SEA Effects in TNFRII Deficient Females:
Measures of Exploratory Behavior in the OF/NO apparatus

a. Percent time in center

b. Percent time in center
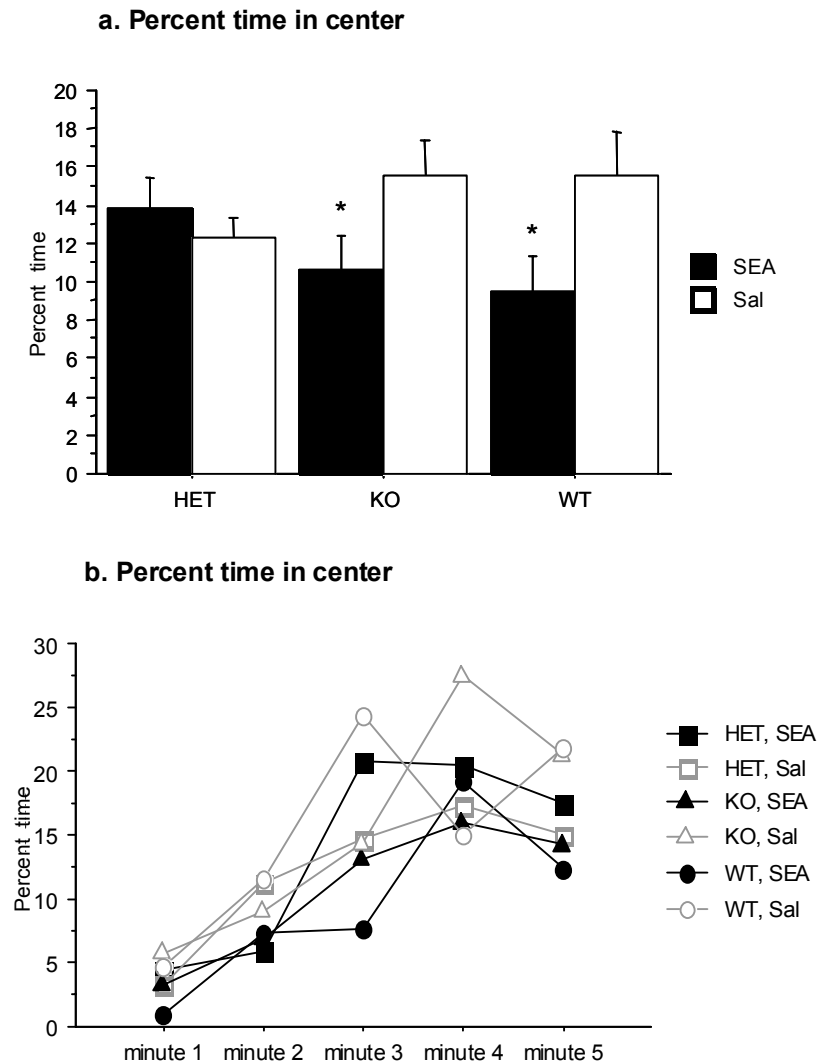
Figure 5.13. a. Mean percent time spent in the center in female TNFRII^{+/-} (HET), TNFRII^{-/-} (KO), and TNFRII^{+/+} (WT) animals treated with SEA or saline. N = 6-12/group; $p<.05$ compared to corresponding salin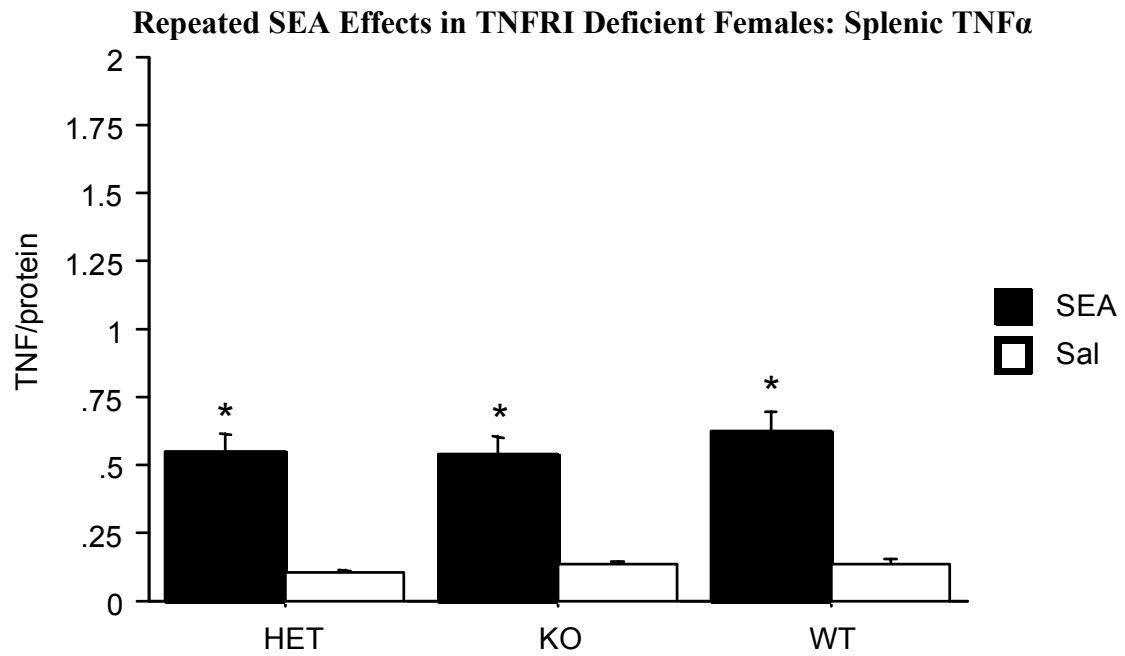e controls. Error bars represent standard error of the mean. b. Mean percent time spent 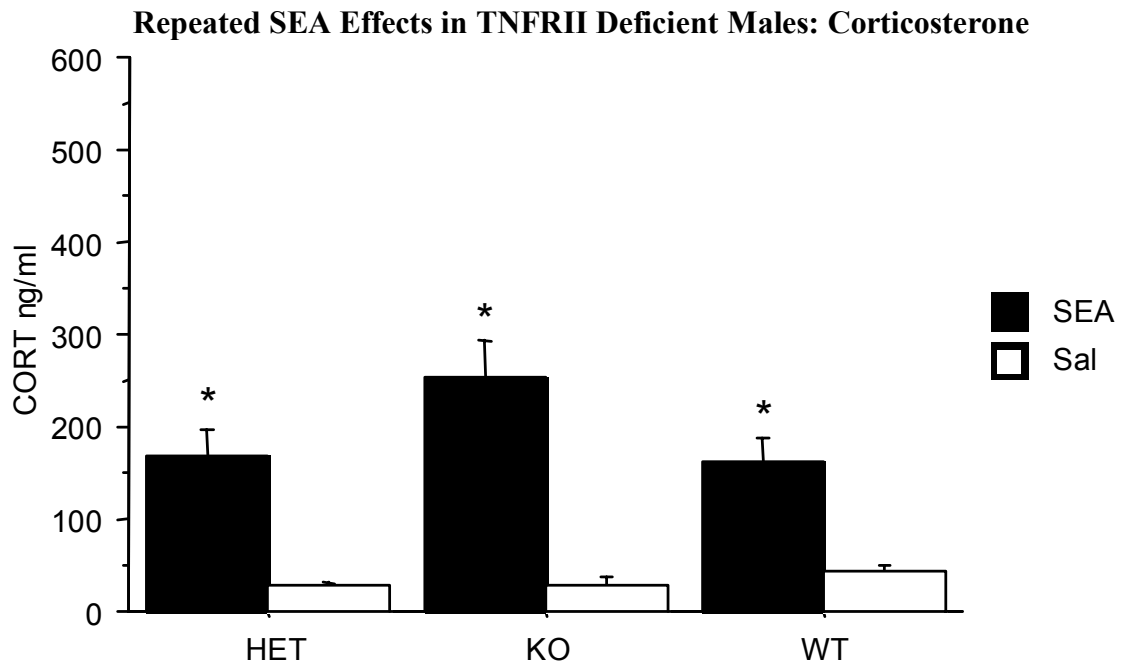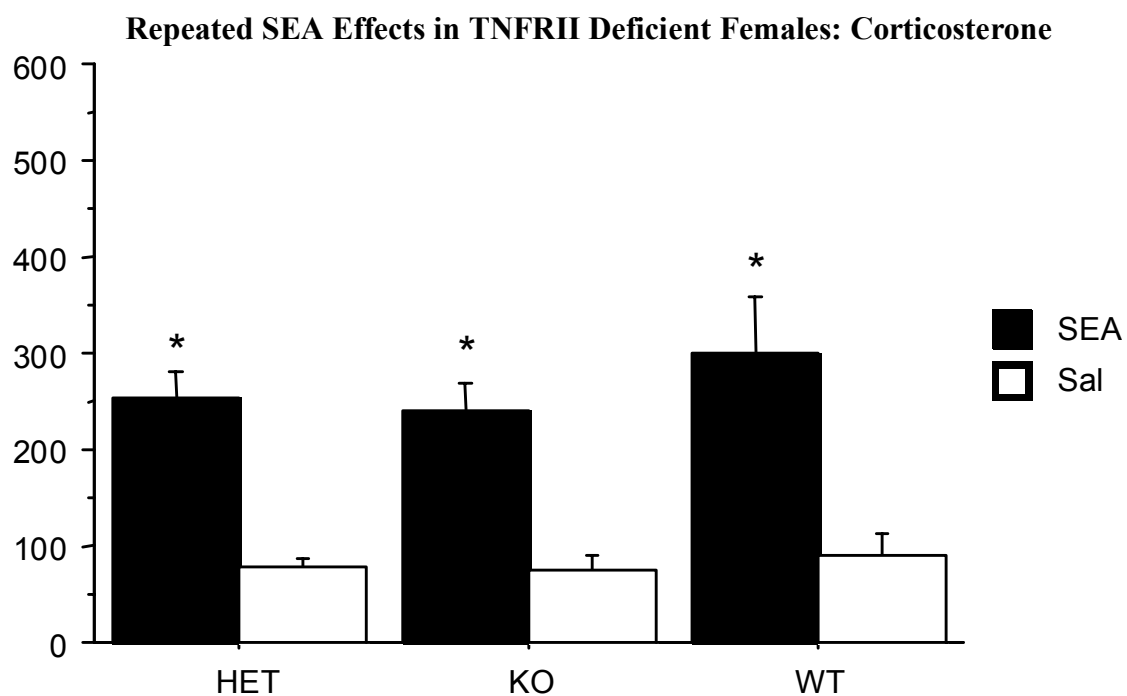in the center in female TNFRII^{+/-} (HET), TNFRII^{-/-} (KO), and TNFRII^{+/+} (WT) animals treated with SEA or saline over the five minute interval. N = 6-12/group; $p<.05$.

SEA Effects in TNFRII Deficient Females:
Measures of Exploratory Behavior in the OF/NO apparatus

a. Total number of entries to center

b. Total number of entries to center

Figure 5.14. a. Mean total number of entries to the center in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 6-12/group; $p<.05$. Error bars represent standard error of the mean. b. Mean total number of entries to the center in TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline over the five minute interval. N = 6-12/group; $p<.05$.

SEA Effects in TNFRII Deficient Females:
Measures of Exploratory Behavior in the OF/NO apparatus

Rears in the periphery
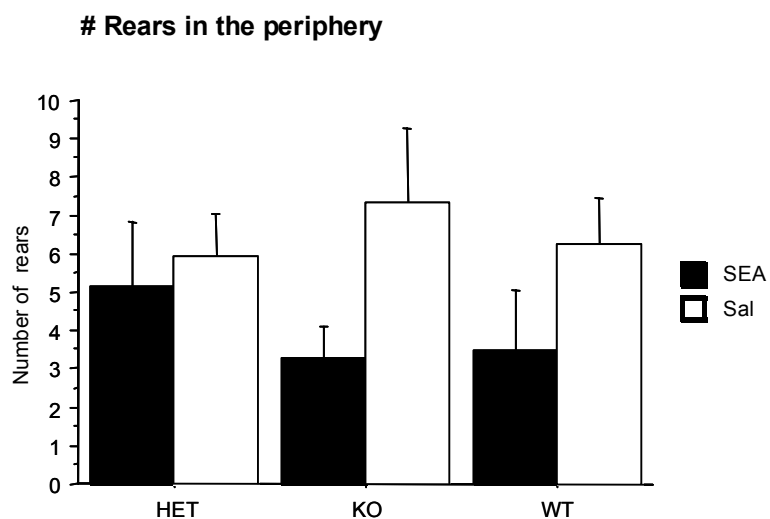
Figure 5.15. Mean total number of rears in the periphery in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with SEA or saline. N = 6-12/group; *p*<.05. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRI Deficient Males: Corticosterone

Figure 6.1. Mean plasma CORT in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals after secondary challenge with SEA or saline. N = 10-14/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

**Repeated SEA Effects in TNFRI Deficient Males: Splenic TNFα**
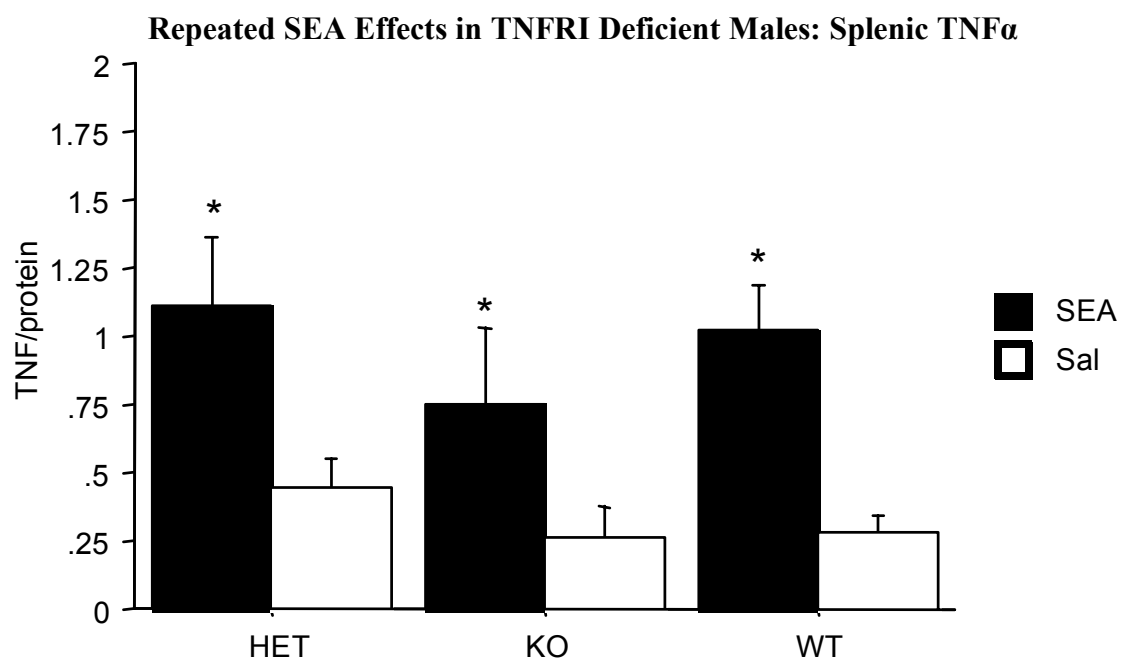
Figure 6.2. Mean splenic TNFα in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals after secondary challenge with SEA or saline. Data is expressed as pg cytokine/µg protein. N = 10-14/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRI Deficient Males: Hypothalamic CRH mRNA
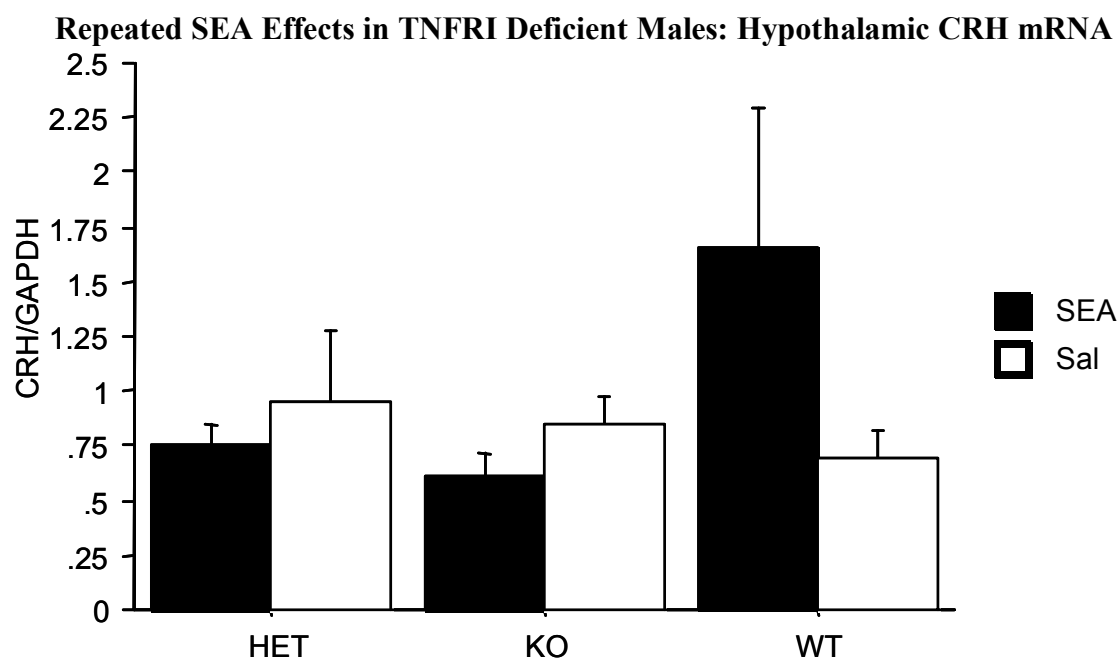
Figure 6.3. Mean hypothalamic CRH mRNA expression in male TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals after secondary challenge with SEA or saline. N = 10-14/group. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRI Deficient Males: Hypothalamic TNFRII mRNA
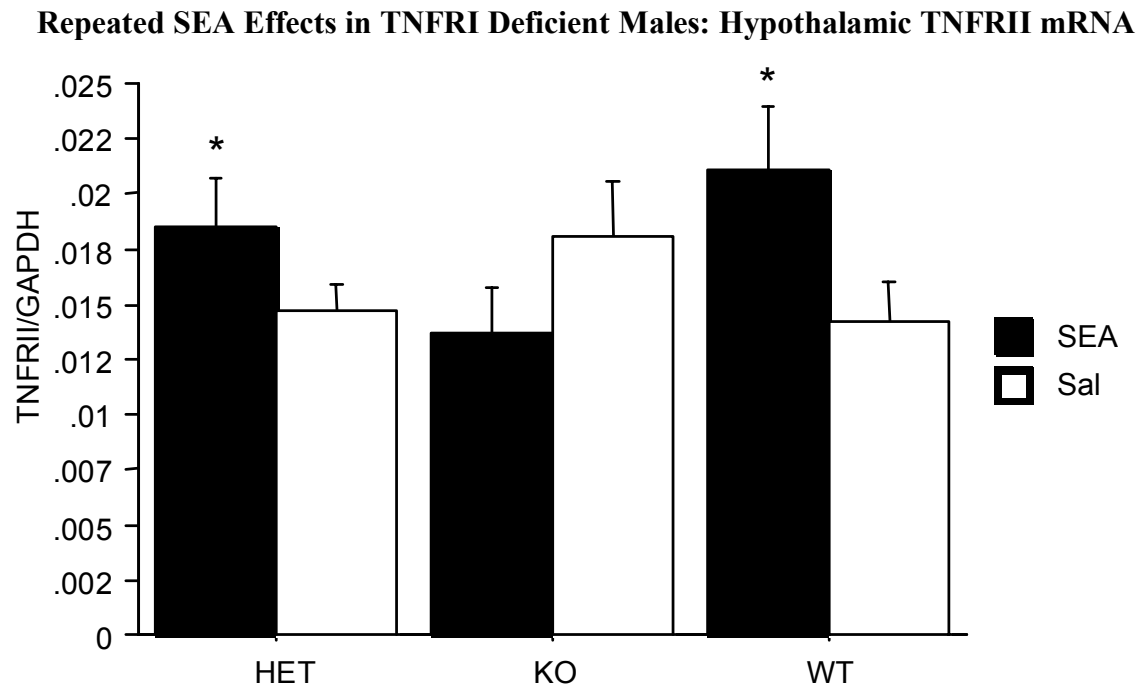
Figure 6.4. Mean hypothalamic TNFRII mRNA expression in TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals after secondary challenge with SEA or saline. N = 10-14/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

**Repeated SEA Effects in Wildtype Males: Hypothalamic TNFRI mRNA**
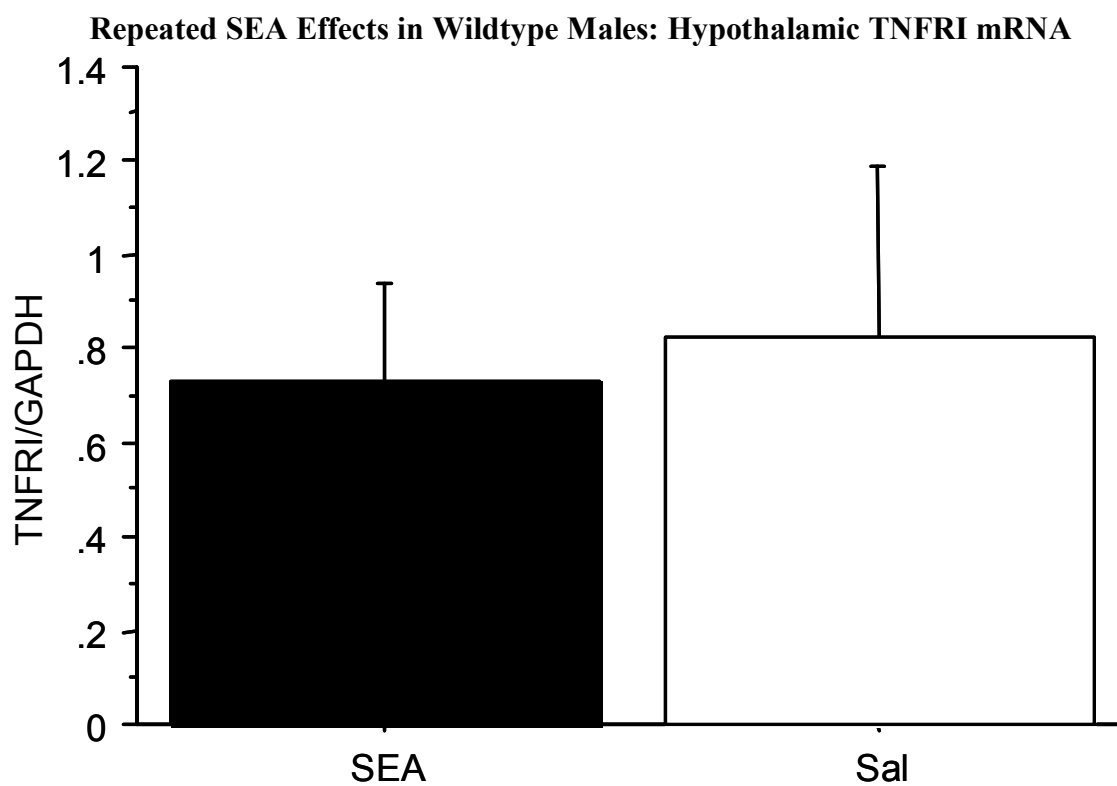
Figure 6.5. Mean hypothalamic TNFRII mRNA expression in male TNFRI$^{+/+}$ animals after secondary challenge with SEA or saline. N = 10-14/group. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRI Deficient Females: Corticosterone
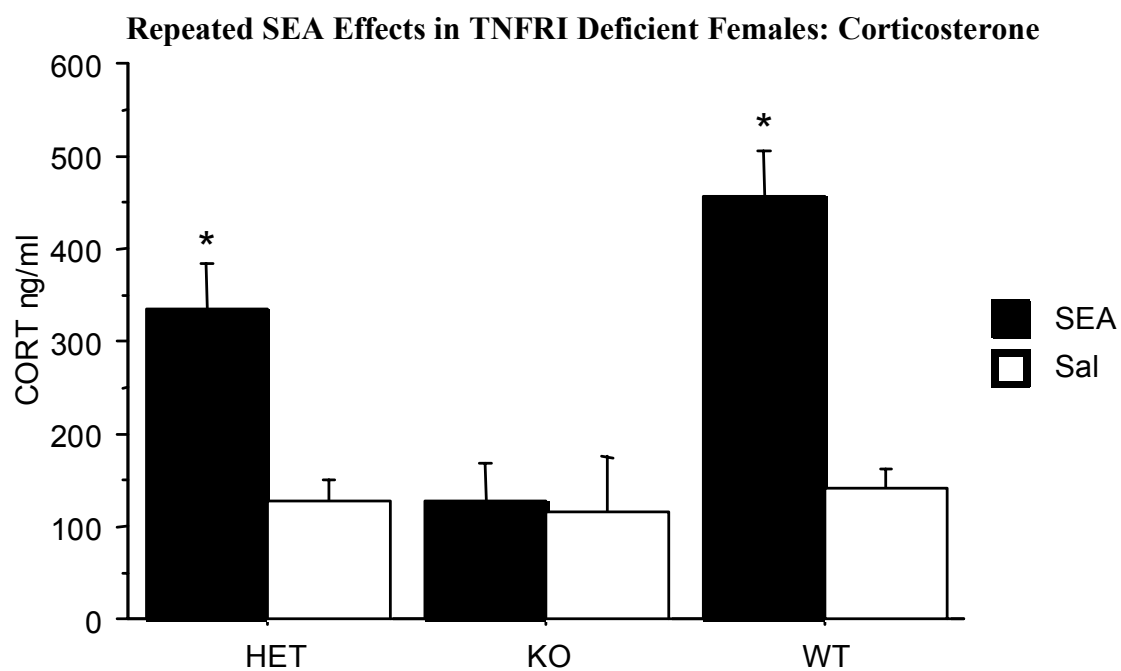
Figure 6.6. Mean plasma CORT in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with secondary SEA or saline. N = 10-15/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

**Repeated SEA Effects in TNFRI Deficient Females: Splenic TNFα**

Figure 6.7. Mean splenic TNFα in female TNFRI$^{+/-}$ (HET), TNFRI$^{-/-}$ (KO), and TNFRI$^{+/+}$ (WT) animals treated with secondary SEA or saline. Data is expressed as pg cytokine/µg protein. N = 10-15/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRII Deficient Males: Corticosterone

Figure 6.8. Mean plasma CORT in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with secondary SEA or saline. N = 10-14/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRII Deficient Males: Splenic TNFα
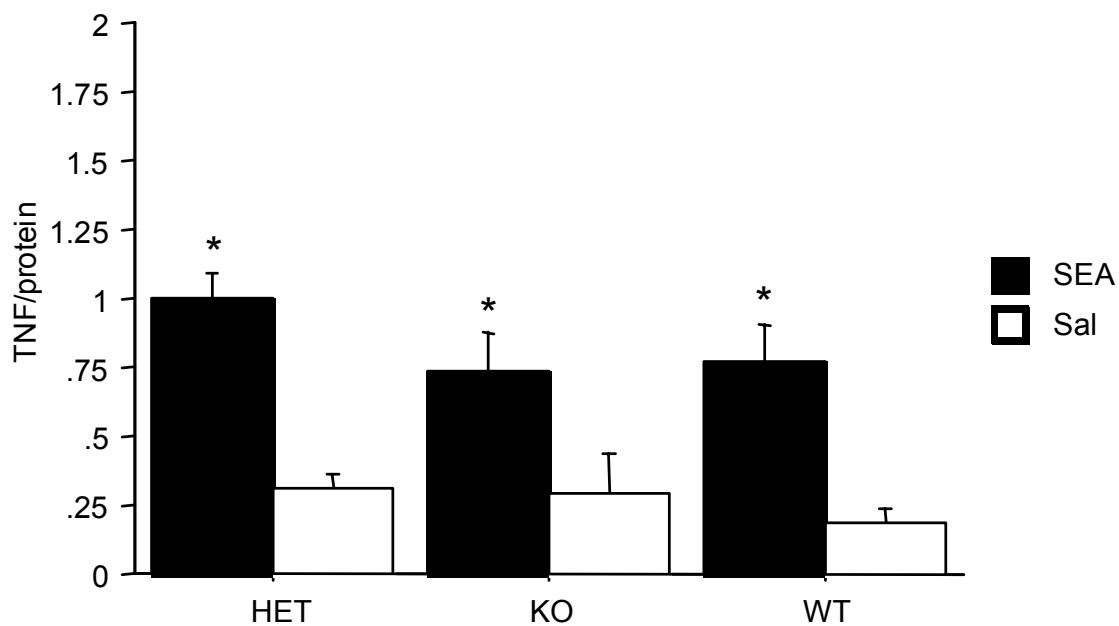
Figure 6.9. Mean splenic TNFα in male TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with secondary SEA or saline. Data is expressed as pg cytokine/μg protein. N = 10-14/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRII Deficient Females: Corticosterone

Figure 6.10. Mean plasma CORT in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with secondary SEA or saline. N = 6-12/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Repeated SEA Effects in TNFRII Deficient Females: Splenic TNFα
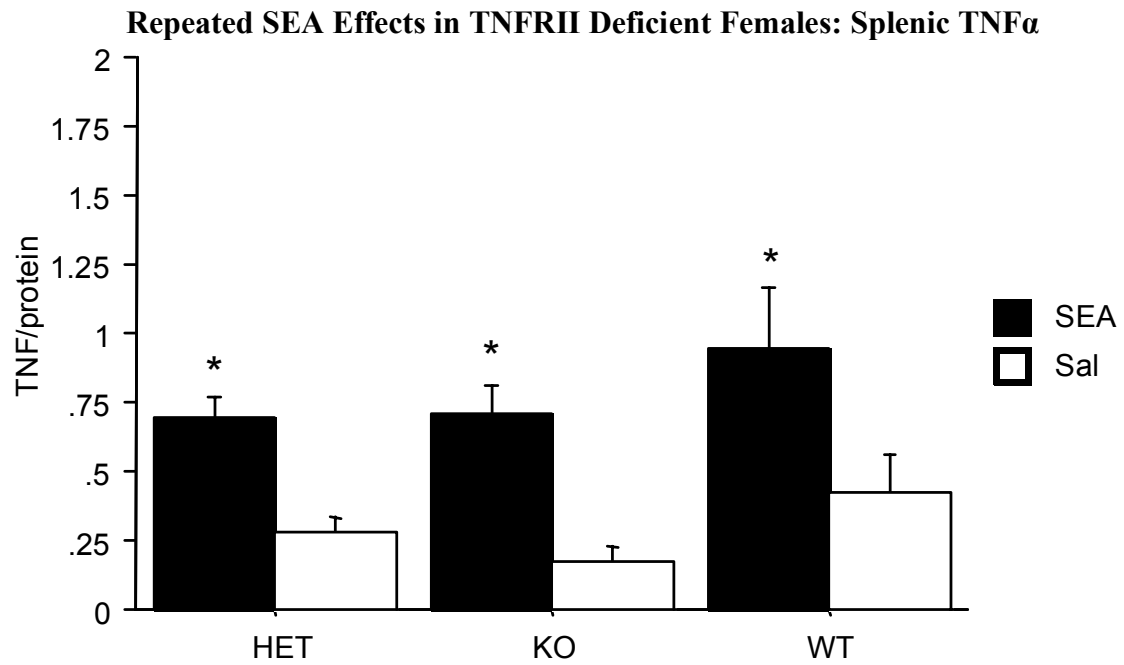
Figure 6.11. Mean splenic TNFα in female TNFRII$^{+/-}$ (HET), TNFRII$^{-/-}$ (KO), and TNFRII$^{+/+}$ (WT) animals treated with secondary SEA or saline. Data is expressed as pg cytokine/µg protein. N = 6-12/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

AMINO Time Course to SEA: Corticosterone
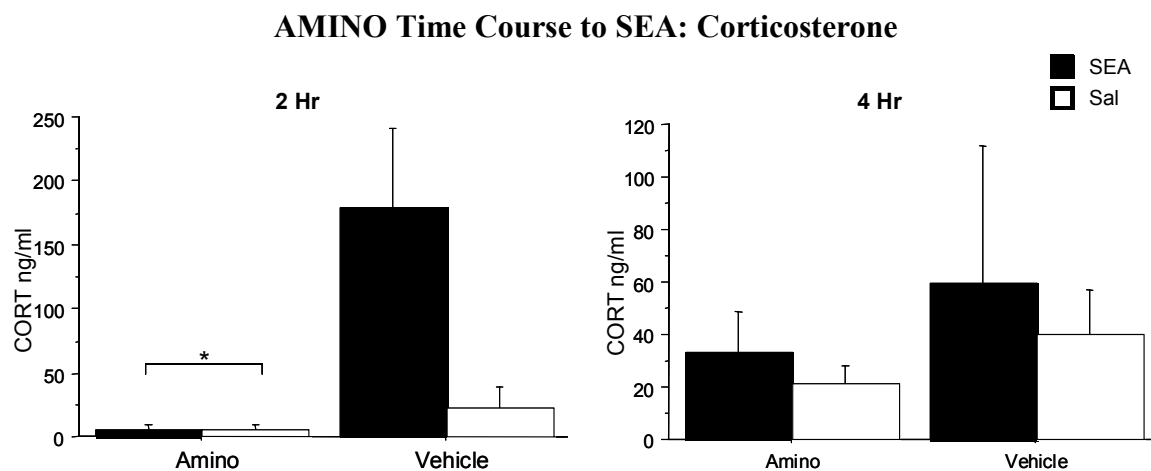
Figure 7.1. Mean plasma CORT after pretreatment with Amino or Veh and sacrificed 2 or 4 hours after treatment with SEA or saline. N = 4-6/group; *p*<.05 compared to Vehicle treated animals. Error bars represent standard error of the mean.

AMINO Time Course to SEA: Hypothalamic TNFα Protein
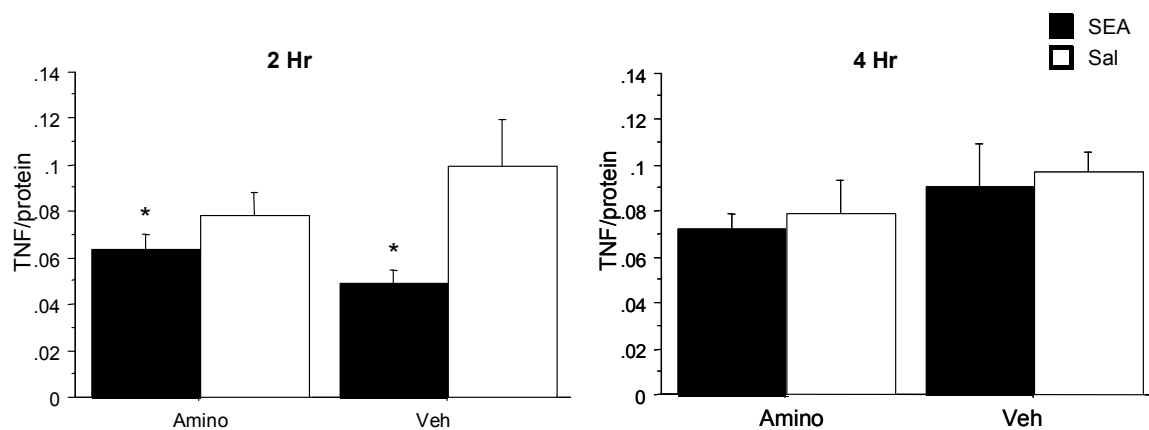
Figure 7.2. Mean hypothalamic TNFα after pretreatment with Amino or Veh and sacrificed 2 or 4 hours after treatment with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 4-6/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

AMINO Time Course to SEA: Hypothalamic IL-1β Protein
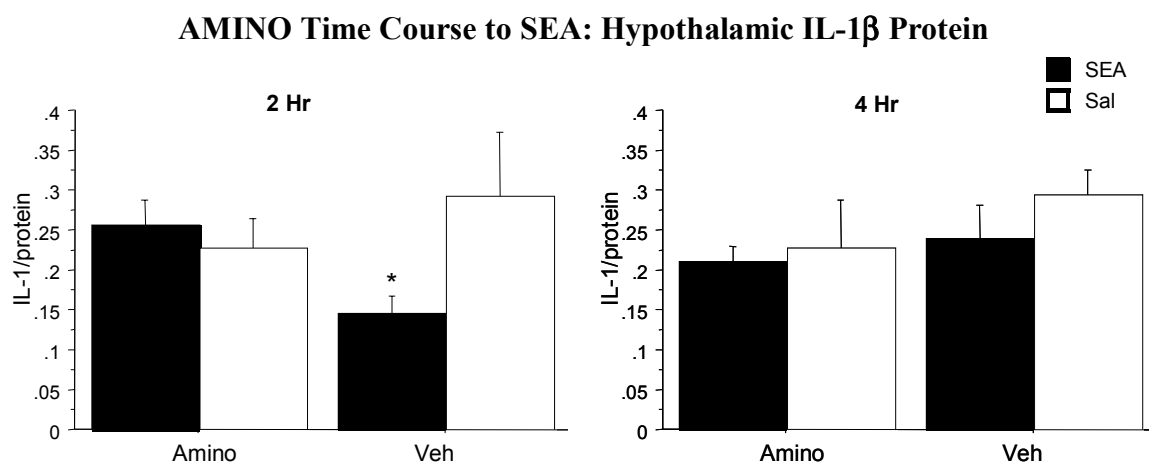
Figure 7.3. Mean hypothalamic IL-1β after pretreatment with Amino or Veh and sacrificed 2 or 4 hours after treatment with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 4-6/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

AMINO Time Course to SEA: Splenic Cytokines
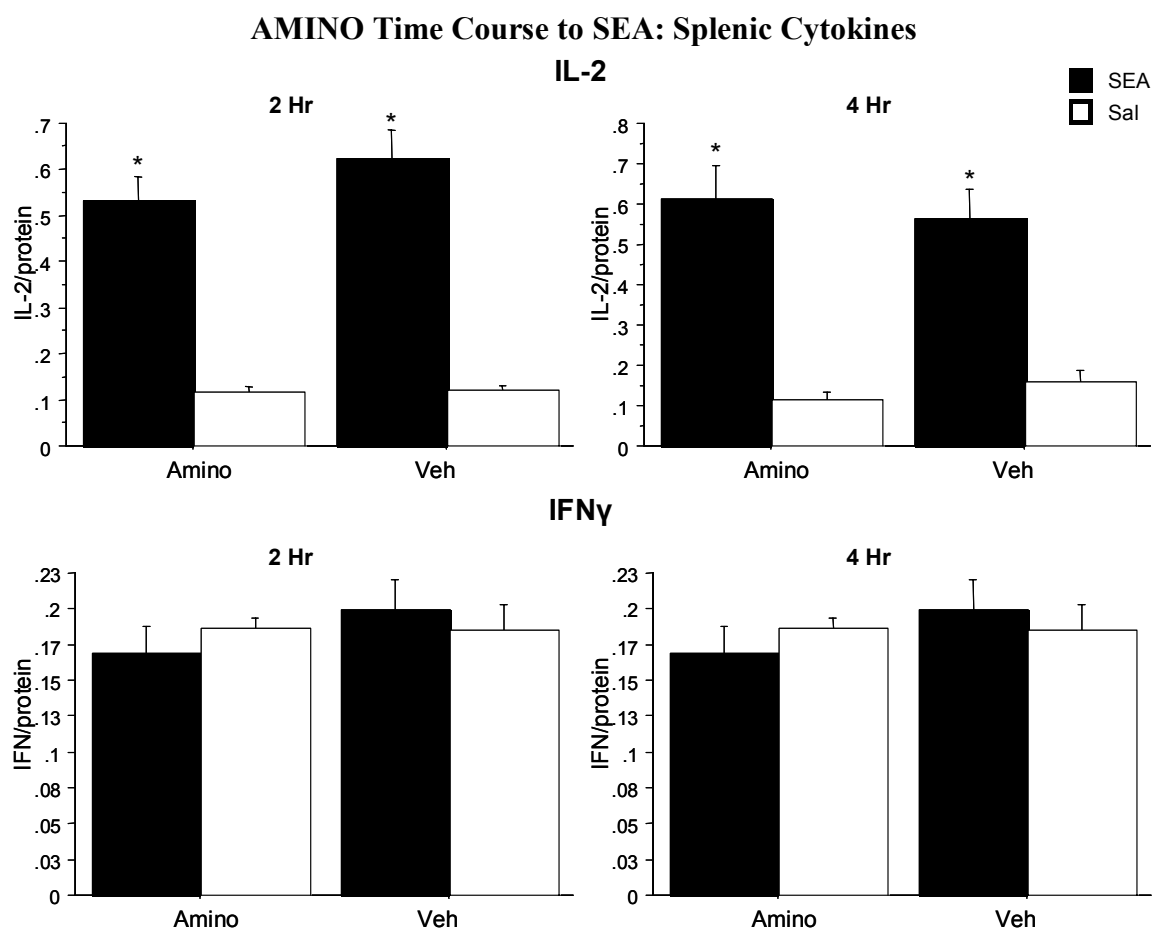
Figure 7.4. Mean IL-2 and IFNγ after pretreatment with Amino or Veh and sacrificed 2 or 4 hours after treatment with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 4-6/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

AMINO Time Course to SEA: Splenic Cytokines

IL-1

IL-6

TNFα

Figure 7.5. Mean IL-1β , IL-6, and TNFα after pretreatment with Amino or Veh and sacrificed 2 or 4 hours after treatment with SEA or saline. Data is expressed as pg cytokine/μg protein. N = 4-6/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

AMINO Time Course to SEA: Plasma Cytokines

IL-6
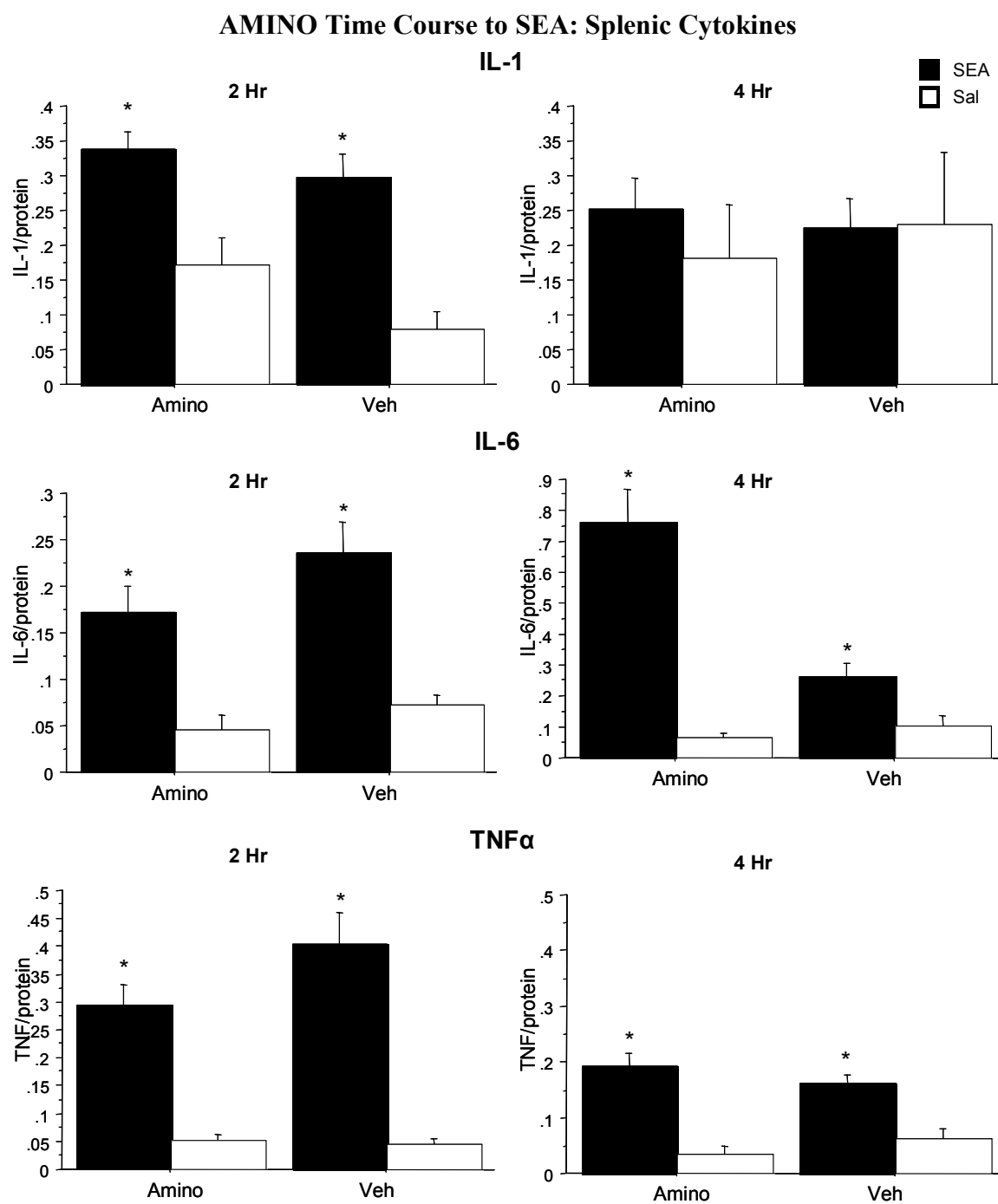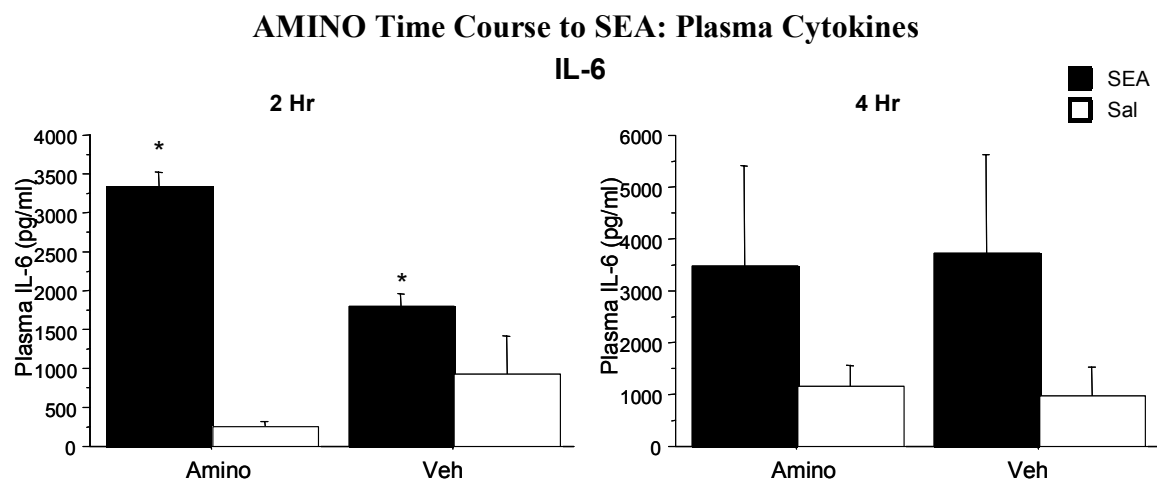
Figure 7.6. Mean plasma IL-6 after pretreatment with Amino or Veh and sacrificed 2 or 4 hours after treatment with SEA or saline. N = 4-6/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

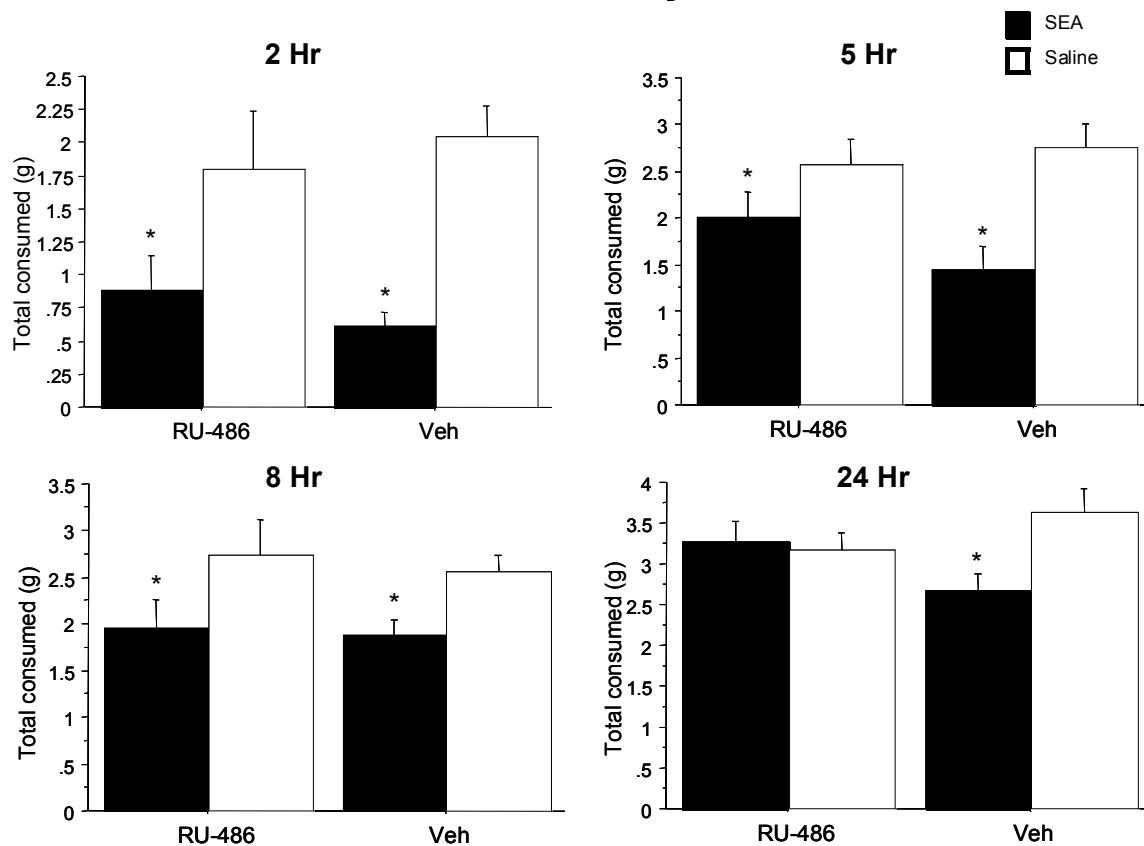**Effect of Glucocorticoid Inhibition Before Primary Exposure to SEA:
Prosobee Consumption**
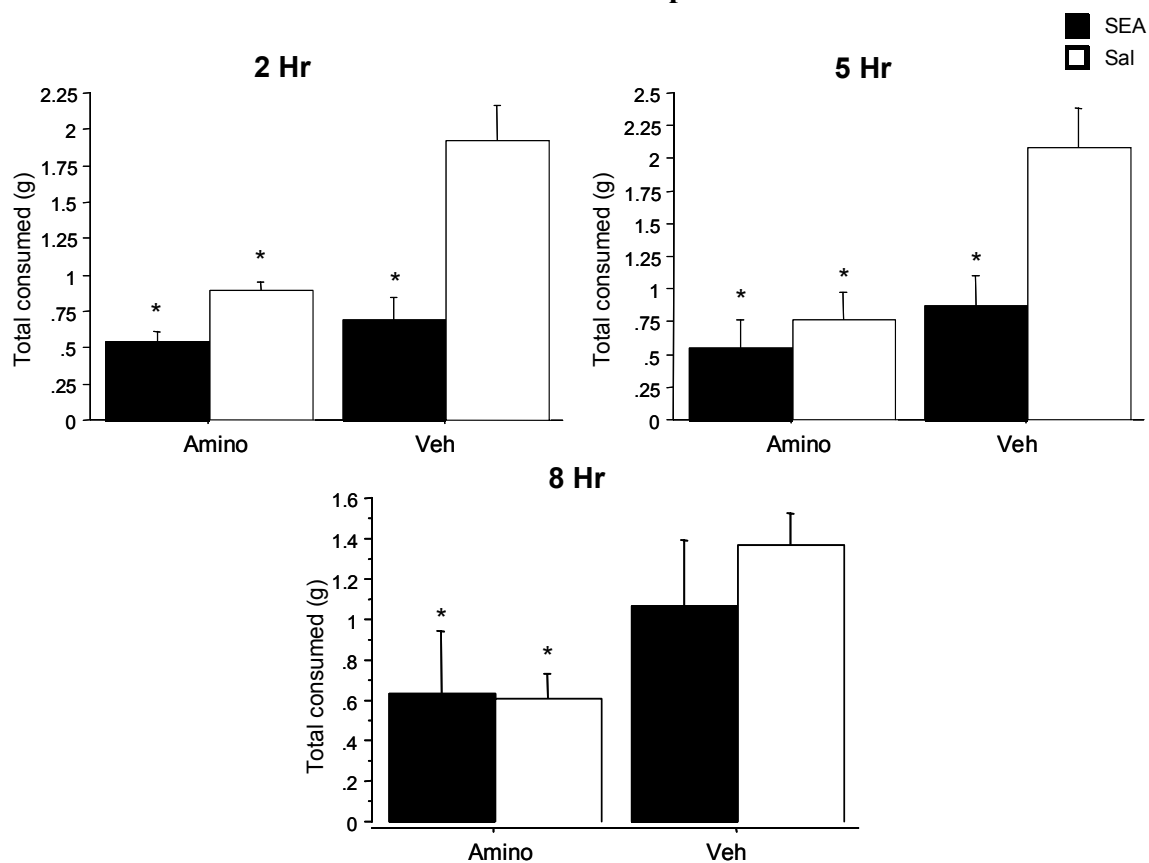
Figure 7.7. The effect of Amino pretreatment on consumption at 2, 5, and 8 hours after SEA. N= 8/group; *p*<.05 compared to corresponding saline controls and Vehicle treated animals. Error bars represent standard error of the mean.

**Effect of Glucocorticoid Inhibition Before Primary Exposure to SEA:
Splenic Cytokines**
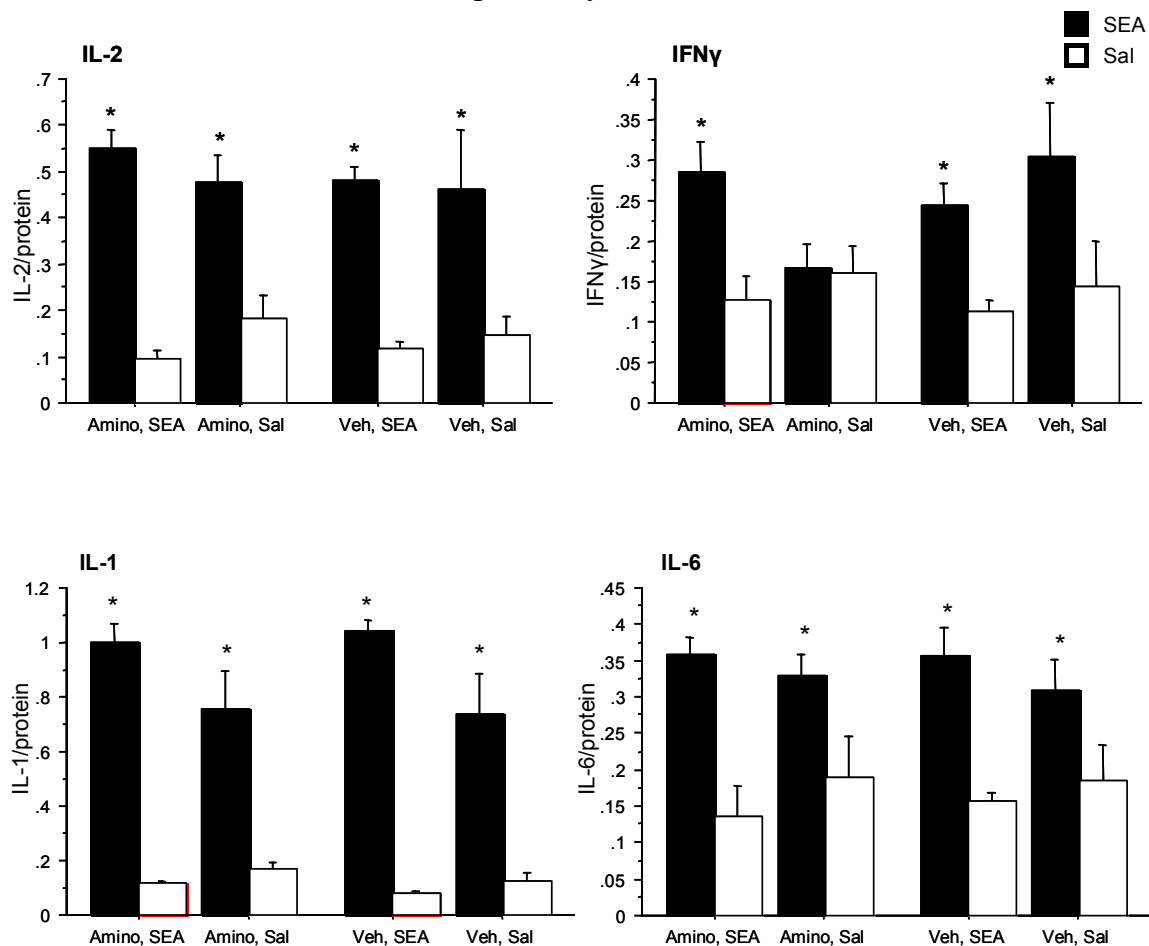
Figure 7.8. The effect of pretreatment with Amino on splenic IL-2, IFNγ, IL-1β, and IL-6 after primary or secondary exposure to SEA. Data is expressed as pg cytokine/μg protein. N= 3-5/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Effect of Glucocorticoid Inhibition Before Primary Exposure to SEA:
TNFα
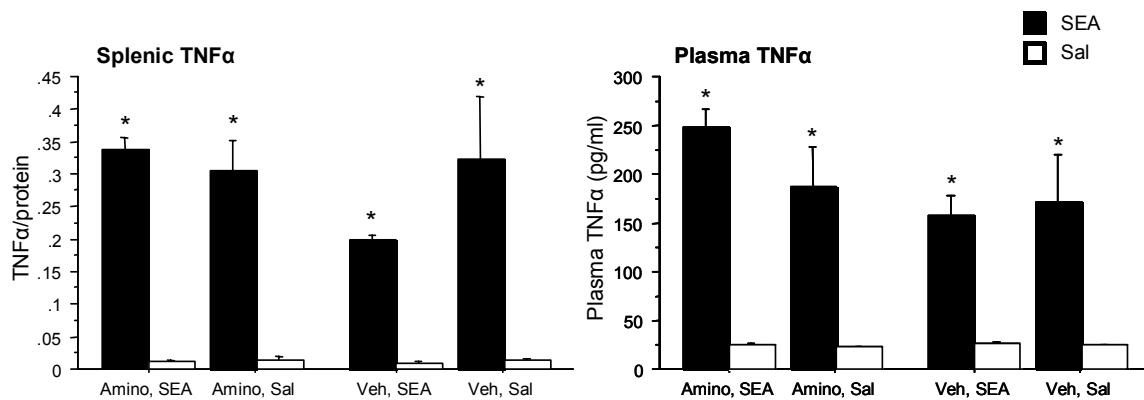
Figure 7.9. The effect of pretreatment with Amino on splenic and plasma TNFα after primary or secondary exposure to SEA. Data is expressed as pg cytokine/μg protein. N= 3-5/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

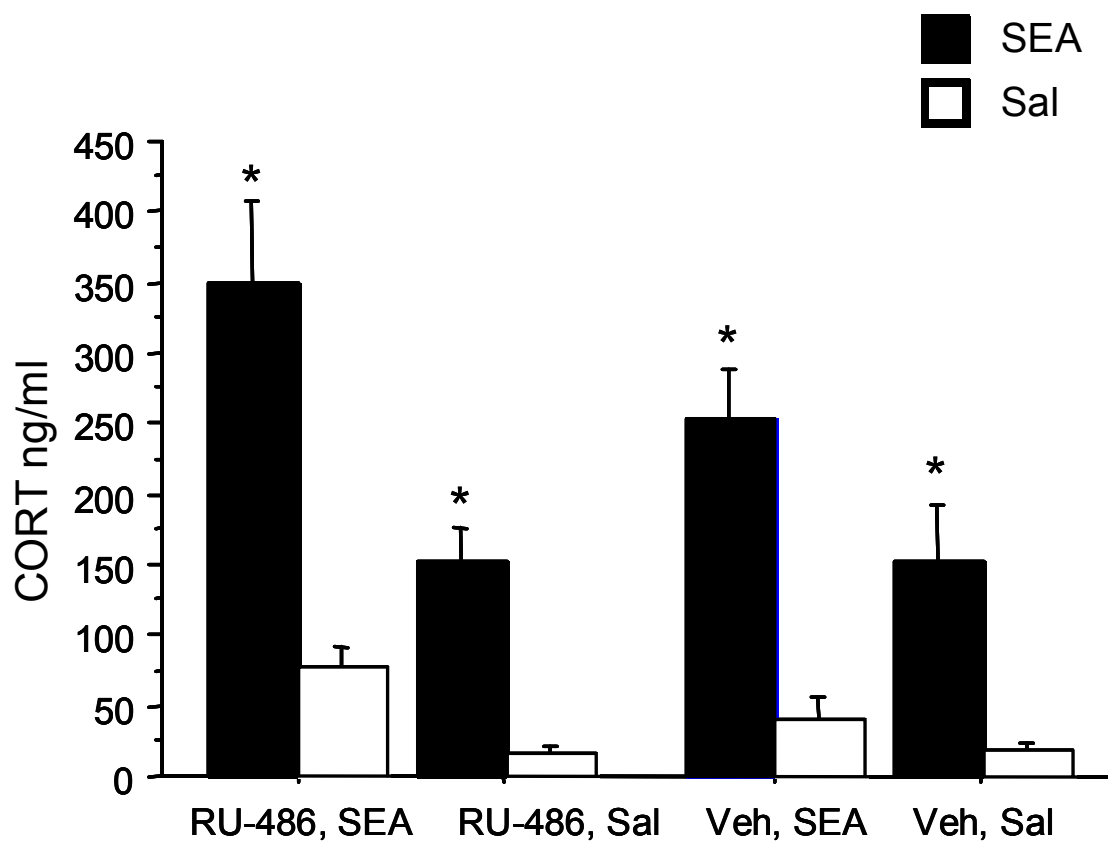**Effect of Glucocorticoid Inhibition Before Primary Exposure to SEA:**
Corticosterone
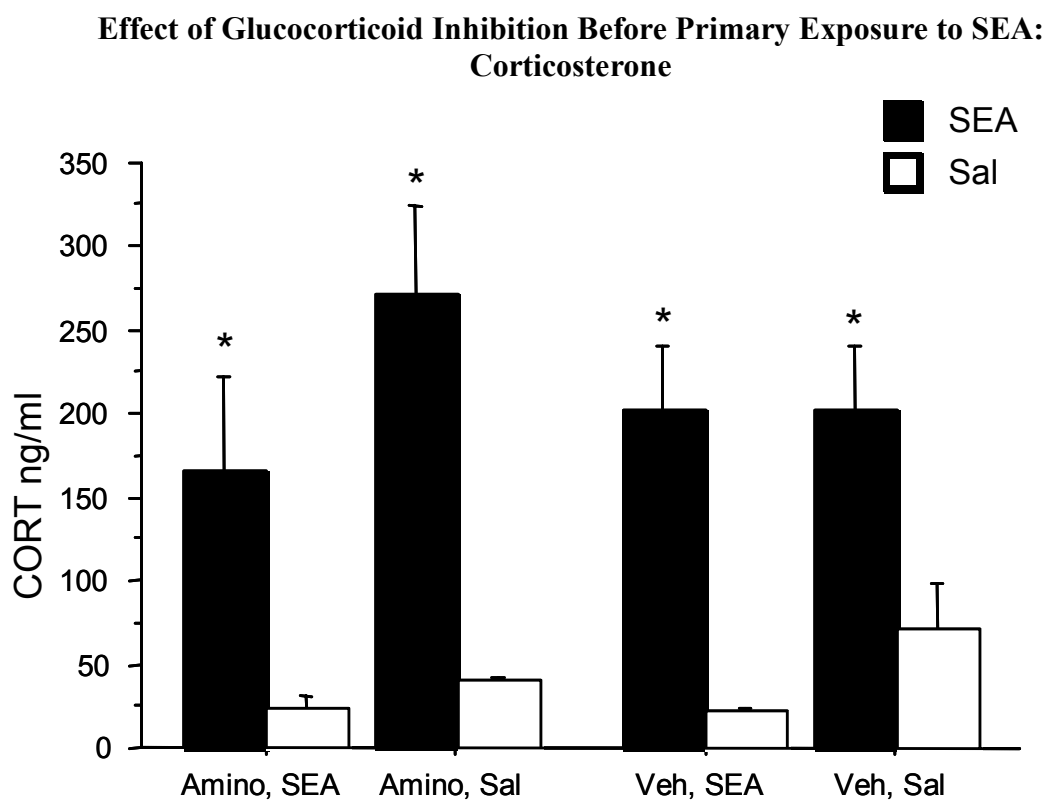
Figure 7.10. The effect of pretreatment with Amino on plasma CORT after primary or secondary exposure to SEA. N= 3-5/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.
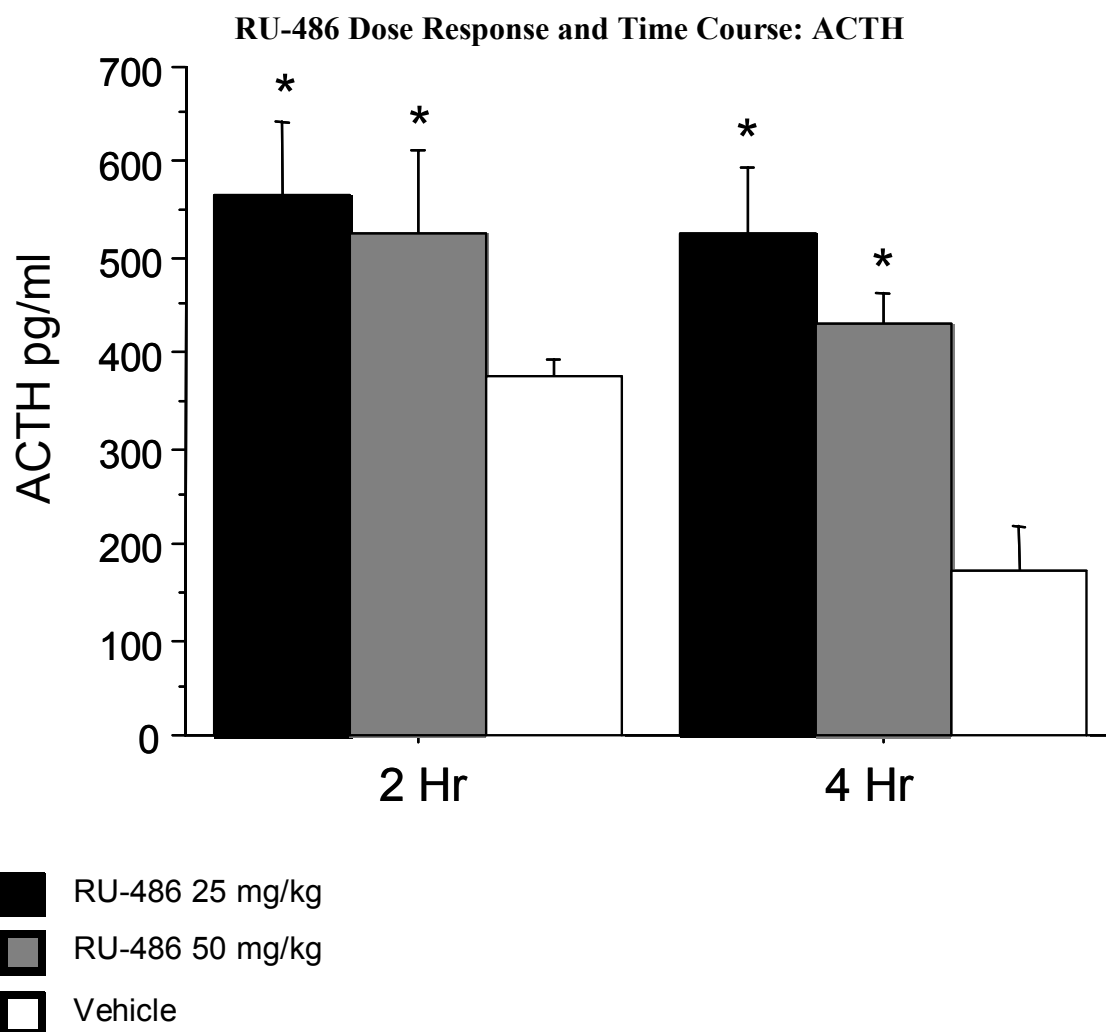
Figure 7.11. Mean ACTH levels at 2 and 4 hours following treatment with 25 mg/kg or 50 mg/kg of RU-486. N= 4-6/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to SEA: Prosobee Consumption
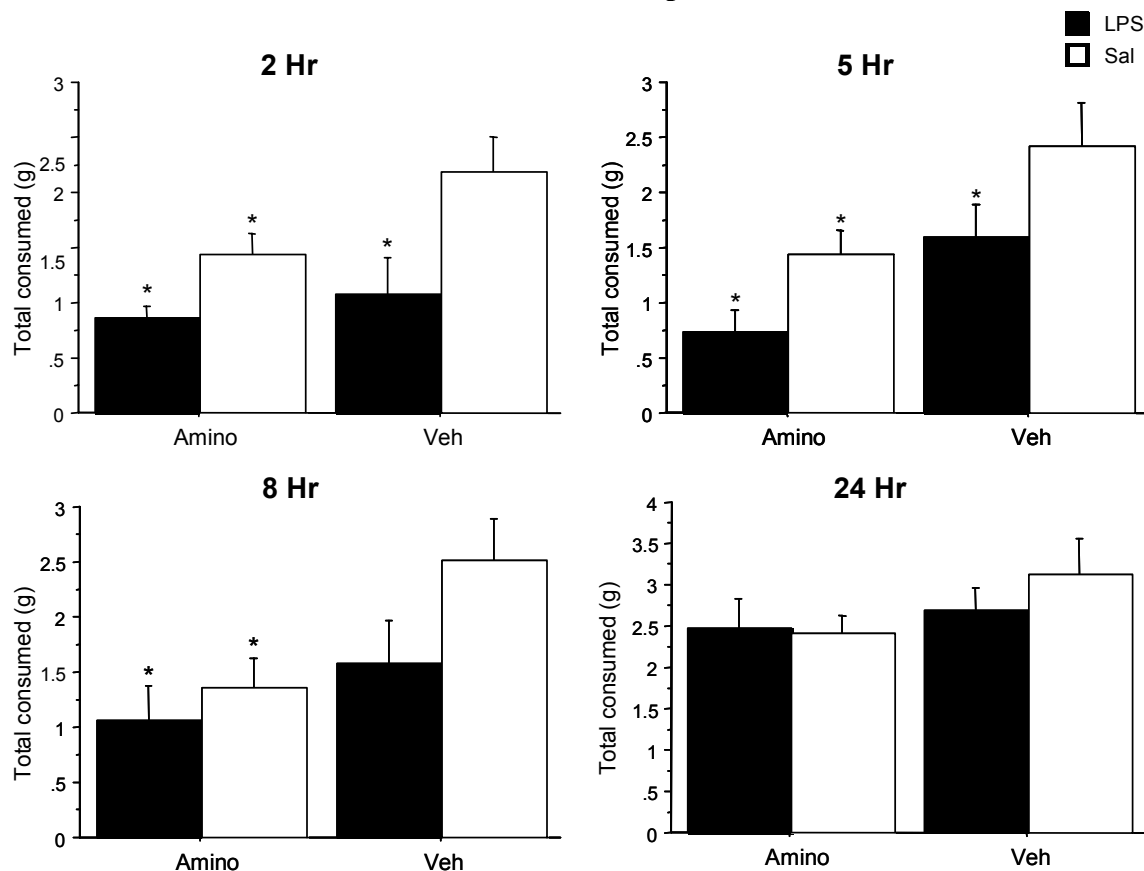

Figure 7.12. The effect of RU-486 pretreatment on consumption at 2, 5, 8, and 24 hours after treatment with SEA. N= 6-8/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

**Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to SEA:
Splenic Cytokines**
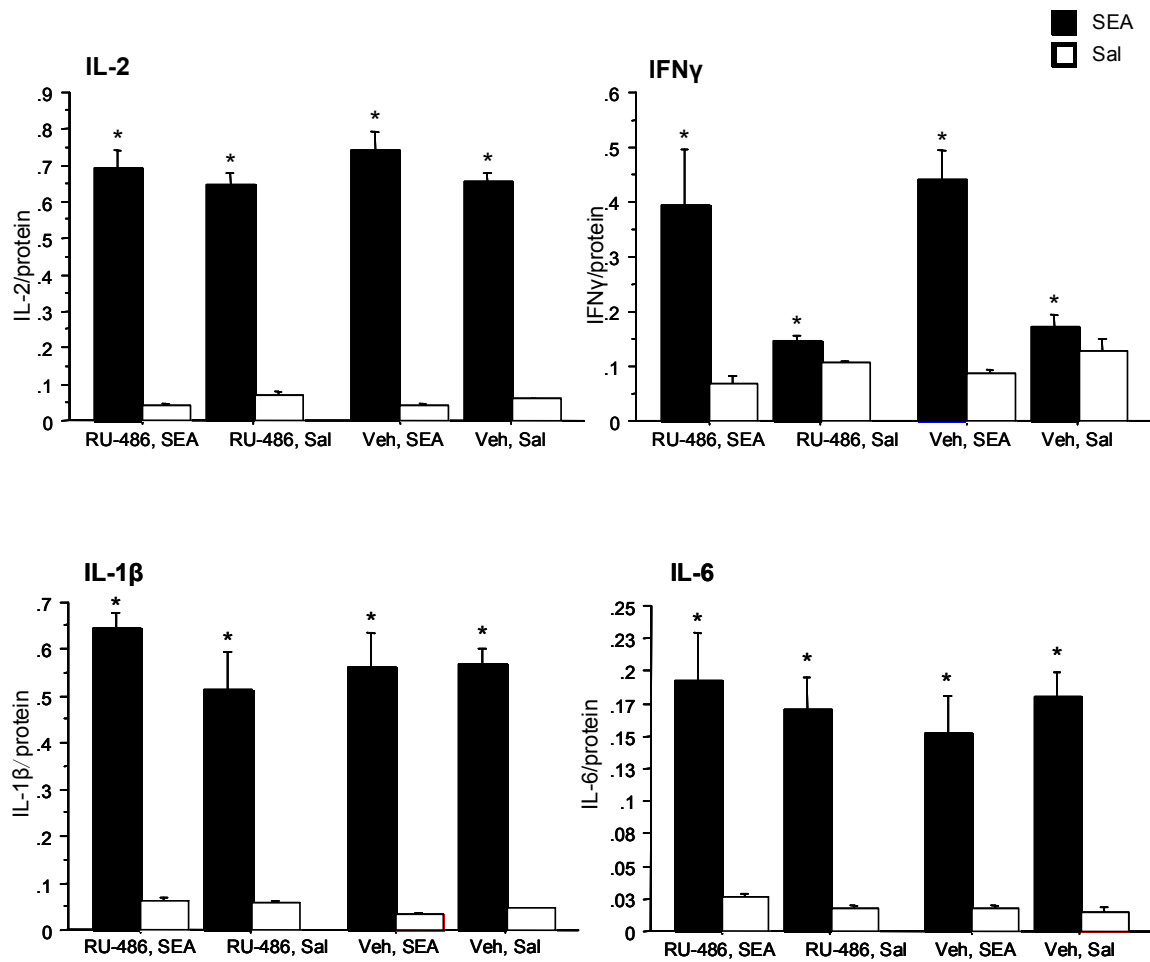
Figure 7.13. The effect of RU-486 pretreatment on splenic IL-2, IFNγ, IL-1β, and IL-6 after primary or secondary exposure to SEA. Data is expressed as pg cytokine/µg protein. N= 2-5/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

**Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to SEA:
Splenic TNFα**
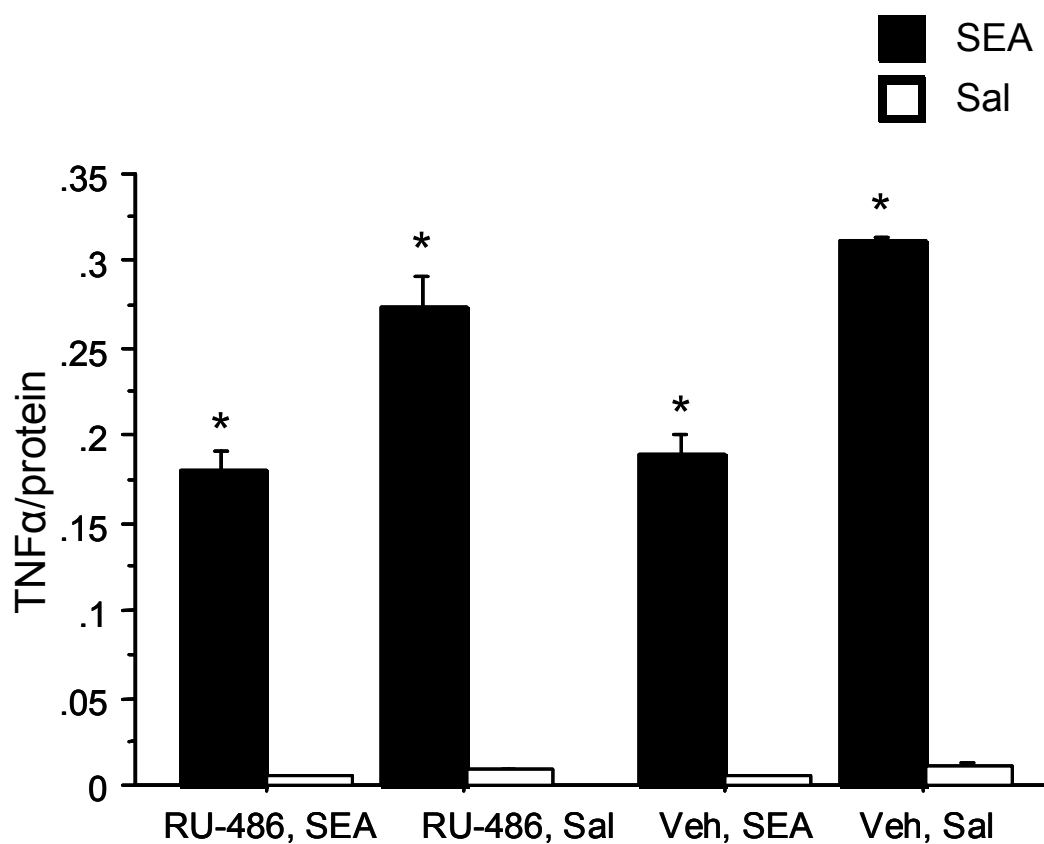
Figure 7.14. The effect of RU-486 pretreatment on splenic TNFα after primary or secondary exposure to SEA. Data is expressed as pg cytokine/μg protein. N= 2-5/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

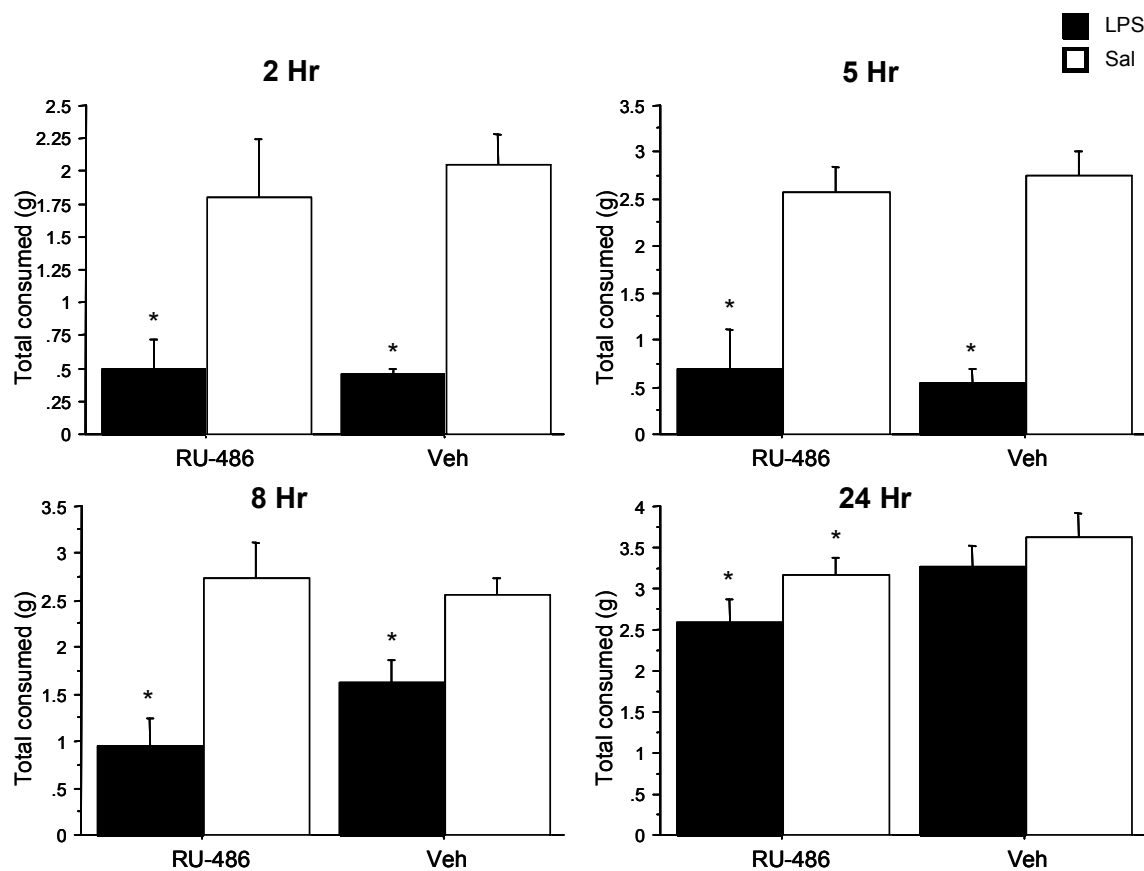**Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to SEA:
Corticosterone**

Figure 7.15. The effect of RU-486 pretreatment on plasma CORT after primary or secondary exposure to SEA. N= 2-5/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

Time Course of AMINO to LPS: Splenic Cytokine and CORT Production
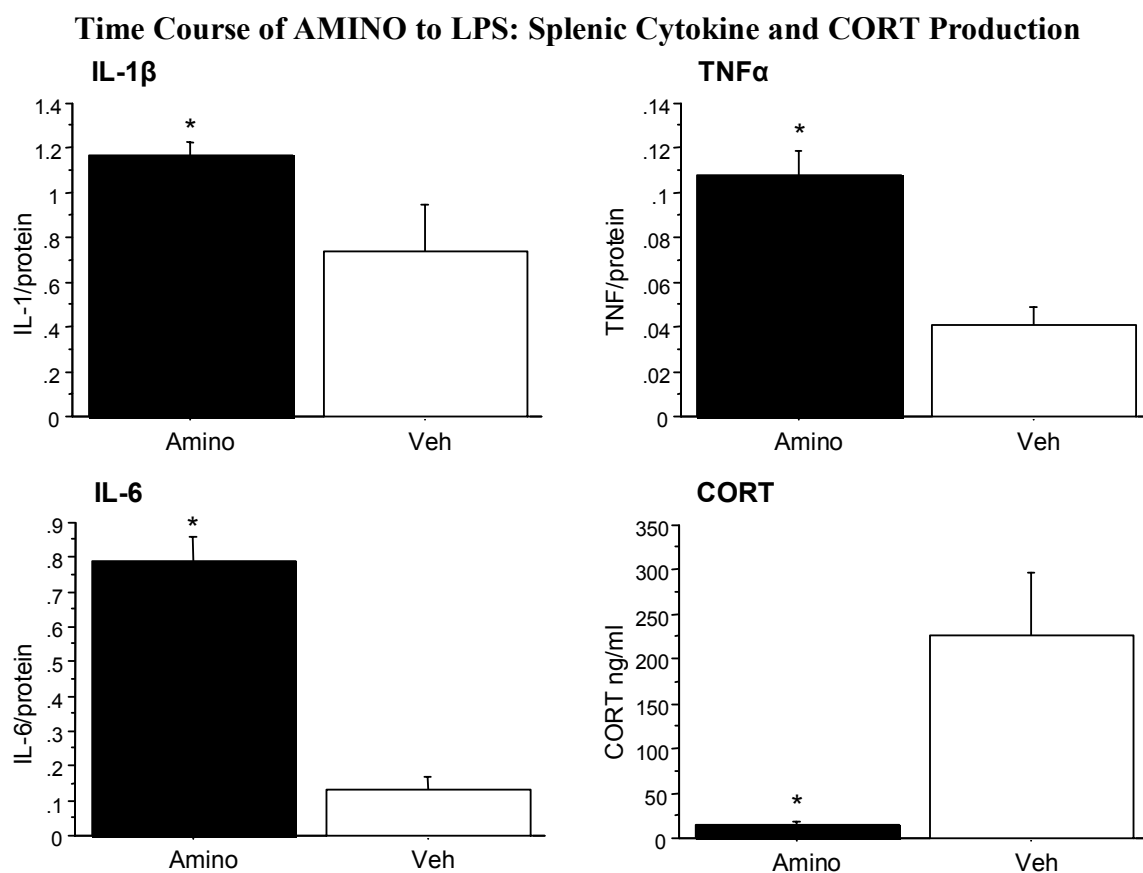
Figure 7.16. The effect of Amino pretreatment on splenic IL-1β, TNFα, IL-6 and plasma CORT after treatment with LPS. Cytokine data is expressed as pg cytokine/μg protein. N= 4-6/group; *p*<.05 compared to Vehicle treated controls. Error bars represent standard error of the mean.

Figure 7.17 The effect of Amino pretreatment on consumption at 2, 5, 8, and 24 hours after treatment with LPS. N= 8/group; $p<.05$ compared to corresponding saline and Vehicle controls. Error bars represent standard error of the mean.

**Effect of Glucocorticoid Inhibition Before Primary Exposure to LPS:
Splenic Cytokines**
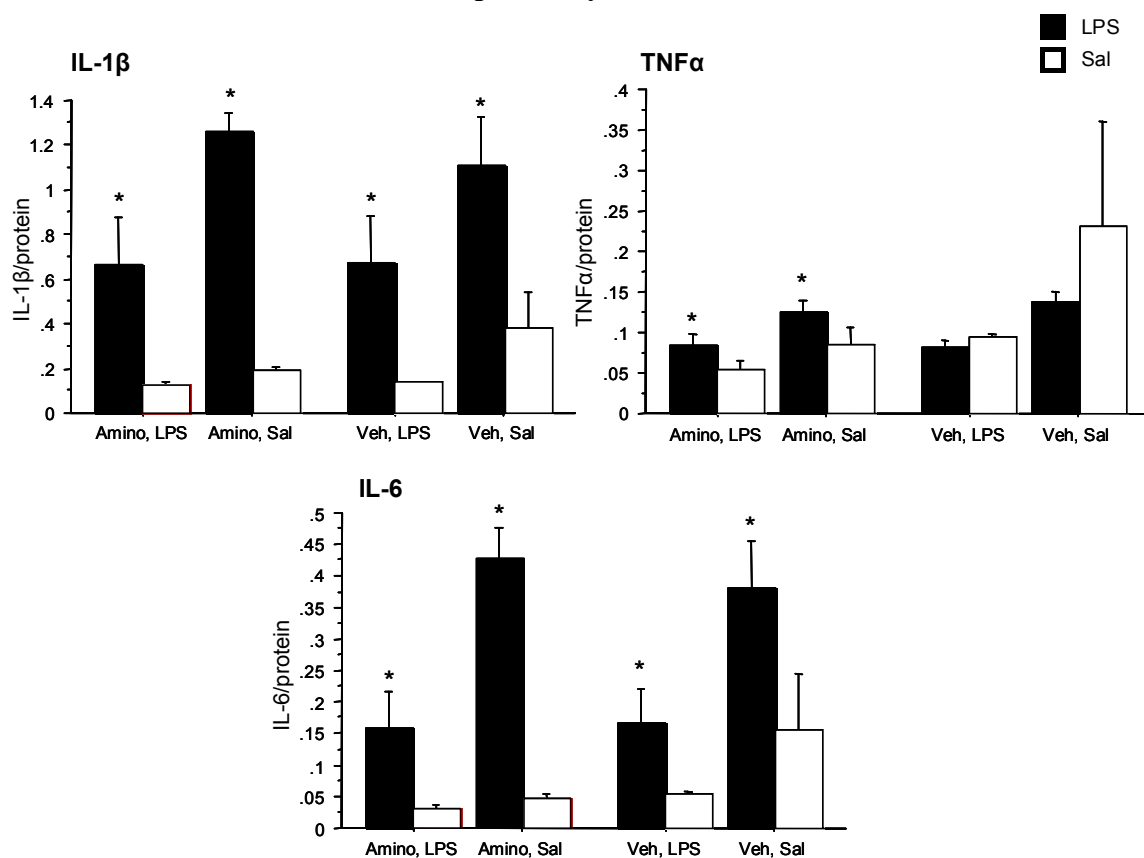
Figure 7.18. The effect of Amino pretreatment on consumption at 2, 5, 8, and 24 hours after treatment with LPS. Data is expressed as pg cytokine/µg of protein. N= 2-5/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.

Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to LPS: Prosobee Consumption

Figure 7.19. The effect of RU-486 pretreatment on consumption at 2, 5, 8, and 24 hours after treatment with LPS. N= 6/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

**Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to LPS:
Splenic Cytokines**
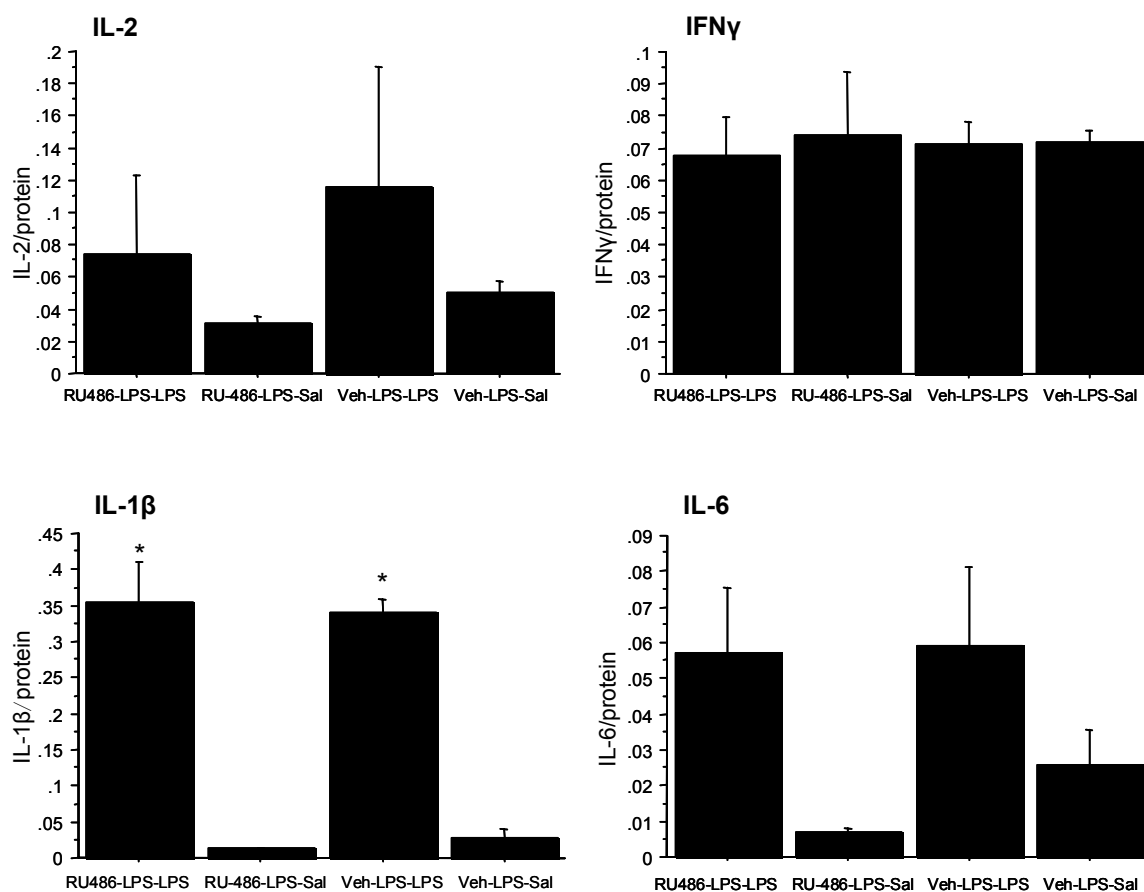
Figure 7.20. The effect of RU-486 pretreatment on splenic IL-2, IFNγ, IL-1β, and IL-6 after primary or secondary exposure to LPS. Data is expressed as pg cytokine/μg of protein. N= 2-4/group; $p<.05$ compared to corresponding saline controls. Error bars represent standard error of the mean.

**Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to LPS:
Splenic TNFα**
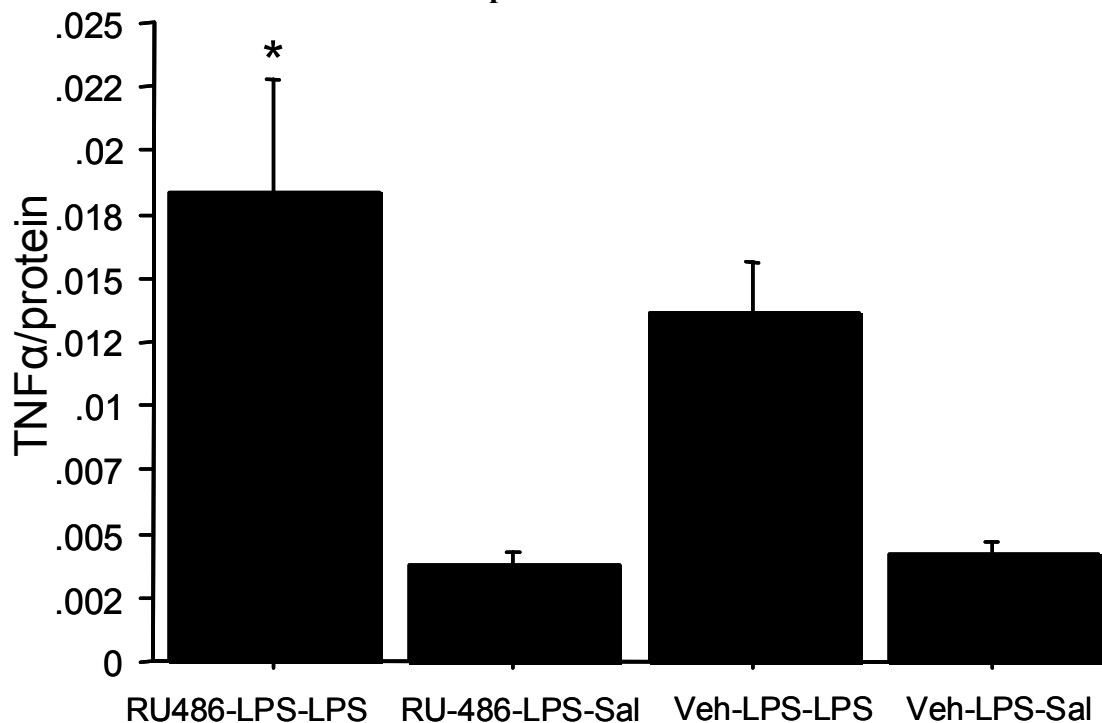
Figure 7.21. The effect of RU-486 pretreatment on splenic TNFα after primary or
secondary exposure to LPS. Data is expressed as pg cytokine/µg of protein. N= 2-4/group;
p<.05 compared to corresponding saline controls. Error bars represent standard error of
the mean.

**Effect of Glucocorticoid Receptor Inhibition Before Primary Exposure to LPS:
Corticosterone**
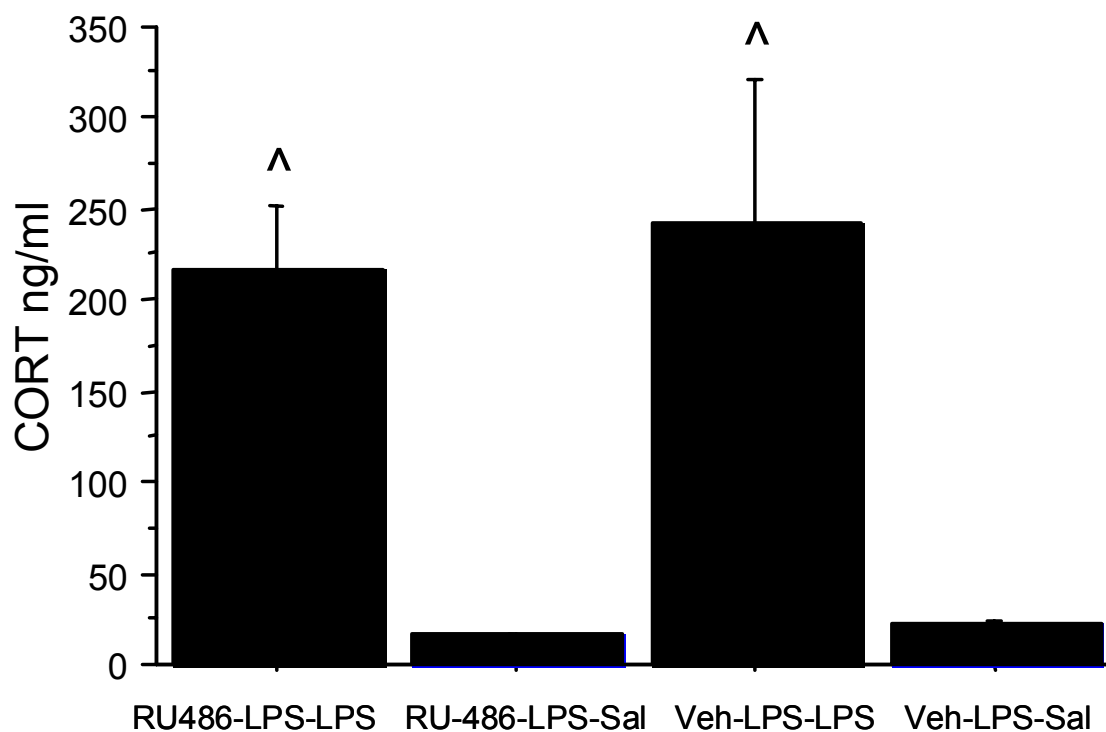
Figure 7.22. The effect of RU-486 pretreatment on CORT after primary or secondary exposure to LPS. N= 2-4/group; ^ = $p<.07$ compared to corresponding saline controls. Error bars represent standard error of the mean.
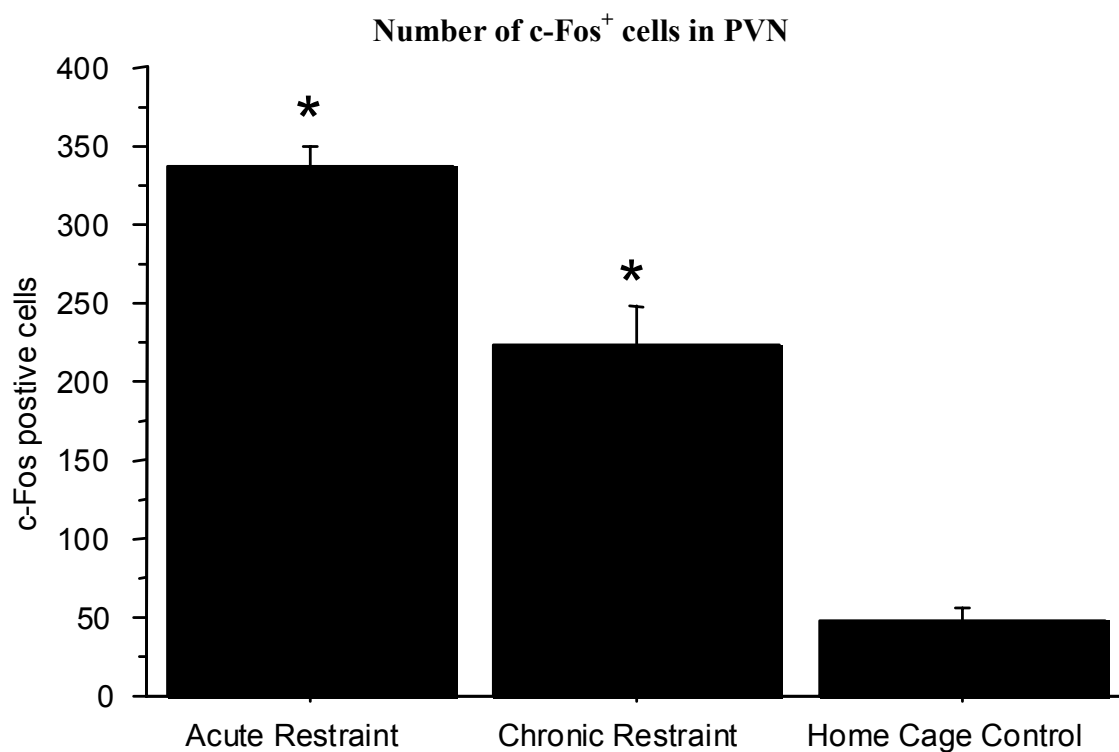
Figure 8.1. Mean number of c-Fos$^+$ cells in the PVN after acute or chronic exposure to restraint stress. N = 6/group; $p<.05$ compared to Home Cage Control. Error bars represent standard error of the mean.

Effect of Acute SEA Following Restraint: Splenic Cytokines
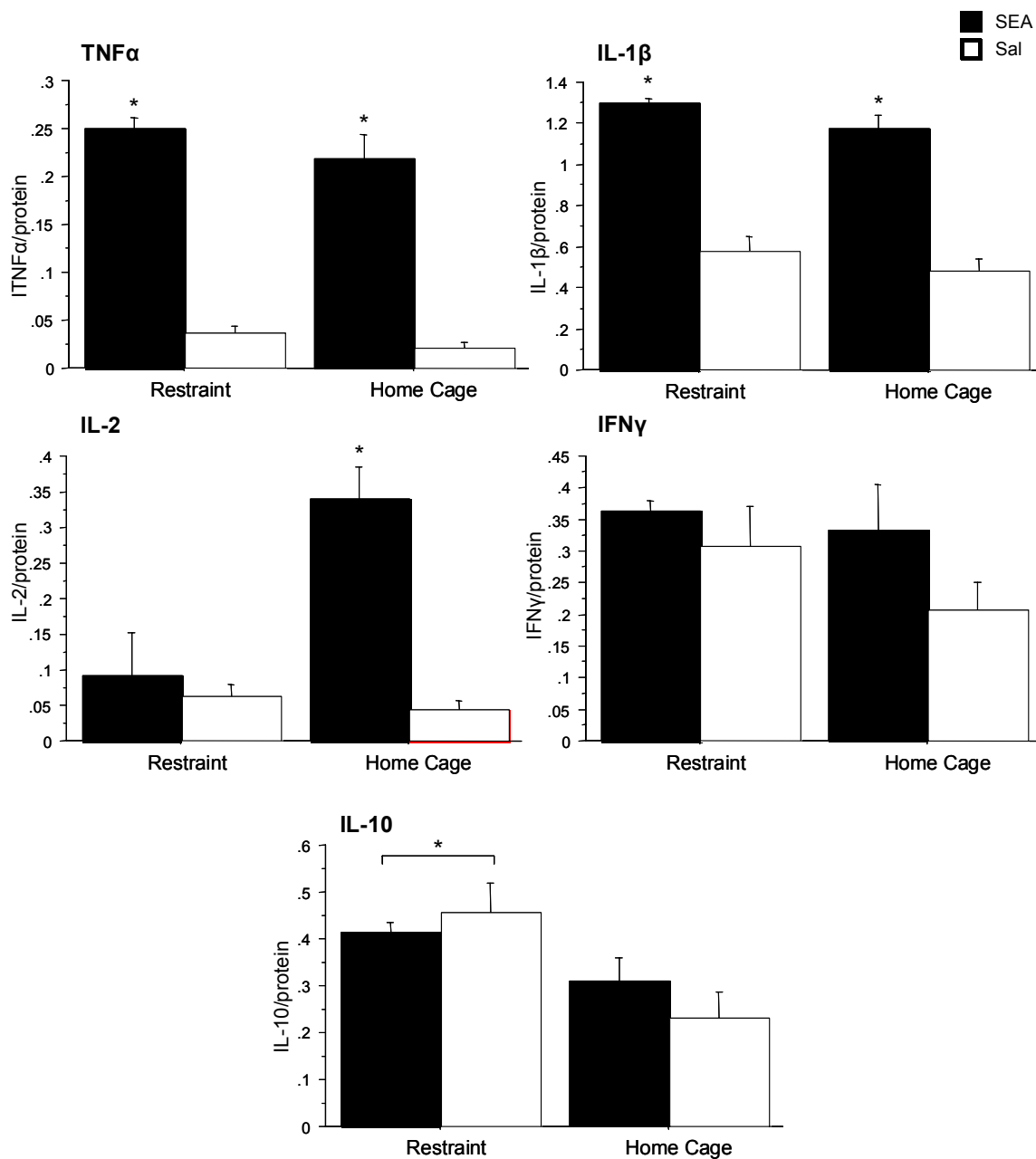
Figure 8.2. Mean splenic TNFα, IL-1β, IL-2, IFNγ, and IL-10 in restrained animals after SEA or saline. Data is expressed as pg cytokine/μg of protein. N = 6/group; *p*<.05 compared to corresponding saline controls or Home Cage controls. Error bars represent standard error of the mean.
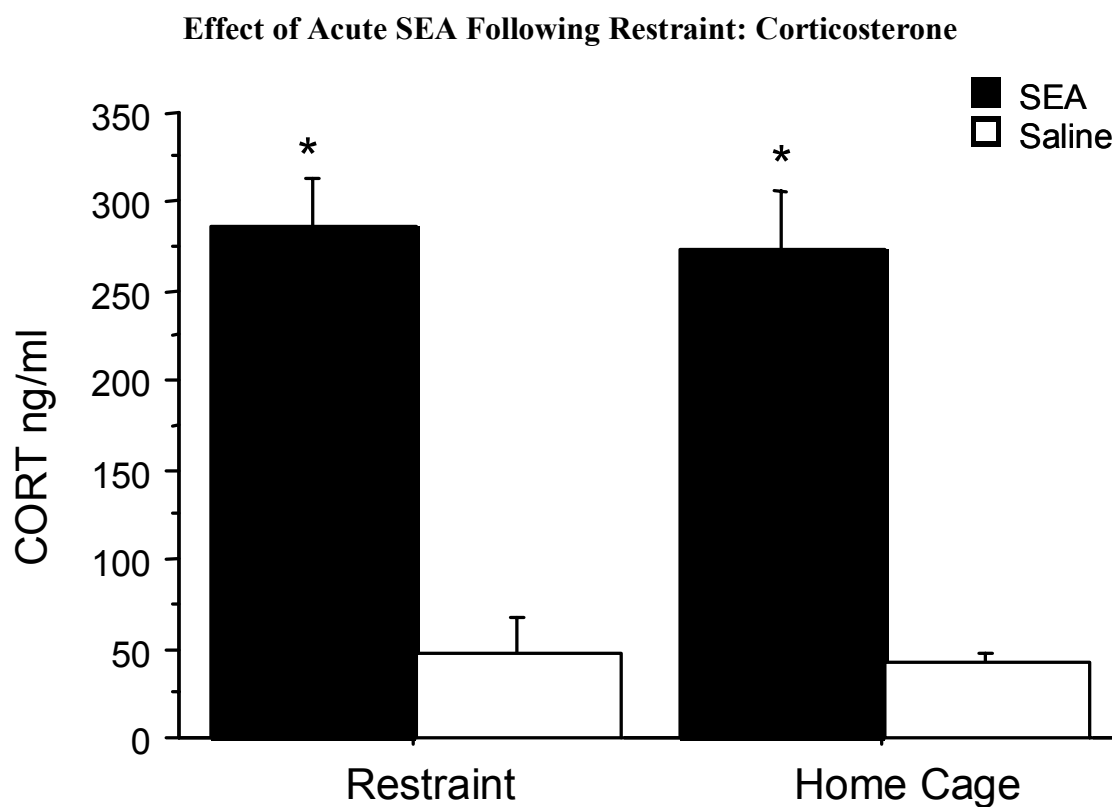
Effect of Acute SEA Following Restraint: Corticosterone

Figure 8.3. Mean CORT in restrained animals after SEA or saline. N = 6/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.
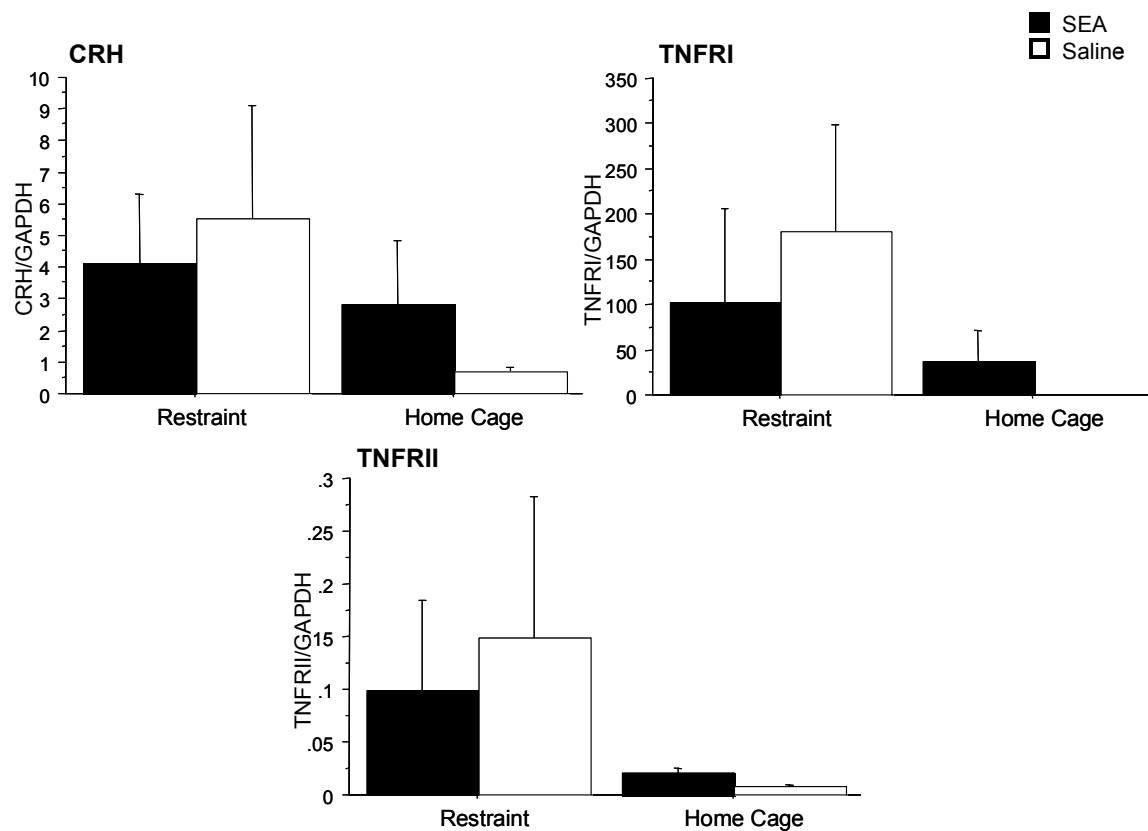
Effect of Acute SEA Following Restraint: Hypothalamic mRNA

Figure 8.4. Mean hypothalamic CRH, TNFRI, and TNFRII mRNA in restraint stressed animals after SEA or saline. N = 6/group. Error bars represent standard error of the mean.

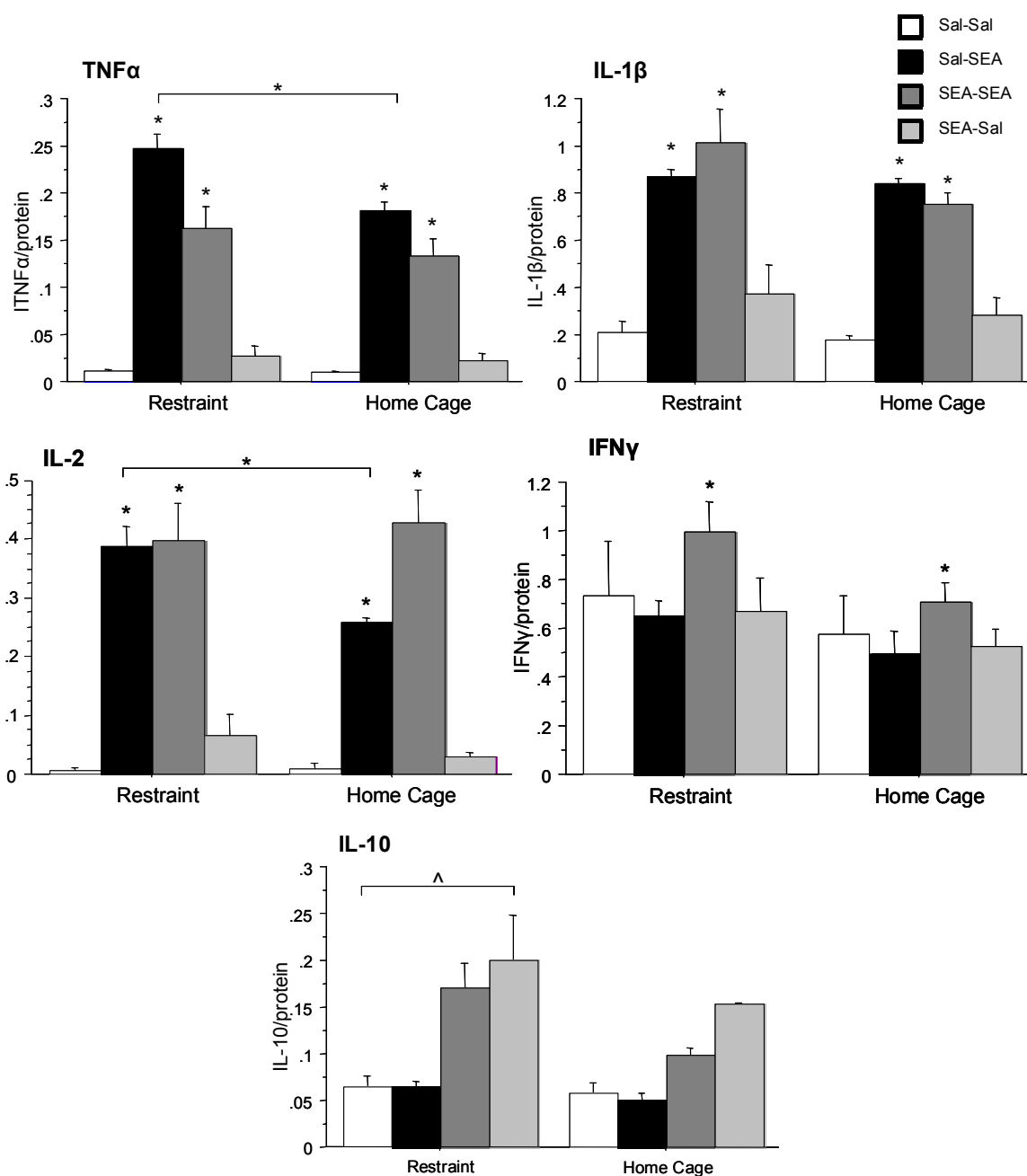**Effect of Acute and Repeated SEA Following Restraint: Splenic Cytokines**

Figure 8.5. Mean splenic TNFα, IL-1β, IL-2, IFNγ, and IL-10 in restrained animals after acute or secondary SEA. Data is expressed as pg cytokine/μg of protein. N = 6/group; *p*<.05 compared to corresponding saline controls or Home Cage controls. Error bars represent standard error of the mean.
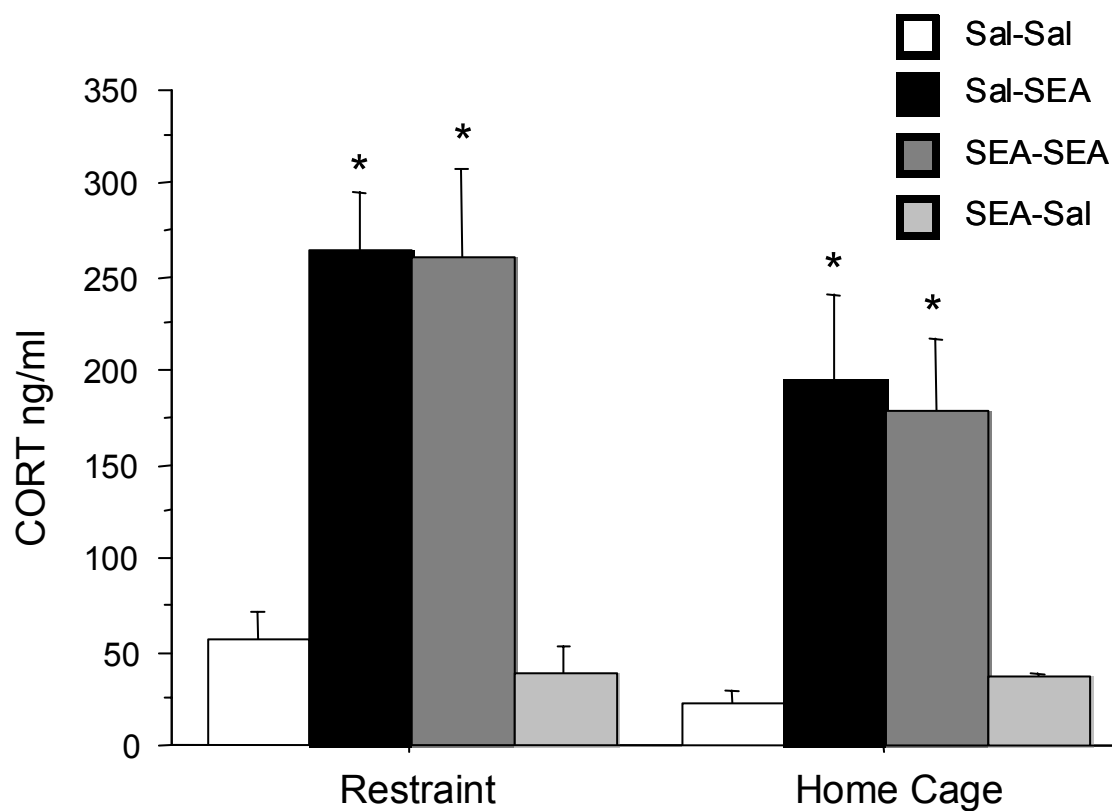
Effect of Acute and Repeated SEA Following Restraint: Corticosterone

Figure 8.6. Mean CORT in restrained animals after acute or secondary SEA. N = 6/group; *p*<.05 compared to corresponding saline controls. Error bars represent standard error of the mean.
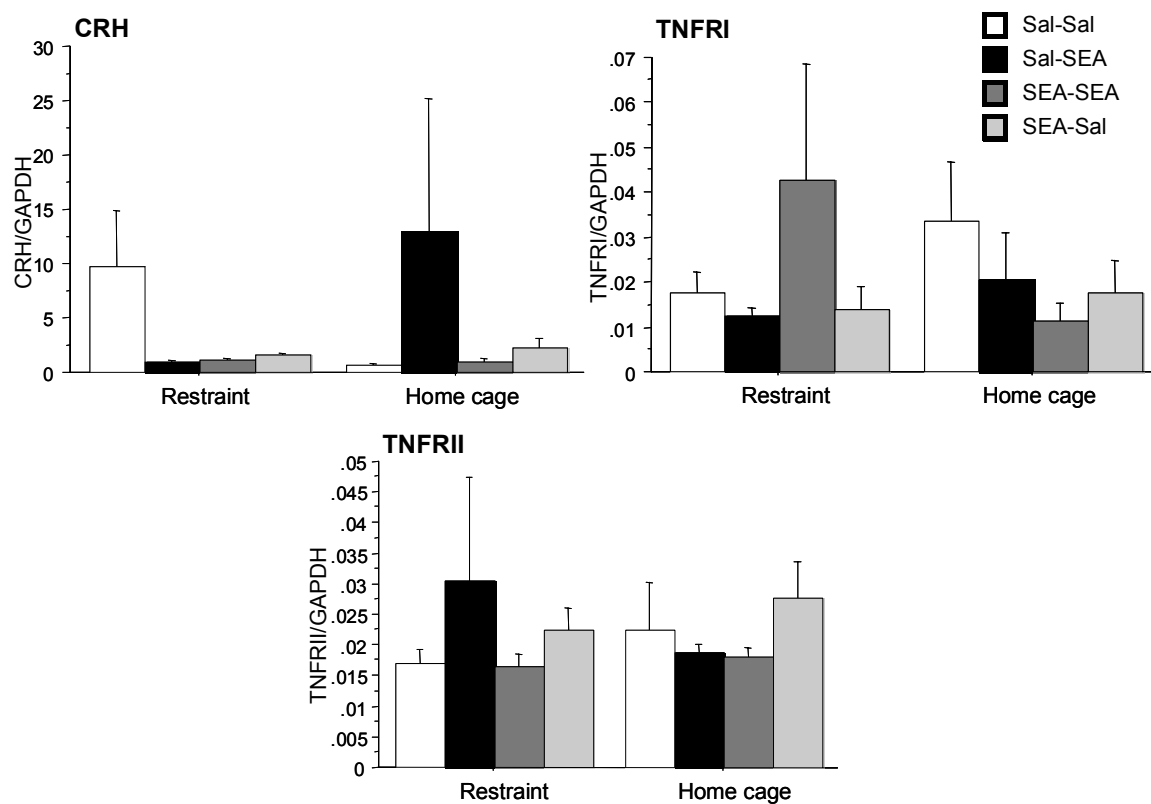
Effect of Acute and Repeated SEA Following Restraint: Hypothalamic mRNA

Figure 8.7. Mean hypothalamic CRH, TNFRI, and TNFRII mRNA in restrained animals after acute or secondary SEA. N = 6/group. Error bars represent standard error of the mean.

Curriculum Vitae

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Education

Ph.D. Toxicology. Rutgers University, New Brunswick, N.J. Expected January 2009.
- Neurotoxicology Track
- <u>Disseration Title</u>: Mechanisms of the Neural and Behavioral Effects of Staphylococcal Enterotoxin A After Acute and Repeated Exposure: The Role of Tumor Necrosis Factor-alpha
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Teaching Experience

Teaching Assistant, Department of Psychology, Rutgers University, Piscataway, NJ, Spring 2002. "Introduction to Psychology and Cognitive Psychology," Dr. Gary Brill.

Volunteer Tutor, Learning Center, Rutgers University, New Brunswick, NJ. Fall 2001-Spring 2002.

Publications

Urbach-Ross, D., Crowell, B., Kusnecov, A.W. (2008). Relationship of varying patterns of cytokine production to the anorexic and neuroendocrine effects of repeated Staphylococcal enterotoxin A exposure. *J Neuroimmunol*. 196, 49-59.

Urbach-Ross, D. & Kusnecov, A.W. (2007). Effects of acute and repeated exposure to lipopolysaccharide on cytokine and corticosterone production during remyelination. *Brain Behav. Immun*. 21, 962-74.

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Rossi-George, A., LeBlanc, F., Kaneta, T., Urbach, D., Kusnecov, A.W. (2004). Effects of bacterial superantigens on behavior of mice in the elevated plus maze and light-dark box. *Brain Behav. Immun*. 18, 46-54.