OPIOID RECEPTOR LIKE-1 RECEPTOR DEFICIENT MICE SHOW DYSREGULATION OF THE HYPOTHALAMIC PITUITARY ADRENAL AXIS FOLLOWING ACUTE IMMUNOLOGIC CHALLENGE WITH STAPHYLOCOCCAL ENTEROTOXIN A

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ABSTRACT OF THE THESIS

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Opioid receptor like-1 receptor (ORL_1) shares considerable sequence identity with the classical μ, δ, and κ opioid receptors yet shows no affinity to the classical opioid ligands. Rather, ORL_1 is selective for its endogenous ligand, orphaninFQ/Nociceptin (OFQ/N). The nociceptin system is integral to many physiological processes (e.g., nociception) and plays a prominent role in regulation of the hypothalamic-pituitary-adrenal (HPA) axis, the stress response, and anxiety. Since immunologic stimuli exert stressor-like effects, the neuroendocrine and behavioral effects of the T-cell superantigen staphylococcal enterotoxin A (SEA) were tested in 129S6, ORL_1 wildtype ($ORL_1^{+/+}$) and knockout ($ORL_1^{-/-}$) mice. Within 2 h of SEA challenge both genotypes showed elevated levels of plasma corticosterone, but only wildtypes remained elevated after 4 h. The effects of SEA on corticosterone levels were determined to be associated with changes in

corticotropin releasing hormone (CRH), CRH receptor 1 (CRH-R1) and CRH-R2 mRNA expression in the hypothalamus. Moreover, SEA-induced changes in CRH and CRH-R1 were dependent on the presence of the ORL_1 gene and suggest that activation of the ORL_1 receptor may modulate positive and negative feedback control of CRH activity in the hypothalamus. These findings are consistent with the idea that ORL_1 activation prolongs stress-induced levels of corticosterone, possibly through ORL_1 dependent modulation of the CRH system. Furthermore, gustatory neophobia due to SEA challenge was augmented in $ORL_1^{-/-}$ mice and is consistent with the anxiolytic role for the nociceptin system. In summary, these results suggest that the ORL_1 gene may be necessary for normal HPA axis activity and may confer resistance to novelty-induced anorexia following acute SEA challenge in 129S6 mice.

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Overview of Thesis

The central nervous system (CNS) reacts to a range of environmental stimuli that can engage various response domains, such as cognition, emotion, motor behavior and basic physiological responses (e.g., autonomic and neuroendocrine function). While many stimuli for CNS function operate through traditional sensory systems, it is well known that endogenous changes induce adaptational and/or homeostatic neuronal responses. Metabolic alterations (e.g., glucose fluctuations) provide one example of an endogenous signaling system, but in recent years, the immune system has also come to represent a source of CNS activation, by virtue of its primary role in responding to infectious agents encountered in the external environment. For example, bacteria and the toxins they produce, strongly activate the immune system, which then mounts an attack against these agents consisting of cellular proliferation, production of antibody, and the release of regulatory molecules called cytokines. The latter are now known to exert neuromodulatory functions, which serve to alter behavior and physiological function. The present thesis will focus on this line of research, testing the hypothesis that the neuroendocrine and behavioral effects of the bacterial toxin, staphylococcal enterotoxin A (SEA), are modulated by Opioid Receptor Like-1 (ORL_1) receptor signaling. As described in detail below, ORL_1 is the endogenous receptor for orphanin FQ/nociceptin (OFQ/N), a neuropeptide implicated in a range of behaviors, including adaptation to stress. However, before reviewing the OFQ/N-ORL_1 (nociceptin) system, a general literature review of the main biological systems addressed in this proposal will be provided.

Chapter 1

General Overview of the Immune System

The immune system is comprised of cells, tissues and organs that distinguish self from non-self and provide host defense through destruction of pathogenic material. All cellular components of the immune system are derived from pluripotent hematopoietic stem cells of the bone marrow (Kumar and Jack 2006). For instance, T and B lymphocytes originate from the common lymphoid progenitor, express antigen-specific receptors and mediate adaptive immunity (Kondo, Weissman et al. 1997). Natural killer (NK) cells are also derived from the common lymphoid progenitor but unlike T and B lymphocytes, NK cells do not express antigen-specific receptors and therefore provide innate immunity (Kondo, Weissman et al. 1997). A second lineage, the common myeloid progenitor, gives rise to granulocytes, macrophages and mast cells that mediate parasitic infection (i.e., eosinophils), phagocytosis (i.e., macrophages and neutrophils) and allergic inflammation, respectively (Akashi, Traver et al. 2000). Lastly dendritic cells (DC) are derived from both common lymphoid and myeloid progenitors and function exclusively as antigen presenting cells (APC) (Kumar and Jack 2006).

Leukocytes limit infection through the production of antibody (by B cells), cytokines and chemokines. Cytokines are small proteins that function as chemical messengers and are produced by most cells of the immune system, in particular macrophages and T cells. For example, activated macrophages secrete proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) and exert local (i.e., activation of vascular endothelium) and systemic (i.e., fever) effects that promote inflammation. In addition, activated macrophages secrete chemokines, or

chemoattractant cytokines such as IL-8 (CXCL8) that induce directed chemotaxis of nearby neutrophils to sites of infection. Cytokines also exert anti-inflammatory effects, for instance, a subset of effector T cells known as helper T (T_h2) cells secrete IL-10 which inhibits cytokine release from activated macrophages (Murphy and Reiner 2002). Through the action of cytokines, leukocytes are able to maintain a balanced immune response and rid the body of infection while minimizing widespread tissue damage.

T cell Immunity and Antigen Presentation

Unlike B cells, immature T cells migrate from the bone marrow to the thymus where they undergo thymic selection (Germain 2002). Specifically, T cells that do not express a functional T cell receptor (TCR) or that exhibit strong reactivity to self-antigen (Ag) are removed via apoptosis (Jameson and Bevan 1998). Developing T cells then differentiate into two main subsets that are characterized by the expression of their T cell co-receptor, CD4 or CD8 (Jameson and Bevan 1998). Mature T cells exit the thymus and re-circulate through the blood and lymph where they bind transiently to each dendritic cell (DC) they encounter.

Immature DCs internalize foreign Ag and receive stimulatory signals (i.e., cytokines) from activated macrophages that prompt their migration to peripheral lymphoid tissue, such as the lymph nodes, and induce their maturation (Mellman and Steinman 2001; Kumar and Jack 2006). As DCs mature they begin to express high levels of the major histocompatibility complex (MHC) class I and class II proteins (Mellman and Steinman 2001). Fragments of digested Ag are then loaded onto MHC proteins and displayed at the cell surface to naïve T lymphocytes (Mellman and Steinman 2001).

Therefore, DCs function as specialized antigen-presenting cells (APCs) and initiate T cell mediated immunity.

Although association of the TCR and MHC molecule is required for Ag presentation, it is insufficient for activation of naïve T cells. Rather, clonal expansion of T lymphocytes requires additional signals from the APC to which it binds (Bretscher and Cohn 1970). Specifically, T cell co-receptors (i.e., CD4 or CD8) must associate with the MHC:TCR complex and co-stimulatory molecules (i.e., B7.1 or B7.2) must signal to their T cell surface receptor, CD28 (Liu and Janeway 1992). In the absence of these co-stimulatory signals naïve T cells become anergic (unresponsive) or die (Bretscher and Cohn 1970). In this way, T cells are precluded from attacking self-Ag and mounting an auto-immune response.

Activated $CD8^+$ and $CD4^+$ T cells target different classes of pathogens and thus, differ in their effector functions (Germain 2002). For example, $CD8^+$ cells recognize and kill intracellular, or cytosolic pathogens complexed to MHC class I molecules whereas $CD4^+$ cells recognize extracellular pathogens presented by MHC class II protein (Germain 2002). Moreover, activated $CD4^+$ lymphocytes differentiate further into T_h1 or T_h2 cells that differ in cytokine production and consequently impact the direction of the adaptive response (i.e., cell-mediated vs. humoral). Specifically, selective production of T_h1 cells results in T cell-mediated immunity that is characterized by cytokine production, phagocytosis and apoptosis (Murphy and Reiner 2002). Conversely, the selective production of T_h2 cells initiates B cell proliferation, Ab production and long lasting, immunological memory (Murphy and Reiner 2002).

Bacterial Superantigens; mechanism of action

During an infection, T lymphocytes secrete cytokines that convey information between immune cells. Usually, Ag is first digested by APC and displayed at its cell surface by MHC protein. Moreover, in order to stimulate T lymphocyte expansion this peptide sequence or epitope must be recognized by the TCR to which it is presented. Although such specificity characterizes adaptive immunity, it inevitably restricts the responsiveness of a mature T cell population and subsequently the magnitude of cytokine production. Nonetheless, in response to infection, cytokines mediate immunological function and alter neuroendocrine and behavioral processes (Assenmacher, Lohning et al. 1998; Kusnecov, Liang et al. 1999; Urbach-Ross, Crowell et al. 2008).

In order to assess CNS function during immunological challenge, previous studies have employed superantigen (SAg) models (Kusnecov, Liang et al. 1999; Kawashima and Kusnecov 2002; Rossi-George, Urbach et al. 2005). Superantigens (Marrack and Kappler 1990) are toxins produced by bacteria (bacterial superantigens) and viruses (viral superantigens). These potent immunogenic proteins are categorized according to the organism from which they originate and can be further classified into subtypes due to variations in their genes (Proft and Fraser 2003). For instance, staphylococcal enterotoxins A (SEA) and B (SEB) are both produced by the bacteria, staphylococcal aureus yet differ from one another in primary amino acid sequence (Proft and Fraser 2003). Moreover, because of the way in which they interact with MHC molecules and the TCR, SAgs produce an exaggerated T cell response that results in excessive cytokine secretion.

Unlike ordinary pathogens, SAgs stimulate T cell proliferation independent of APC processing and instead bind directly to the external domains of the MHC molecule and TCR (Proft and Fraser, 2003; Hong, Waterbury and Janeway, 1996). Specifically, bacterial SAgs bind to the α-region of the MHC II molecule (Proft and Fraser 2003). However, because of variations in structure, the way in which bacterial SAgs bind MHC molecules can vary. For example, SEB binds to the side of an MHC II peptide, away from the Ag recognition site whereas toxic shock syndrome toxin (TSST) extends over the top of the MHC-peptide binding groove (Proft and Fraser 2003). Moreover, while most SAgs bind only to the α-region of the MHC molecule (i.e., SEB and TSST) others such as SEA also bind a high-affinity site located on the β-chain. In this way, MHC molecules crosslink at the surface of an APC resulting in increased production of proinflammatory cytokines such as TNFα (Proft and Fraser 2003). Thus, the MHC-SAg interaction determines the affinity of SAgs for MHC molecules and the magnitude of cytokine secretion.

Similarly, immunological reactivity to SAgs is dependent on where SAgs bind the TCR and the distribution of T cell subsets. Briefly, the TCR is a disulfide-linked heterodimer composed of one α and one β chain that each expresses a variable (V) and constant (C) region. The juxtaposition of the V domains form the Ag binding site and through combinatorial diversity this peptide binding region (PBR) acquires specificity toward only one epitope (clonal specificity) that it subsequently encounters during Ag presentation. Therefore, many clonally-specific TCRs are generated that can recognize a vast array of pathogens.

Despite this Ag specificity relatively invariant sequence motifs are also present on the Vβ chain (20-50; mice and humans) that are encoded by several different genes (i.e., Vβ1, Vβ2, Vβ3 etc). Accordingly, T cells bearing clonal-specificity toward different Ag can be encoded by the same Vβ gene (i.e., Vβ1, Vβ2, Vβ3 etc) and share common, sequence motifs on their TCRs. Thus, bacterial SAgs differ from ordinary Ag in that they bind with varying affinities to the common motifs encoded by the Vβ gene rather than at the traditional PBR (Hong, Waterbury et al. 1996). For example, SEA binds with high affinity to and stimulates $V\beta3^+$ and $V\beta11^+$ T cells while SEB preferentially binds to and activates $V\beta8^+$ T cells (Hong, Waterbury et al. 1996; Proft and Fraser 2003). Thus, regardless of Ag specificity activation of T lymphocytes in response to SAgs is dependent upon the sequence motif that is encoded by each Vβ gene and ultimately expressed on the TCR. Moreover, because the number of possible Vβ gene products is somewhat restricted SAgs engage a considerably larger percentage of all mature T cells (up to 20% vs. less than 1%) than common pathogens (Proft and Fraser 2003).

2. Stress and the Hypothalamic-Pituitary-Adrenal (HPA) axis

Stress may be of psychogenic (processive stressors) or physiological (systemic stressors) origin and is differentially processed by the brain. Whereas processive stress requires interpretation by higher brain structures a systemic stressor circumvents cognitive processing and exerts its effects directly (Herman and Cullinan 1997). Ultimately, stress signals converge in the paraventricular nucleus (PVN) of the hypothalamus where they modulate activation of the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA axis is a major branch of the neuroendocrine system that mediates the physiological and behavioral responses to stress. First, complex signals (i.e., excitatory and inhibitory) are integrated in the hypothalamus and result in either activation or inhibition of the PVN. Activation of the PVN stimulates neurosecretory cells of the hypothalamus to secrete corticotropin-releasing hormone (CRH) into the median eminence. From here CRH travels through the hypophyseal portal system of the pituitary stalk and enters the adenohypophysis (anterior pituitary). In this way, CRH indirectly activates the anterior pituitary which in turn secretes adrenocorticotropic (ACTH) releasing hormone into circulation. ACTH then stimulates the adrenal glands to produce stress hormones, or glucocorticoids.

The complex signals mediating HPA axis activity comprise both stress-inhibitory and excitatory circuits that originate from limbic and brainstem nuclei (Herman and Cullinan 1997). Moreover, these descending and ascending circuits convey information in a stressor-specific manner and are integrated in the PVN. For example, catecholaminergic projections from the brainstem provide direct stimulation of the PVN

in response to systemic stressors such as hypoxia or cytokine administration and activate the HPA axis (Herman and Cullinan 1997; Wang, Wang et al. 2004). Conversely, limbic-forebrain stress circuits process psychogenic stressors (i.e., restraint stress) and activate or inhibit the HPA axis in a region specific manner (Herman and Cullinan 1997). Activation of the HPA axis in response to psychogenic stress is mediated through stimulation of the medial (MeA) and basolateral (BLA) nuclei of the amygdala whereas inhibition of the HPA axis is mediated by the hippocampus. However, the hippocampus and amygdala exhibit sparse connections with the hypothalamus and must relay information to the PVN indirectly through forebrain structures such as the bed nucleus of the stria terminalis (BNST) (Herman and Cullinan 1997).

Despite such stimulus-specific activation and region-specific modulation of the HPA axis, systemic stress (e.g., cytokines) is also processed by limbic-forebrain circuits (Xu, Day et al. 1999). In rats, bilateral lesions of the CeA significantly reduced ACTH release and expression of Fos positive-CRH cells in the PVN, 30 minutes after IL-1β injection (Xu, Day et al. 1999). Moreover, bilateral lesions of the CeA significantly attenuated IL-1β-induced c-fos expression in the BNST, suggesting that in response to immune stress the CeA may mediate HPA axis output indirectly, similar to that of psychogenic stress.

Furthermore, the magnitude and duration of the stress response is mediated by complex feedback signals that alter the synthesis and release of CRH. For example, high levels of circulating glucocorticoids repress CRH gene expression in the PVN but simultaneously increase CRH transcript levels in the CeA (Makino, Gold et al. 1994). While ligand availability and glucocorticoid receptor (GR) expression are important

features of this response, Van der laan et al (2008) determined that GR-mediated repression of the CRH gene is also modulated by the type of transcriptional coregulator that is recruited. Specifically, whereas steroid receptor coactivator (SRC) 1a increased the efficacy of GR-mediated-repression of CRH gene transcription, SRC1e significantly reduced glucocorticoid-dependent repression (van der Laan, Lachize et al. 2008). Importantly, because the expression of these coregulators has been shown to vary across brain regions (Meijer, Steenbergen et al. 2000), the findings of Van der laan et al (2008) are consistent with the opposing actions of glucocorticoids on CRH gene transcription in the PVN and CeA. In this way stress hormones provide negative feedback to the hypothalamus and inhibit further activation of the HPA axis and glucocorticoid release.

The activity of CRH neurons is also regulated by sources of positive feedback during stress. For instance, in rats, acute LPS up-regulates mRNA expression of CRH and CRH receptor 1 (CRH-R1) in CRH neurons of the PVN (Rivest, Laflamme et al. 1995). Moreover, rats pretreated with the CRH-R1 antagonist, CP-154,526 showed a ~50% reduction in ACTH secretion following acute restraint stress (Imaki, Katsumata et al. 2001). Together, these findings suggest that the activity of hypothalamic CRH neurons is regulated, in part, by CRH and CRH-R1, and provides support for the existence of ultra-short loop positive feedback control of CRH in the hypothalamus (Ono, Bedran de Castro et al. 1985). For example, local CRHergic projections originating from the hypothalamus may be augmenting CRH release in response to stress (Ono, Bedran de Castro et al. 1985; Drolet and Rivest 2001). Moreover, upon binding to CRH-R1, CRH can stimulate its own transcription by increasing intracellular levels of the second messenger, cyclic adenosine monophosphate (cAMP). In turn, cAMP phosphorylates

protein kinase A (PKA), an enzyme that modulates protein synthesis by activating the transcription factor cAMP response element binding (CREB) protein (Shepard, Liu et al. 2005).

In addition to activating CRH gene transcription, (Shepard, Liu et al. 2005) argue that increases in intracellular cAMP, in response to stimulation of CRH-R1, also inhibit CRH gene expression by progressively increasing intracellular levels of the repressor protein, inducible cAMP early repressor (ICER). Therefore, increased activity of CRH neurons in response to stress may provide both positive and negative feedback control by initially stimulating and then later repressing CREB-mediated CRH gene transcription (Shepard, Liu et al. 2005; Aguilera, Kiss et al. 2007).

Finally, while CRH and CRH-R1 are critical for activation of the HPA axis (Smith, Aubry et al. 1998; Timpl, Spanagel et al. 1998) and play a large role in controlling the activity of CRH neurons during stress, recovery of the stress response is also mediated by CRH receptor 2 (CRH-R2) (Coste, Kesterson et al. 2000). For instance, relative to wildtype mice, corticosterone levels were significantly increased 90 minutes after acute restraint stress in CRH-R2 deficient (CRH-R2$^{-/-}$) mice (Coste, Kesterson et al. 2000). Moreover, immunological challenge with the proinflammatory cytokine TNFα, acute restraint stress and corticosterone treatment down regulated CRH-R2 expression in mouse cardiomyocytes (Coste, Heldwein et al. 2001). Thus, down-regulation of CRH-R2 in response to acute stress appears to maintain elevated levels of corticosterone that in the context of immunologic stimuli may serve a protective role by exerting anti-inflammatory effects.

In conclusion, central and peripheral signals mediating the stress response are complex and proper control over HPA axis activity likely involves cooperation between several sources of positive and negative feedback. This type of feedback control is important for stress adaptation and restoration of homeostasis as chronic CRH overproduction can lead to HPA axis dysfunction, and can increase some aspects of anxiogenic behavior in mice (van Gaalen, Stenzel-Poore et al. 2002). Moreover, in the context of systemic immune stress, stimulation of glucocorticoid release is important for restraining the inflammatory response but also must be tightly controlled as chronically elevated levels of glucocorticoids can suppress immune function and increase ones vulnerability to infection.

Superantigens and the CNS; neuroendocrine and behavioral effects

Bacterial SAgs produce systemic stress signals that alter neuroendocrine function (Gonzalo, Gonzalez-Garcia et al. 1993; Goehler, Gaykema et al. 2001; Kawashima and Kusnecov 2002; Kaneta and Kusnecov 2005). For instance, acute SEB increased plasma ACTH and corticosterone in BALB/c mice (Shurin, Shanks et al. 1997) and rats (Goehler, Gaykema et al. 2001). In the same way, acute SEA challenge elevated plasma ACTH and corticosterone in immunologically responsive, C57BL/6J mice (Kawashima and Kusnecov 2002). Importantly, the peripheral effects of acute SEB or SEA (i.e., corticosterone secretion) are associated with neuroendocrine changes in the hypothalamus such as increased c-fos expression (Goehler, Gaykema et al. 2001; Rossi-George, Urbach et al. 2005) and up-regulation of CRH mRNA (Kusnecov, Liang et al. 1999) in the PVN. Moreover, SEB and SEA-induced activation of the HPA axis is T cell dependent as mice lacking functional T cells exhibit significantly attenuated, SAg-induced elevations in plasma ACTH and corticosterone (Shurin, Shanks et al. 1997; Kawashima and Kusnecov 2002). Thus, bacterial SAgs such as SEA and SEB exert stimulatory effects on neuroendocrine function in a T cell-dependent manner.

Exaggerated secretion of proinflammatory cytokines in response to SAgs overwhelms the host and suppresses the adaptive immune system from mounting an Ab-specific response. Consequently, heightened secretion of proinflammatory cytokines, namely TNFα can result in systemic toxicity. However, because the sensitivity of rodents to SAgs is orders of magnitude lower than that of humans, bacterial SAgs such as SEA and SEB can be administered at doses in which toxicity is not reached and provide a useful means of assessing how cytokines mediate the neurobiological and behavioral

effects of systemic (immune) stress. For example, it was determined that removal of endogenous TNFα abrogates SEA-induced elevations of ACTH and corticosterone (Rossi-George, Urbach et al. 2005). Moreover, activation of hypothalamic nuclei, such as the PVN and arcuate nucleus (ARC) is significantly attenuated in TNFα deficient ($TNF\alpha^{-/-}$) mice following acute SEA challenge but not psychogenic (restraint) stress (Rossi-George, Urbach et al. 2005).

Changes in behavioral functioning subsequent to SAg exposure are also mediated by cytokines (Kusnecov, Liang et al. 1999; Kawashima and Kusnecov 2002; Rossi-George, Urbach et al. 2005) and indirect activation of the HPA axis (i.e., limbic stress circuits) subsequent to immune stress is of great importance considering that limbic structures (i.e., amygdala) are associated with stress-related behaviors, such as fear responses. For example, mice placed into a novel (or stressful) context and presented with a novel liquid solution exhibit profound anorexia, 2 h after acute SEA (Kawashima and Kusnecov 2002; Kaneta and Kusnecov 2005). However, when endogenous TNFα was neutralized or removed ($TNF\alpha^{-/-}$ mice) SEA-induced anorexia was significantly attenuated (Rossi-George, Urbach et al. 2005). Similarly, in the presence of a novel, non-appetitive stimulus, SEA treated mice exhibit augmented anxiogenic behavior in an open field apparatus, 90 min after injection (Kawashima and Kusnecov 2002). Importantly, unlike other toxins such as LPS, appetitive and non-appetitive neophobia in response to SAgs is not due to general malaise (Kusnecov, Liang et al. 1999; Kawashima and Kusnecov 2002). Thus, under conditions of acute SEA challenge systemic stress signals synergize with psychogenic stress and augment reactivity to novel stimuli.

However, SAgs (and cytokines) may not always exert anxiogenic effects (Zalcman 2001; Rossi-George, LeBlanc et al. 2004). In addition to the open field/novel object test, behavioral assays such as the EPM and light-dark box (LD box) provide reliable measures of anxiety-related behaviors. Briefly, when tested in the EPM or LD box animals with a more anxiogenic phenotype will spend a greater percentage of their time in the closed arms (or dark compartment) of the maze (or LD box) relative to the open, unsafe arms (or light compartment). Moreover, the latency to exit the closed arms of the EPM or dark compartment of the LD box will be significantly greater than that of an animal that is not anxious.

Despite the fact that SEA produced anxiogenic behavior during novel object testing, acute challenge with SEA or SEB did not augment anxiogenic behavior in the EPM or LD box, 2 h after injection (Rossi-George, LeBlanc et al. 2004). Surprisingly, mice injected with SEA or SEB spent a greater percentage of their time in the most distal and middle segments of the open arms during EPM testing than saline treated controls. Moreover, SAg-treated mice did not differ from saline controls on measures of anxiety-related behaviors during the LD box test (Rossi-George, LeBlanc et al. 2004). Although these findings are in direct opposition to idea that SAgs exert stressor-like effects, increased exploration in the EPM may be dependent upon the behavioral effects of IL-2. Both SEA and SEB stimulate production of IL-2 which can enhance exploratory activity in response to a novel context through modulation of dopaminergic transmission (Zalcman 2001).

Overview of the nociceptin system; Opioid Receptor Like-1 Receptor (ORL_1) and OrphaninFQ/ Nociceptin (OFQ/N)

The Opioid Receptor Like-1 Receptor (ORL_1) is a G protein-coupled receptor (GPCR) composed of seven transmembrane-spanning domains (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Nishi, Takeshima et al. 1994; Wick, Minnerath et al. 1994). The ORL_1 receptor shares substantial sequence homology with the classical opioid receptors (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Wick, Minnerath et al. 1994) yet early experiments confirmed that ORL_1 does not bind any known opioid ligand (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994). Thus, the ORL_1 receptor was classified as an orphan receptor. However, shortly after its isolation an endogenous ligand for the ORL_1 receptor was identified; OrphaninFQ/Nociceptin (OFQ/N). Like the ORL_1 receptor, OFQ/N resembles the endogenous opioid peptides in structure and does not interact with components of the classical opioid system. Rather, OFQ/N is selective for the ORL_1 receptor (Bunzow, Saez et al. 1994; Mollereau, Parmentier et al. 1994; Reinscheid, Ardati et al. 1996).

Both ORL_1 and OFQ/N are expressed throughout the central nervous system (CNS) and periphery (Bunzow, Saez et al. 1994; Nothacker, Reinscheid et al. 1996; Neal, Mansour et al. 1999; Neal, Mansour et al. 1999; Pampusch, Serie et al. 2000) and previous studies have proposed a modulatory role for the nociceptin system (ORL_1 and OFQ/N) in several physiological and behavioral processes such as nociception (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995), consummatory behavior (Pomonis, Billington et al. 1996; Polidori, de Caro et al. 2000), anxiogenic behavior (Koster, Montkowski et al. 1999; Jenck, Wichmann et al. 2000), as well as immune

(Kawashima, Fugate et al. 2002; Leggett, Dawe et al. 2009) and endocrine function (Devine, Watson et al. 2001; Fernandez, Misilmeri et al. 2004; Green, Barbieri et al. 2007).

ORL_1 Receptor Distribution and Function

As summarized in table 1, the ORL_1 receptor is widely expressed throughout the CNS (Bunzow, Saez et al. 1994; Chen, Fan et al. 1994; Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994; Wick, Minnerath et al. 1994; Lachowicz, Shen et al. 1995). Specifically, moderate to high ORL_1 receptor distribution is observed in limbic structures such as the hippocampus and amygdala (Bunzow, Saez et al. 1994; Mollereau, Parmentier et al. 1994; Lachowicz, Shen et al. 1995) and in the hypothalamus (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994; Lachowicz, Shen et al. 1995). High expression of ORL_1 receptor mRNA is also exhibited in the bed nucleus of stria terminalis (Lachowicz, Shen et al. 1995), septal nuclei (Mollereau, Parmentier et al. 1994; Lachowicz, Shen et al. 1995), and dorsal and ventral horns of the spinal cord (Bunzow, Saez et al. 1994; Mollereau, Parmentier et al. 1994). Moderate ORL_1 expression has been detected in olfactory nuclei (Mollereau, Parmentier et al. 1994), superficial and deep layers of the cerebral cortex (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994; Lachowicz, Shen et al. 1995), nucleus accumbens shell (Mollereau, Parmentier et al. 1994), thalamus (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Wang, Johnson et al. 1994; Lachowicz, Shen et al. 1995) and globus pallidus (Bunzow, Saez et al. 1994;

Lachowicz, Shen et al. 1995). Finally, low to near absent expression of ORL_1 receptor mRNA is observed in the caudate-putamen (Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Lachowicz, Shen et al. 1995), nucleus accumbens core, corpus callosum, white matter of the anterior commissure, (Mollereau, Parmentier et al. 1994), and cerebellum (Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994).

Characterization of peripheral ORL_1 receptor distribution has been less extensive, however, ORL_1 receptor mRNA has been detected in several tissues (see table 1) (Wang, Johnson et al. 1994; Halford, Gebhardt et al. 1995; Lachowicz, Shen et al. 1995). In the rat, ORL_1 receptor mRNA is observed in the intestine, skeletal muscle, vas deferens and spleen (Wang, Johnson et al. 1994) as well as the liver and adrenal gland (Lachowicz, Shen et al. 1995). Conversely, ORL_1 receptor expression is absent in the heart, kidney, lung, ovary, retina and pancreas (Lachowicz, Shen et al. 1995). Therefore, broad yet restricted expression of ORL_1 throughout the CNS suggests a role for this receptor in modulation of cognitive processes, pain perception, and neuroendocrine function (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Lachowicz, Shen et al. 1995). Furthermore, peripheral expression of the ORL_1 receptor implicates its signaling in many physiological processes, including, modulation of immune function (Halford, Gebhardt et al. 1995; Peluso, LaForge et al. 1998; Finley, Happel et al. 2008).

Early attempts at isolating an endogenous ligand for the ORL_1 receptor were unsuccessful as ORL_1 did not show an affinity to any known opioid receptor ligands (Bunzow, Saez et al. 1994; Fukuda, Kato et al. 1994; Wick, Minnerath et al. 1994;

Lachowicz, Shen et al. 1995). For example, ORL_1 did not bind μ opioid-selective agonists [D-Ala^2, $MePhe^4$, Gly-Ol^5] enkephalin (DAMGO), buprenorphine, or phenylalanine-cysteine-tyrosine-D-tryptophan-ornithine-threonine-penicillamine-threonine (CTOP) (Bunzow, Saez et al. 1994; Chen, Fan et al. 1994; Fukuda, Kato et al. 1994; Wang, Johnson et al. 1994; Lachowicz, Shen et al. 1995). Similarly, ORL_1 showed no specific binding activity for κ opioid-selective agonists ethylketocylazocine, bremazocine, U69,593, U50488, U-62066 or benzenacelamide, nor δ opioid-receptor agonists [D-Ala^2, D-Leu^5] enkephalin (DADLE), [D-$penicillamine^2$, D-$penicillamine^3$] enkephalin (DPDPE) or naltrindole (Bunzow, Saez et al. 1994; Chen, Fan et al. 1994; Fukuda, Kato et al. 1994; Wang, Johnson et al. 1994; Wick, Minnerath et al. 1994; Lachowicz, Shen et al. 1995).

Universal, non-selective opiate ligands etorphine, diprenorphine, naloxone and β-endorphin were also ineffective in ORL_1 receptor stimulation (Bunzow, Saez et al. 1994; Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994; Wick, Minnerath et al. 1994; Lachowicz, Shen et al. 1995). Furthermore, no specific binding of σ opioid receptor ligands (haloperidol, SKF-10,047 and 1,3-di-O-tolylguanidine) or somatostatin receptor agonists (somatostatin-14 and somatostatin-28) to the ORL_1 receptor could be detected (Fukuda, Kato et al. 1994; Mollereau, Parmentier et al. 1994; Lachowicz, Shen et al. 1995). Consequently, ORL_1 was classified as an orphan, opioid-like receptor.

Orphanin FQ/Nociceptin (OFQ/N); Endogenous ligand for the ORL_1 receptor

High sequence identity between ORL_1 and opioid receptors prompted further investigation into the identification of an endogenous ligand for the newly discovered, orphan receptor. In particular structural similarities of extracellular loop 2, a domain required for high-affinity binding of dynorphins (Wang, Johnson et al. 1994; Xue, Chen et al. 1994), suggested that the endogenous ligand to ORL_1 may resemble a dynorphin in structure (Meunier, Mollereau et al. 1995). Indeed, shortly after isolation of ORL_1 a 17 amino acid, heptadecapeptide, most closely related to opioid peptide dynorphin A, was identified (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995). This novel peptide was termed nociceptin, for its pro-nociceptive properties (Meunier, Mollereau et al. 1995) or orphaninFQ, for its affinity to the orphan opioid receptor, ORL_1 and amino acid sequence (Reinscheid, Nothacker et al. 1995).

OrphaninFQ/Nociceptin (OFQ/N) binds selectively to ORL_1 receptors and inhibits forskolin-induced accumulation of cyclic adenosine monophosphate (cAMP) (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995). Moreover, OFQ/N lacks affinity for opioid receptors and upon binding to ORL_1 is not affected by opioid agonists or antagonists (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995). Thus, similar to ORL_1, OFQ/N does not interact with components of the traditional opioid system (Reinscheid, Nothacker et al. 1995; Mogil, Grisel et al. 1996; Mogil and Pasternak 2001).

OFQ/N is derived from a larger precursor protein, preproOFQ/N (see Figure 1; ppOFQ/N) (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995; Pan, Xu et al. 1996) that, in addition to coding for OFQ/N, is cleaved to form other biologically

active peptides (Meunier, Mollereau et al. 1995; Okuda-Ashitaka, Minami et al. 1998; Reinscheid, Nothacker et al. 2000; Mogil and Pasternak 2001). For example, nocistatin, a 35 amino acid peptide lies immediately upstream and NocII/OFQII, a 17 amino acid peptide is found immediately downstream of OFQ/N (Meunier, Mollereau et al. 1995; Okuda-Ashitaka, Minami et al. 1998; Reinscheid, Nothacker et al. 2000). In this way ppOFQ/N is similar to pro-opiomelanocortin (POMC), the precursor to several unrelated bioactive peptides (Meunier, Mollereau et al. 1995). Thus, similarities observed between the derivation and structure of traditional opioid peptides and OFQ/N suggest a possible evolutionary relation. However, lack of interaction between OFQ/N and opioid receptors implies a pharmacological divergence between these two systems (Reinscheid, Nothacker et al. 1995).

Distribution of ppOFQ/N and OFQ/N mRNA has been characterized throughout the CNS and a good correlation exists between precursor and protein (see table 1) (Reinscheid, Nothacker et al. 2000). Light to moderate expression of ppOFQ/N mRNA has been observed throughout the neocortex with the density of staining varying across regions (Neal, Mansour et al. 1999). Distribution of ppOFQ/N positive cells is most abundant in prefrontal and frontal cortical regions, orbital, piriform and entorhinal cortices (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000). Conversely, sparse expression of ppOFQ/N is exhibited in temporal and parietal regions (Neal, Mansour et al. 1999).

Moderate expression of ppOFQ/N mRNA is found throughout the nucleus accumbens shell while a light to scattered distribution of OFQ/N-containing neurons is detected in the nucleus accumbens core, ventral pallidum and olfactory nucleus (Neal,

Mansour et al. 1999). Within the forebrain, ppOFQ/N mRNA expression is highest in the basal telencephalon, specifically in the bed nucleus of stria terminalis (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000). Moreover, OFQ/N immunoreactivity is prominent throughout the septum and substantia nigra (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000). Finally, moderate and intense expression of OFQ/N is exhibited in the locus coeruleus and spinal cord, respectively (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000).

Discrepancies in the distribution of ppOFQ/N mRNA are evident (Mollereau, Simons et al. 1996; Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000). For example, Neal et al (1999) report intense ppOFQ/N expression throughout the globus pallidus and light mRNA expression in the nucleus accumbens. Alternatively, Houtani et al (2000) found no appreciable amount of ppOFQ/N mRNA in these same regions. This discrepancy in mRNA expression may be attributable to the species used, mouse (Houtani, Nishi et al. 2000) versus rat (Neal, Mansour et al. 1999), rather than methodology. Nevertheless, structures such as the fornix, caudaute putamen, cerebellum, pituitary and pineal glands are devoid of ppOFQ/N expressing cells (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000).

Despite such discrepancies in region-specific ppOFQ/N distribution, the most notable mRNA expression is found in limbic regions, a finding that is consistent across studies (Mollereau, Simons et al. 1996; Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000). For example, high expression of ppOFQ/N mRNA is found in central and medial nuclei of the amygdala (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000). Moreover, a moderate to

strong distribution of ppOFQ/N positive neurons is exhibited in the granule cell layer of the dentate gyrus (DG) with a strong yet scattered pattern of mRNA expression throughout *Cornu Ammonis* (CA) regions 1 and 3 of the hippocampus (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000). Similarly, heavy mRNA expression is observed throughout the hypothalamus, specifically in the medial zone of the anteroventral preoptic nucleus and the arcuate nucleus (Neal, Mansour et al. 1999; Houtani, Nishi et al. 2000; Reinscheid, Nothacker et al. 2000).

Although not as pronounced as its cognate receptor, expression of ppOFQ/N has also been observed in the periphery (see table 1) (Mollereau, Simons et al. 1996; Pampusch, Serie et al. 2000; Reinscheid, Nothacker et al. 2000). Specifically, ppOFQ/N mRNA has been detected in porcine splenic and thymic tissue (Pampusch, Serie et al. 2000), human adult spleen and peripheral blood leukocytes (PBL), fetal kidney (Nothacker, Reinscheid et al. 1996) and rat ovary (Mollereau, Simons et al. 1996). However, ppOFQ/N is absent from rat intestine, liver, stomach, lung, adrenal gland and testis (Mollereau, Simons et al. 1996). Overall, expression of OFQ/N and ORL_1 correlate well in the rodent CNS (Mollereau, Simons et al. 1996). However, peripheral expression of OFQ/N is less consistent with that of ORL_1, in particular in rodent immune tissue (Mollereau, Simons et al. 1996). Nevertheless, disparity between expression of OFQ/N and ORL_1 may represent differences between OFQ/N sites of synthesis and sites of action as a more restricted distribution of OFQ/N (relative to ORL_1) has been suggested (Reinscheid, Nothacker et al. 2000).

Functional Role of the Nociceptin System

Widespread expression of ORL_1 and OFQ/N throughout the CNS suggests a modulatory role for this system in CNS function and a variety of physiological processes (Mogil and Pasternak 2001). Much attention has been placed on the involvement of this peptide in the mediation of nociception via supraspinal, spinal and peripheral mechanisms (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995; Rossi, Perlmutter et al. 1998; Mogil and Pasternak 2001). However, ORL_1 signaling modulates the development of drug addiction (Ciccocioppo, Economidou et al. 2004; Lutfy, Lam et al. 2008; Marquez, Nguyen et al. 2008), locomotion (Reinscheid, Nothacker et al. 1995; Florin, Suaudeau et al. 1997; Reiss, Wichmann et al. 2008), cognitive function (Goda and Mutneja 1998; Mamiya, Noda et al. 1998; Nabeshima, Noda et al. 1999), energy homeostasis (Pomonis, Billington et al. 1996; Olszewski, Billington et al. 2000; Polidori, de Caro et al. 2000), and in particular, hypothalamic-pituitary-adrenal (HPA) axis function, and anxiogenic behavior (Jenck, Moreau et al. 1997; Devine, Watson et al. 2001; Ciccocioppo, Biondini et al. 2002; Fernandez, Misilmeri et al. 2004). Moreover, an immunomodulatory for ORL_1 signaling has been proposed (Buzas, Rosenberger et al. 2002; Kawashima, Fugate et al. 2002; Zhao, Huang et al. 2002; Goldfarb, Reinscheid et al. 2006).

Nociception

Upon its isolation, OFQ/N was first shown to mediate nociception in a manner opposite to that of the endogenous opioids (Meunier, Mollereau et al. 1995; Reinscheid, Nothacker et al. 1995). For example, in mice, intracerebroventricular (icv) administration of 100ng OFQ/N significantly decreased latencies to rearing and escape jumping in a hot plate test (Meunier, Mollereau et al. 1995). Moreover, Reinscheid et al (1995) found that mice treated with OFQ/N (0.3 to 10 nmol, icv) had significantly reduced reaction times in the tail flick assay, relative to that of saline treated animals. Thus, it was concluded that exogenous stimulation of ORL_1 produced hyperalgesia, however, subsequent studies have revealed the complex manner in which ORL_1 activation modulates pain transmission (Mogil, Grisel et al. 1996; Rossi, Perlmutter et al. 1998; Vanderah, Raffa et al. 1998; Heinricher 2005).

For example, in opposition to the findings of Meunier et al (1995) and Reinscheid et al (1995), icv or intrathecal (IT) administration of OFQ/N was shown to have either no effect on pain sensitivity (Vanderah, Raffa et al. 1998) in mice or rats, or to produce analgesia in rats (Rossi, Perlmutter et al. 1998). Furthermore, Mogil et al (1996) suggest that icv administration of OFQ/N does not produce hyperalgesia but rather reverses opioid-mediated, stress-induced antinociception. Specifically, when tested for nociceptive responses in three different algesiometric assays, OFQ/N-treated animals did not differ from un-injected animals in their sensitivity to a noxious stimulus. However, vehicle-treated mice displayed significantly reduced nociceptive sensitivity, a finding that was attributed to stress-induced antinociception and reversed by administration of OFQ/N (Mogil, Grisel et al. 1996). Therefore, care must be taken when interpreting the impact

of ORL_1 signaling on nociception as results may vary with factors such as route of administration, species and testing conditions (Heinricher 2005).

Energy Homeostasis

Several studies have shown that the nociceptin system modulates consummatory behavior similar to that of other members of the opioid family (Pomonis, Billington et al. 1996; Stratford, Holahan et al. 1997; Polidori, de Caro et al. 2000). Specifically, icv administration of OFQ/N (1-10 nmol) into the lateral or third ventricles stimulates feeding in non-food deprived rats (Pomonis, Billington et al. 1996; Polidori, de Caro et al. 2000). Moreover, site specific microinjection of OFQ/N into areas such as the ventromedial hypothalamus (VMH), nucleus accumbens shell (AcbSh) (Stratford, Holahan et al. 1997), and hypothalamic arcuate nucleus (ARC) induce hyperphagia in free-feeding rats (Polidori, de Caro et al. 2000). The hyperphagia observed following site-specific OFQ/N infusion is not surprising as both OFQ/N and ORL_1 are highly expressed in brain regions implicated in the regulation of food intake (Houtani, Nishi et al. 2000). Still, discrepancies exist with regard to the sensitivity of specific nuclei to the hyperphagic effect of OFQ/N (Stratford, Holahan et al. 1997; Polidori, de Caro et al. 2000) and further experimentation is necessary in order to elucidate the role(s) of such potential regions.

In extension of these findings, Olszewski et al (2000) determined that several brain regions displayed changes in c-Fos immunoreactivity (Fos-IR), an indirect measure of neuronal activity, subsequent to icv administration of OFQ/N. Specifically, pronounced increases in Fos-IR were evident in the rostrocaudal portion of the nucleus of

the solitary tract (NTS), paraventricular nucleus of the hypothalamus (PVN), lateral habenular nucleus (LHb), lateral septal nucleus (LSN) and central nucleus of the amygdala (CeA) (Olszewski, Billington et al. 2000). Moreover, these regions are responsible for the integration of feeding-associated information and rewarding stimulation, and the initiation of feeding behavior (Olszewski, Billington et al. 2000). Interpretation of these results suggests that the NTS, PVN, LHb and LSN may serve as putative sites of OFQ/N induced hyperphagia, and possibly through the blockade of POMC-derived peptides that exert an inhibitory control on feeding (Olszewski, Billington et al. 2000).

HPA axis activity and the stress response; modulation of anxiogenic behavior

Among the many hypothesized roles of the nociceptin system, a large but controversial literature exists with respect to the anxiolytic properties of ORL_1 signaling (Jenck, Moreau et al. 1997; Koster, Montkowski et al. 1999; Fernandez, Misilmeri et al. 2004; Gavioli, Rizzi et al. 2007; Green, Barbieri et al. 2007). In mice and rats OFQ/N (0.03-1.0 nmol, icv) decreased anxiogenic behavior in behavioral tests such as the light-dark box, elevated plus maze and operant conflict procedure (Jenck, Moreau et al. 1997). Conversely, $OFQ/N^{-/-}$ mice spent less time in the center-most region of an open field and the open arms of an elevated plus maze (Koster, Montkowski et al. 1999). Moreover, $OFQ/N^{-/-}$ mice made significantly fewer entries into the lit compartment of a dark-light box and displayed increased latencies to first exit the preferred, dark compartment, when compared to wildtype mice (Koster, Montkowski et al. 1999). Still, the anxiolytic role of the nociceptin system is open to debate, as others report that OFQ/N does not effect

behavior in the elevated plus maze (0.3 or 0.5 nmol/rat, icv) or increased anxiogenic behavior in similar animal models of anxiety (Fernandez, Misilmeri et al. 2004; Vitale, Arletti et al. 2006; Green, Barbieri et al. 2007).

The role of the nociceptin system in modulation of anxiety-related behaviors becomes more complex when removal of the ORL_1 gene is considered (Mamiya, Noda et al. 1998; Gavioli, Rizzi et al. 2007). For example, Gavioli et al (2007) reported an anxiogenic phenotype for $ORL_1^{-/-}$ mice when assessed by traditional behavioral tests of anxiety (i.e., elevated plus-maze, light-dark box) that is consistent with the findings of Koster et al (1999). Conversely, Mamiya et al (1998) argued that $ORL_1^{-/-}$ mice did not exhibit any behavioral differences in the elevated plus-maze test when compared to $ORL_1^{+/+}$ mice. Moreover, phenotypic differences between receptor knockouts and neuropeptide knockouts have been noted and are attributed to the presence of additional receptor subtypes, related ligand molecules (e.g., nocistatin and OFQ/N II), and differences in genetic background (Koster, Montkowski et al. 1999).

Activation of the HPA axis induces elevations in circulating adrenocorticotropin releasing hormone (ACTH) and corticosterone. These changes represent physiological components of the stress response and are largely associated with anxiety states (Herman and Cullinan 1997). Several studies have elucidated a role for the nociceptin system in modulation of HPA axis activity in response to stress (Koster, Montkowski et al. 1999; Devine, Watson et al. 2001; Fernandez, Misilmeri et al. 2004; Leggett, Harbuz et al. 2006; Vitale, Arletti et al. 2006; Leggett, Jessop et al. 2007; Leggett, Dawe et al. 2009). For example, in rats exposed to mild (novel environment) but not severe (restraint) stress icv administration of OFQ/N enhanced stress-induced elevations of ACTH and prolonged

stress-induced levels of plasma corticosterone, 30 minutes after infusion (Devine, Watson et al. 2001). In unstressed rats OFQ/N (icv) produced dose-dependent elevations in plasma ACTH and corticosterone concentrations (Devine, Watson et al. 2001; Nicholson, Akil et al. 2002; Leggett, Harbuz et al. 2006) and significantly increased expression of corticotropin releasing hormone (CRH) mRNA in the hypothalamus (Leggett, Harbuz et al. 2006). Moreover, Leggett et al (2006) determined that OFQ/N induced stimulation of the HPA axis is ORL_1 dependent as co-administration of ORL_1 selective antagonist, [Nphe1,Arg14,Lys15] Nociceptin/OrphaninFQ-NH2 (UFP-101) and OFQ/N blocked ORL_1 dependent elevations in plasma corticosterone and hypothalamic expression of CRH mRNA.

Conversely, Koster et al (1999) report increased basal and post-stress levels of corticosterone in $OFQ/N^{-/-}$ mice as compared to wild-type littermates ($OFQ/N^{+/+}$). This finding is in direct opposition to those of Devine et al (2001), Leggett et al (2006) and others (Fernandez, Misilmeri et al. 2004; Green, Barbieri et al. 2007) and suggests that ORL_1 signaling provides an inhibitory input to the HPA axis (Koster, Montkowski et al. 1999). Although much evidence supports a stimulatory role for ORL_1 signaling with respect to HPA axis activation, such discrepancies in nociceptin-dependent modulation of HPA output may be attributable to differential responding across species. Still, ORL_1-dependent activation of HPA axis activity is inconsistent with its hypothesized anxiolytic role. Rather, stimulation of the HPA axis suggests an anxiogenic role for the nociceptin system (Fernandez, Misilmeri et al. 2004; Green, Barbieri et al. 2007). For example, Fernandez et al (2004) report increased latencies to enter the unprotected arms of an

elevated plus maze and lit compartment of a dark-light box following OFQ/N administration (icv).

Alternatively, stimulation of the HPA axis by the nociceptin system may represent physiological mechanisms of homeostasis. For example, Ciccocioppo et al (2001; 2004) determined that OFQ/N inhibited stress and CRH-induced anorexia. Interestingly, the hyperphagic effect of OFQ/N is mediated by corticosterone and central glucocorticoid receptors (Nicholson, Akil et al. 2002). Specifically, OFQ/N induced hyperphagia was abrogated upon blockade of CRH signaling or adrenalectomy and was restored upon high dose corticosterone replacement (Nicholson, Akil et al. 2002). Moreover, Leggett et al (2009) determined that antagonism of the ORL_1 receptor prevented lipopolysaccharide (LPS) induced increases in ACTH and corticosterone 30 min after injection. In this way, OFQ/N activation of the HPA axis serves to protect an organism from the detrimental effects of stress (e.g., stress-induced anorexia, prolonged inflammation). Thus, the stimulatory effect of the nociceptin system on HPA axis output may be dissociable from its anxiolytic effects. This is not uncommon as other recognized anxiolytic compounds, such as diazepam, have also been shown to activate the HPA axis (Fernandez, Misilmeri et al. 2004). In sum, the nociceptin system modulates HPA axis activity and may be central to restoring homeostasis during anxiety states.

Immune Function

ORL_1 and OFQ/N are expressed in various peripheral tissues (Lachowicz, Shen et al. 1995; Mollereau, Simons et al. 1996) including immune cells and organs (Wang, Johnson et al. 1994). Similar to endogenous opioids, the nociceptin system plays a role

in immunomodulation (Halford, Gebhardt et al. 1995; Kawashima, Fugate et al. 2002; Goldfarb, Reinscheid et al. 2006; Miller and Fulford 2007; Finley, Happel et al. 2008; Leggett, Dawe et al. 2009). Specifically, stimulation of ORL_1 enhances or suppresses expression of proinflammatory cytokines (Kawashima, Fugate et al. 2002; Zhao, Huang et al. 2002; Fu, Zhu et al. 2007) or chemokines (Finley, Happel et al. 2008), stimulates chemotaxis (Serhan, Fierro et al. 2001; Trombella, Vergura et al. 2005), and modulates activation of the HPA axis in response to inflammatory stimuli (Leggett, Dawe et al. 2009). In general, the hypothesized role of the nociceptin system is believed to be anti-inflammatory and exert its effects primarily through regulation of cytokine expression.

Previously, Zhao et al (2002) determined that ORL_1 mRNA is co-localized with neurons, astrocytes and microglia. In rats, treatment with OFQ/N (icv) significantly reduced the number of activated microglia following neural trauma (Zhao, Huang et al. 2002). *in vitro*, LPS induced upregulation of IL-1β mRNA in microglia and was inhibited by application of OFQ/N (Zhao, Huang et al. 2002). Moreover, this effect was blocked by co-administration of an ORL_1 receptor antagonist, confirming that downregulation of Il-1β mRNA transcripts in microglia of the CNS is ORL_1 dependent. However, when applied to cultured neurons, OFQ/N significantly increased LPS-induced upregulation of IL-1β mRNA (Zhao, Huang et al. 2002). The differential effect of OFQ/N on IL-1β gene expression was suggested to reflect a disturbance in the endogenous synaptic structural arrangement between microglia and neurons. Thus, *in vivo* microglia may exert a direct inhibitory action on neurons and when this connection

is disturbed (e.g., *in vitro* preparations) neurons may upregulate IL-1β mRNA instead (Zhao, Huang et al. 2002).

Although Zhao et al (2002) did not observe OFQ/N mediated changes in astrocyte number following neural trauma or IL-1β gene expression subsequent to LPS stimulation they did determine that OFQ/N is co-localized with astrocytes in the CNS. Similarly, Fu et al (2007) detected the expression of ORL_1 receptor mRNA and protein on astrocytes of rat spinal cord. Moreover, proinflammatory cytokine expression was inhibited in astrocyte cultures exposed to OFQ/N and was significantly blocked by an ORL_1 receptor antagonist (Fu, Zhu et al. 2007). This finding is in opposition to that of Zhao et al (2002) but may reflect differences in methodology. Specifically, Zhao et al (2002) isolated astrocytes of the brain whereas Fu et al (2007) cultured astrocytes from the spinal cord. The difference in astrocyte population may have contributed to the effects of OFQ/N on regulation of cytokine gene expression. OFQ/N modulation of cytokine gene expression, as proposed by Fu et al (2007), would be in agreement with the anti-nociceptive role for OFQ/N when delivered into the spinal cord. Perhaps ORL_1 activation within the spinal cord modulates inflammation via inhibition of proinflammatory cytokine production from astrocytes. Alternatively, astrocytes of the brain may be considered sites of synthesis rather than targets of OFQ/N as Buzas et al (2002) determined that LPS stimulated OFQ/N gene expression *in vitro*.

In the same way Miller et al (2007) determined that OFQ/N secretion from lymphocytes and splenocytes (*in vitro*) is increased following exposure to various inflammatory mediators such as, T (Concanavalin A; Con A) and B cell (LPS) mitogens,

proinflammatory cytokines IL-1β, and TNFα, and synthetic glucocorticoids. Moreover, OFQ/N suppressed production of IL-2 from cultured splenocytes and significantly attenuated splenocyte proliferation in response to Con A stimulation, an effect that was inhibited by ORL_1 receptor antagonists (Miller and Fulford 2007). A relationship between the immune system and OFQ/N is evident *in vivo* as well. For example, challenge with T cell-activating-bacterial superantigen, staphylococcal enterotoxin A (SEA) significantly increased precursor mRNA for OFQ/N (ppOFQ/N) in hypothalamic and amygdaloid regions of mice (Kawashima et al, 2002).

Intraperitoneal injection (ip) of nociceptin to mice, prior to SEA challenge produced an enhancement of splenic TNFα and IFNα mRNA expression and increased plasma levels of TNFα (Goldfarb, Reinscheid et al. 2006). Enhancement of cytokine secretion by OFQ/N may serve to augment certain aspects of the inflammatory response such as HPA axis activity. For example, Rossi-George et al (2005) determined that HPA activation subsequent to SEA exposure is TNFα dependent. If OFQ/N enhances TNFα production this mechanism may serve to augment the HPA stimulating effects of T-cell activating antigens. In addition, ORL_1 signaling may mediate LPS-induced stimulation of the HPA axis. For example, Leggett et al (2009) determined that blockade of ORL_1 receptors eliminated LPS- induced, ACTH and corticosterone elevations 30 min after treatment. ORL_1 modulation of HPA axis activity may serve a protective role against prolonged inflammation and tissue damage as corticosterone is immunosuppressive. In conclusion, an immunomodulatory role for the nociceptin system is evident and may ultimately exert anti-inflammatory effects.

Specific Aims

In conclusion, the nociceptin system has been hypothesized to modulate the neuroendocrine and behavioral response to stress (Jenck, Moreau et al. 1997; Koster, Montkowski et al. 1999; Ciccocioppo, Cippitelli et al. 2004) and plays a prominent role in modulating the HPA axis during stress (Devine, Watson et al. 2001; Fernandez, Misilmeri et al. 2004; Leggett, Dawe et al. 2009). Moreover, mRNA levels for ppOFQ/N are increased in response to immunological challenge (Kawashima, Fugate et al. 2002) and OFQ/N modulates peripheral cytokine release in response to SEA (Goldfarb, Reinscheid et al. 2006). Therefore, activation of the ORL_1 receptor may be necessary to negatively regulate HPA axis activity and attenuate anxiogenic behavior subsequent to systemic immune stress. Accordingly, the present set of experiments sought to determine the role of ORL_1 in modulating HPA axis function, splenic TNFα and IL-2 release and novelty-induced anorexia 2 and 4 h after acute SEA exposure. Specifically, we hypothesized that mice lacking the ORL_1 gene ($ORL_1^{-/-}$) would show heightened corticosterone release, augmented TNFα and IL-2 production and enhanced novelty-induced anorexia relative to similarly treated, wildtype mice ($ORL_1^{+/+}$) mice, following acute SEA injection.

Chapter 2: Materials and Methods

1.1 Animals

Male 129S6, ORL1$^{+/+}$ and ORL1$^{-/-}$ mice, were initially bred in the vivarium of Robert Wood Johnson Medical School, University of Medicine and Dentistry, New Jersey. After weaning, animals were housed 3-4 per cage under a 12:12 h light:dark cycle (lights on 0600h) in the Psychology Department, Rutgers University. Food and water were available ad libitum and animals were allowed two weeks acclimation prior to the start of testing. Animals were 5-6 months of age at the time of experimentation. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and approved by the Rutgers Institutional Animal Care and Guidance Committee.

1.2 Experimental Procedures

In all experiments animals were injected intraperitoneally (IP) with either staphylococcal enterotoxin A (5µg/animal) or physiological saline in a volume of 0.2 mL. All injections were given between 0900 and 1000 h. For studies involving biological measures, animals were sacrificed 2 or 4 h after a single injection. In one experiment, two sessions of behavioral testing occurred after an initial injection with SEA or Saline. This involved food intake measures at 2 and 4 h after injection and exposure to an open field and novel object on Day 4 (Urbach-Ross, Dissertation 2009; see below for behavioral methodology).

1.3 Endocrine and Immune Assays

1.3.1 Corticosterone measures

After injection of SEA or saline, animals were sacrificed by rapid decapitation and trunk blood was collected into EDTA-treated vacutainer tubes. Blood was then centrifuged for 20 minutes at 2500 RPM, and plasma was collected and stored at -70°C until ready for corticosterone assay using a commercially available, radioimmunoassay kit (MP Biomedicals, Solon, OH, USA).

1.3.2 Splenic Protein and Cytokine Quantification

Spleens were dissected and placed on dry ice until storage at -70°C. For protein extraction, spleens were homogenized in1 ml of 1mM phenylmethanesulfonyl fluoride (PMSF) in 0.1 M phosphate buffer to inhibit protease activity and centrifuged for 30 min at 3500 RPM. The supernatant was collected and measured for protein and cytokine concentration. Total protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Absorbance was read at 562 nm (using EL800 universal BioTek microplate reader) with concentrations (μg/ml) calculated from a standard curve generated using bovine serum albumin (BSA).

Following protein quantification, tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2) was determined using ELISA kits purchased from eBiosciences. The supernatants from the spleen homogenates (see above) were assayed at a 1:4 dilution for detection of TNFα and at a 1:40 dilution for IL-2. All cytokine standards and samples were run in duplicate and concentrations calculated from a standard curve using KC

Junior software (BioTek) and expressed as pg/ml. Subsequently, cytokine concentrations were adjusted per μg of protein.

1.4 Brain dissection and RNA extraction

Upon sacrifice of animals via rapid decapitation, brains were removed and immediately frozen in 2-methylbutane, placed on dry ice and stored at -70°C until ready for dissection of the hypothalamus, amygdala and hippocampus as previously described (Kawashima, Fugate et al. 2002). These brain regions were subjected to total RNA extraction using TRIzol® reagent as per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, tissue was homogenized in Trizol solution, incubated (for five minutes) at room temperature and then chloroform extracted (.2mL chloroform/1mL Trizol). This was followed by centrifugation at 3500 RPM for 15 min, collection of supernatant and addition of isopropanol for RNA precipitation (.5mL isopropanol/1mL Trizol). The RNA was pelleted by centrifugation, washed in 75% ethanol and finally dissolved in RNAse-free DEPC treated water. The concentration of RNA was quantified (ng/ul) using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at -70°C until ready to be assayed.

1.5 Reverse Transcription and qRT-PCR

Following RNA extraction 1 μg of RNA was reversed transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). cDNA was then assayed by quantitative real-time PCR (Applied Biosystems 7900HT system) for quantification of mRNA expression for corticotropin releasing hormone (CRH), CRH receptor 1 (CRHR1), CRH receptor 2 (CRHR2), TNFα,

and TNF receptor 1 (TNFR1) (Rossi-George, Urbach et al. 2005; Kohman, Crowell et al. 2009; Westphal, Evans et al. 2009). Sample values were calculated from standard curves generated using a twofold serial dilution of undiluted control cDNA obtained from splenic, hypothalamic or amygdaloid tissue of an animal that was given SEA. This method allowed for relative quantification of mRNA expression within each sample for each gene of interest. Thus, for all samples mRNA was expressed as a ratio of the gene of interest to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in arbitrary units based on the standard curve.

1.6 Behavioral Testing

1.6.1 Food Consumption

Animals were injected with SEA or saline and tested for consumption of a novel liquid diet (Prosobee baby formula) in a novel context. This testing situation has previously been shown to elevate plasma ACTH, likely due to neophobia (Kusnecov, Liang et al. 1999). No food or water deprivation was necessary prior to testing, since untreated and non-deprived mice can drink up to 1 ml of the Prosobee liquid over a 1 hr period. Therefore, given that water deprivation may produce stressor-like effects, no deprivation schedule was instituted. For testing, animals were removed from their home cages and placed into individual, opaque, shoe box cages. A tube of Prosobee solution was then placed through the cage top and made easily accessible to the animal. Consumption was measured across two consecutive tests. Each test lasted 1 hr with the first commencing 2 hr after injection and the second test commencing 4 hr after injection. Animals remained in the novel cages between consumption periods. At the end of each

consumption test, tubes were weighed and subtracted from pre-test weights. At the end of the second consumption test, animals were returned to their home cages.

1.7 Data Analysis

Data were analyzed by ANOVA, and where appropriate using repeated measures. Fisher's LSD was used to determine significant differences in the case of a main effect. Significance was set at $p < 0.05$.

Chapter 3: Results

The 2 hr and 4 hr acute experiments were conducted separately and therefore separate ANOVAs were conducted on the data.

3.1 Effects of SEA on Immunological Function in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ Mice 2 and 4 hours following Acute Injection

ORL_1 modulates splenic TNFα and IL-2 production 4 but not 2 hours after acute SEA exposure

Two hours after acute SEA injection, splenic TNFα was equally elevated in both genotypes ($F_{(1,20)} = 413.98$, $p < .0001$) (Figure 2A). By four hours after acute SEA or saline injection splenic TNFα was significantly lower relative to 2 h levels, with SEA induced TNFα slightly higher in $ORL_1^{+/+}$ mice than $ORL_1^{-/-}$ mice ($F_{(1,18)} = 4.31$, $p = .0524$) (Figure 2B). Splenic IL-2 was dramatically elevated in SEA treated mice relative to saline injected controls at both 2 ($F_{(1,20)} = 373.09$, $p < .0001$) and 4 ($F_{(1,18)} = 151.16$, $p < .0001$) hours after injection (Figures 3A and 3B). Further, although at 2 h there was no difference between genotypes, at 4 h SEA-injected $ORL_1^{-/-}$ mice had higher splenic IL-2 levels than $ORL_1^{+/+}$ mice ($F_{(1,18)} = 6.85$, $p < .05$).

3.2 Effects of SEA on Neuroendocrine Function in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ Mice 2 and 4 hours following Acute Injection

3.2.1 ORL_1 modulates HPA axis activity 4 but not 2 hours after acute SEA exposure

Two hours following acute injection, plasma corticosterone of SEA treated mice was significantly higher than that of saline injected controls ($F_{(1,20)} = 11.35$, $p < .01$) (See Figure 4A). However, the main effect of genotype was non-significant as corticosterone levels were similarly elevated between genotypes ($F_{(1,20)} = 3.15$, $p > .05$). Figure 4B shows plasma corticosterone 4 h after injection. SEA exposure significantly increased plasma corticosterone in $ORL_1^{+/+}$ mice relative to $ORL_1^{-/-}$ mice ($F_{(1,18)} = 13.26$, $p < .01$). The main effect of toxin was non-significant ($F_{(1,18)} = 1.70$, $p > .05$) and was likely attributable to the lack of an SEA effect in $ORL_1^{-/-}$ mice. However, there was a Genotype x Toxin interaction ($F_{(1,18)} = 6.19$, $p < .05$) indicating that $ORL_1^{+/+}$ mice administered SEA had significantly higher levels of plasma corticosterone than similarly treated, $ORL_1^{-/-}$ mice.

3.2.2 Expression of CRH, CRHR1, CRHR2, TNFα, and TNFR1mRNA in the Hypothalamus and Amygdala of 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ Mice

3.2.2.1 SEA increased hypothalamic CRH mRNA expression in $ORL_1^{-/-}$ mice 2 h after injection and decreased hypothalamic CRH mRNA expression in $ORL^{-/-}$ mice 4 hours after injection

Hypothalamus

Figure 5 depicts hypothalamic CRH mRNA expression, 2 and 4 h after acute SEA or saline injection. Overall, CRH mRNA levels did not differ between genotypes ($F_{(1,20)} < 1$, $p = .498$) or between SEA and saline treated mice ($F_{(1,20)} = 1.90$, $p > .05$), 2 h after injection. However, there was a significant Genotype x Toxin interaction ($F_{(1,20)} = 5.83$, $p < .05$), indicating that acute SEA challenge increased hypothalamic expression of CRH mRNA in $ORL_1^{-/-}$ mice but not similarly treated wildtype mice, 2 h after injection (see Figure 5A). Figure 5B shows CRH mRNA expression in the hypothalamus 4 h after acute injection. Analyses revealed main effects of genotype ($F_{(1,18)}= 6.12$, $p < .05$) and toxin ($F_{(1,18)}=10.25$, $p < .01$) indicating that $ORL_1^{-/-}$ mice had significantly higher levels of CRH mRNA than $ORL_1^{+/+}$ mice and that SEA reduced CRH mRNA levels in the hypothalamus, 4 h after injection . However, this was due to SEA significantly reducing CRH mRNA in $ORL_1^{+/+}$ mice (see Figure 5B), which was suggested by a significant Genotype x Toxin effect ($F_{(1,18)}= 3.92$, $p < .05$).

Amygdala

Figure 6 shows CRH mRNA expression in the amygdala, 2 and 4 h after acute SEA or saline administration. While at 2 h after injection CRH mRNA levels appeared to be greater in $ORL_1^{-/-}$ mice, the main effect of genotype did not reach statistical significance ($F_{(1,20)} = 3.99$, $p > .05$). Moreover, SEA did not alter CRH mRNA expression in the amygdala, at this same time point ($F_{(1,20)} < 1$, $p > .05$) (see Figure 6A). However, irrespective of genotype ($F_{(1,18)}<1$, $p > .05$), SEA increased CRH mRNA levels 4 h after injection ($F_{(1,18)}= 7.56$, $p < .05$) (see Figure 6B).

3.2.3.2 SEA up-regulates hypothalamic CRH-R1 mRNA expression in $ORL_1^{+/+}$ mice and down-regulates CRH-R1 mRNA expression in $ORL_1^{-/-}$mice 4 h after injection

Hypothalamus

Overall, CRH-R1 mRNA expression in the hypothalamus did not differ between genotypes (2 h: $F_{(1,20)} < 1$, $p > .05$; 4 h: $F_{(1,17)} < 1$, $p > .05$) or between SEA and saline-treated mice (2 h: $F_{(1,20)} <$, $p > .05$; 4 h: $F_{(1,17)} < 1$, $p > .05$) at either time point tested (see Figure 7). However, a significant Genotype x Condition interaction indicated that SEA differentially affected the expression of CRH-R1 mRNA in ORL_1 wildtype and knockout mice. Specifically, SEA increased CRH-R1 mRNA levels in $ORL_1^{+/+}$ mice and decreased CRH-R1 mRNA expression in $ORL_1^{-/-}$ mice 4 h after injection ($F_{(1,18)}= 7.98$, $p <.05$) (see Figure 7B). Moreover, while this pattern of results appeared to emerge 2 h after injection, the Genotype x Toxin interaction was non-significant at this time point ($F_{(1,20)}= 4.10$, $p > .05$) (see Figure 7A).

Amygdala

Figure 8A shows CRH-R1 mRNA expression in the amygdala 2 h after acute SEA or saline administration. Overall, CRH-R1 mRNA expression did not differ between genotypes ($F_{(1,20)} < 1$, $p > .05$) and was not altered by SEA ($F_{(1,20)} < 1$, $p > .05$). Similar to the hypothalamus, SEA tended to decrease CRH-R1 mRNA expression in $ORL_1^{-/-}$ mice 2 h after injection. However, this too did not reach statistical significance ($F_{(1,20)}= 3.75$, $p > .05$). Figure 8B shows CRH-R1 mRNA expression 4 h after injection. At this time point neither main nor interaction effects were observed for CRH-R1 mRNA expression.

3.2.3.3. SEA down-regulates CRH-R2 mRNA expression in the hypothalamus 4 hours after injection

Hypothalamus

Two hours after injection, CRH-R2 mRNA expression did not differ between $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice ($F_{(1,18)} < 1$, $p > .05$) and was not altered by SEA ($F_{(1,18)} = 2.28$, $p > .05$) (see Figure 9A). Similarly, CRH-R2 mRNA expression did not differ between genotypes 4 h after injection ($F_{(1,17)}= 1.10$, $p > .05$). However, SEA significantly reduced CRH-R2 mRNA expression in the hypothalamus 4 h after injection ($F_{(1,17)}= 12.10$, $p < .01$) (see Figure 9B).

Amygdala

Neither main nor interaction effects were observed for amygdaloid, CRH-R2 mRNA expression 2 or 4 h after injection (See Figure 10).

3.2.3.4 TNFα mRNA expression in the amygdala is up-regulated 4 hours after acute SEA injection

Hypothalamus

Figure 11A shows hypothalamic TNFα mRNA expression, which did not differ between $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice ($F_{(1,18)}= 2.22$, $p > .05$) nor between SEA and saline treated mice ($F_{(1,18)}< 1$, $p > .05$) 4 h after acute injection.

Amygdala

Overall TNFα mRNA expression in the amygdala (Figure 11B) did not differ between $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice ($F_{(1,18)} < 1$, $p > .05$), although exposure to SEA significantly increased TNFα mRNA expression ($F_{(1,18)}= 5.66$, $p < .05$). While it appears that TNFα mRNA is higher in $ORL_1^{+/+}$ mice, the Genotype x Toxin interaction was non-significant.

3.2.3.5 ORL_1 modulates endogenous TNFR1 mRNA expression in the hypothalamus

Hypothalamus

Figure 12A shows hypothalamic TNFR1 mRNA expression, which was significantly lower in $ORL_1^{+/+}$ mice relative to $ORL_1^{-/-}$ mice ($F_{(1,18)}= 4.68$, $p < .05$). However, Toxin exposure did not affect TNFR1 mRNA expression ($F_{(1,18)}= 2.23$, $p > .05$) in the hypothalamus.

Amygdala

Neither main nor interaction effects were observed for amygdaloid, TNFR1 mRNA expression (See Figure 12B).

3.3 Behavioral Effects of Acute SEA Exposure in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ Mice

$ORL_1^{-/-}$ mice exhibit marked novelty-induced anorexia 2 and 4 hours following acute SEA challenge

Previous studies have determined that following SEA exposure, elevated levels of plasma corticosterone are accompanied by novelty induced hypophagia (viz., Prosobee; see Materials and Methods) 2 hours after injection (Kawashima and Kusnecov 2002). Figure 14 shows total Prosobee consumed 2 and 4 h after SEA or saline injection for 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Analyses revealed a main effect of genotype ($F_{(1,17)} = 25.98$, $p < .0001$) indicating that $ORL_1^{-/-}$ mice consumed significantly more Prosobee than $ORL_1^{+/+}$ mice. Moreover, a main effect of Toxin was found as SEA-injected mice consumed significantly less Prosobee than saline treated mice ($F_{(1,17)} = 5.76$, $p < .05$), a finding that is largely attributable to the SEA-induced anorexia evident 4 h after injection (Time x Toxin interaction: $F_{(1,17)} = 5.81$, $p < .05$). Finally, there was a significant Genotype x Toxin interaction ($F_{(1,17)} = 9.07$, $p < .01$), suggesting that SEA treatment differentially affected the consumption of wildtype and knockout mice. As seen in Figure 13, it is clear that SEA-induced reduction in consumption is more strongly evident in $ORL_1^{-/-}$ mice for both time points. However, SEA increased consumption by $ORL_1^{+/+}$ mice at 2 h, but this subsequently declined below saline-treated mice by 4 h (See Figure

13). Overall, irrespective of treatment, consumption was greater in $ORL_1^{-/-}$ mice across both time points.

Chapter 4: Discussion

ORL_1 Prolongs Stress-Induced Levels of Corticosterone following Acute SEA Challenge

The present study tested whether exposure of 129S6, $ORL_1^{-/-}$ mice to an immunologic stressor, the T cell superantigen SEA, alters various parameters of neuroendocrine, behavioral and cytokine responsiveness. A prominent feature of SEA challenge is activation of the HPA axis, with elevations of plasma corticosterone being CRH-dependent (Kaneta, 2005; Rossi-George, 2005). In the present study, two hours following SEA challenge, plasma corticosterone was equally elevated in both wildtype and ORL_1 knockout mice; however, four hours after injection SEA increased plasma corticosterone in $ORL_1^{+/+}$ mice but not $ORL_1^{-/-}$ mice. Therefore, under conditions of acute immunologic stress, the ORL_1 gene appears to confer prolonged stimulation of the HPA axis. This conclusion is in keeping with other evidence that intracerebroventricular (icv) administration of OFQ/N prolonged stress-induced elevations of plasma corticosterone following acute, mild stress (Devine, Watson et al. 2001; Fernandez, Misilmeri et al. 2004) and that blockade of the ORL_1 receptor significantly attenuated the ACTH and corticosterone response to LPS (Leggett, Dawe et al. 2009). Together, these findings suggest that OFQ/N exerts stimulatory effects on the HPA axis in an ORL_1dependent manner.

The mechanism(s) through which the nociceptin system activates the HPA axis is currently under investigation. Still, it has been hypothesized that endogenous ORL_1 signaling may modulate HPA axis activity by stimulating the release of CRH (Olszewski, Billington et al. 2000; Devine, Watson et al. 2001; Leggett, Harbuz et al. 2006). For instance, OFQ/N (icv) increased c-Fos immunoreactivity in the PVN (Olszewski,

Billington et al. 2000) and stimulated ACTH and corticosterone release in unstressed rats (Leggett, Harbuz et al. 2006). However, at the cellular level the ORL_1 receptor exerts inhibitory effects as it is negatively coupled to adenylyl cyclase (Mollereau, Parmentier et al. 1994; Wang, Johnson et al. 1994), stimulates K^+ channels (Hawes, Graziano et al. 2000) and inhibits high-voltage activated (HVA) N-type Ca^{2+} channels (Altier and Zamponi 2008). In fact, OFQ/N hyperpolarized magnocellular and parvocellular neurons of the PVN (*in vitro*) (Shirasaka, Miyahara et al. 2001) and inhibited neurotransmitter release in the amygdala (Meis and Pape 2001). Therefore, it has been hypothesized that endogenous ORL_1 signaling stimulates the release of CRH through dis-inhibition of PVN neurons (Devine, Watson et al. 2001; Leggett, Harbuz et al. 2006). For example, by disrupting limbic feedback to the hypothalamus (i.e., from the hippocampus), ORL_1 may be able to enhance the release of CRH and ACTH in response to stress and prolong elevated levels of corticosterone (Devine, Watson et al. 2001).

Similarly, it could be hypothesized that endogenous ORL_1 signaling prolongs SEA-induced activation of the HPA axis via dis-inhibition of PVN neurons. The ORL_1 receptor is highly expressed throughout the limbic system, in particular within the posteromedial BNST (pmBNST) (Neal, Mansour et al. 1999). Moreover, because limbic regions such as the hippocampus and amygdala, must relay information to the PVN indirectly through the BNST (Ulrich-Lai and Herman 2009) and because SEA increased c-Fos immunoreactivity in this same region (Rossi-George, Urbach et al. 2005), the BNST may be an important neuroanatomical substrate for ORL_1 mediated, dis-inhibition of the PVN during stress. For example, GABAergic cells originating in the pmBNST send afferents to the PVN that inhibit CRH release (Ulrich-Lai and Herman 2009).

However, subsequent to acute SEA challenge activation of ORL_1 receptors within the pmBNST could inhibit the release of GABA and, in turn, enhance (or sustain) CRH release in the PVN (see Figure 14). In this way, endogenous ORL_1 signaling could prolong stress-induced levels of corticosterone. Consistent with this hypothesis is the finding that, in unstressed rats, unilateral, intra-BNST infusion of OFQ/N significantly increased plasma corticosterone in a dose dependent manner (Green, Barbieri et al. 2007).

ORL_1 Limits Hypothalamic CRH Synthesis following Acute SEA Injection

The neuropeptide CRH is a primary ACTH secretagogue, and was shown to be responsible for HPA axis activation in response to various T cell superantigens, including SEA (Kusnecov, Liang et al. 1999; Rossi-George, Urbach et al. 2005). Moreover, while stimulation of CRH-R1 is critical for activation of the HPA axis (Smith, Aubry et al. 1998; Timpl, Spanagel et al. 1998) and plays a role in controlling the activity of CRH neurons during stress (Imaki, Katsumata et al. 2001) recovery of the stress response is mediated by CRH receptor 2 (CRH-R2) (Coste, Kesterson et al. 2000). Therefore, in the current study quantitative real-time PCR was conducted on the hypothalamus and amygdala at both 2 and 4 h after acute SEA in order to determine whether the corticosterone responses to SEA in 129S6, $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice were associated with variations in CRH, CRH-R1 and CRH-R2 mRNA expression.

Two hours after saline-injection, SEA significantly increased hypothalamic CRH mRNA expression in $ORL_1^{-/-}$ mice only. Conversely, SEA dramatically reduced hypothalamic CRH mRNA expression in $ORL_1^{+/+}$ but not $ORL_1^{-/-}$ mice, 4 h after

injection. Finally, SEA increased CRH mRNA expression in the amygdala 4 h but not 2 h after injection, although this did not appear to rely on the presence of the ORL_1 gene. These findings are in partial agreement with those of Kusnecov et al (1999) in that SEA increased CRH mRNA expression in the amygdala 4 h after injection. However, our data contrast with that of Kusnecov et al (1999) in that changes in hypothalamic CRH mRNA were observed as early as 2 h after SEA and by 4 h after injection were significantly reduced in normal mice. This discrepancy may reflect strain-dependent responsiveness to SEA and requires furthers investigation.

Still, our findings are consistent with others and may reflect the onset of negative feedback processes that control CRH synthesis (Shepard, Liu et al. 2005; Aguilera, Kiss et al. 2007). This explanation is conceivable in light of the fact that in $ORL_1^{-/-}$ mice SEA-treatment did not significantly change the level of hypothalamic CRH mRNA at 4 h, which is a time point that coincided with significantly lower corticosterone levels (and hence less negative feedback) than that observed in SEA-treated $ORL_1^{+/+}$ mice. Moreover, the pattern of CRH mRNA expression that was observed in wildtype mice (i.e., down regulation in the hypothalamus and up-regulation in the amygdala) is in agreement with region-specific expression of CRH mRNA in response to stress (Makino, Gold et al. 1994), a finding that is in part dependent on the type of transcriptional coregulator that is recruited (van der Laan, Lachize et al. 2008). Therefore, lower hypothalamic CRH mRNA expression in $ORL_1^{+/+}$ mice may have been due to glucocorticoid-mediated repression of the CRH gene 4 h after SEA injection.

An alternative conceptualization is that the reduced CRH mRNA levels in SEA-treated $ORL_1^{+/+}$ mice reflected CRH-R1 mediated, negative feedback control of CRH

(Aguilera, Kiss et al. 2007). Stress increases CRH-R1 mRNA expression in the hypothalamus (Rivest, Laflamme et al. 1995; Imaki, Katsumata et al. 2001) and activation of CRH-R1 during stress mediates CRH synthesis. Initially, CRH-R1 signaling provides positive feedback to CRH neurons by increasing cAMP levels and CREB-stimulated CRH gene transcription. However, over time high levels of cAMP progressively increase the production of proteins (i.e., inducible cAMP early repressor; ICER) that provide negative feedback to CRH neurons by inhibiting the activity of CREB (Shepard, Liu et al. 2005). Moreover, because the corticosterone response to SEA is, in part, dependent upon central CRH receptors (Kaneta and Kusnecov 2005), and because high levels of cAMP are associated with up-regulation of ppOFQ/N (Buzas, Rosenberger et al. 1998), the present study sought to determine whether SEA altered CRH receptor mRNA expression and, whether these changes were modulated by the ORL_1 gene.

Interestingly, acute SEA increased hypothalamic CRH-R1 mRNA expression in wildtype mice and decreased CRH-R1 mRNA levels in $ORL_1^{-/-}$ mice 4 h after injection. These findings are consistent with the idea that stress increases hypothalamic CRH-R1 mRNA expression (Rivest, Laflamme et al. 1995; Imaki, Katsumata et al. 2001) and also suggests that subsequent to acute SEA injection, up-regulation of CRH-R1 may be dependent on the presence of the ORL_1 gene. With respect to classical negative feedback control, increased receptor expression is usually associated with compensatory decreases in ligand availability. Moreover, up-regulation of mRNA levels could reflect low levels of available protein due to increased activity. The present results are consistent with this idea in that 4 h after injection, SEA up-regulated hypothalamic CRH-R1 mRNA expression and down-regulation of CRH mRNA levels in wildtype mice.

Therefore, it could be hypothesized that the ORL_1 gene is associated with CRH-R1signaling at that in response to systemic stress ORL_1 is important for proper feedback regulation of CRH and CRH-R1. Moreover, while ORL_1 may be necessary for SEA-induced up-regulation of CRH-R1, it could also be inhibiting CRH synthesis via this same mechanism. Specifically, activation of the ORL_1 receptor may be inhibiting CRH-R1 dependent accumulation of cAMP and CREB-stimulated CRH gene transcription (see Figure 15). In fact, application of OFQ/N to accumbal and striatal tissue inhibited the activity of dopamine (DA) neurons that are coupled to similar intracellular signaling cascades (Olianas, Dedoni et al. 2008). More specifically, OFQ/N inhibited DA-dopamine receptor 1 (D1) dependent increases of cAMP and reduced DA-D1 dependent phosphorylation of CREB (Olianas, Dedoni et al. 2008). Moreover, OFQ/N (*in vitro*) inhibited CRH-stimulated adenylyl cyclase activity in the olfactory bulb glomerular layer (Onali, Ingianni et al. 2001). Thus, subsequent to acute SEA injection, the ORL_1 receptor may limit CRH mRNA expression directly by inhibiting CRH-R1 signaling cascades in the hypothalamus.

Finally, SEA decreased CRH-R2 mRNA expression in the hypothalamus 4 h but not 2 h after injection. This is in agreement with other data showing down-regulation of CRH-R2 mRNA expression subsequent to immunological challenge (Coste, Heldwein et al. 2001) and supports the idea that CRH-R2 is important for recovery from the stress response (Coste, Kesterson et al. 2000). For example, relative to wildtype mice, corticosterone levels remained elevated 90 minutes after infusion (icv) of the stress-related hormone, urocortin in CRH-R2 deficient ($CRH\text{-}R2^{-/-}$) mice (Coste, Kesterson et al. 2000). Therefore, in the present study decreased expression of CRH-R2 following

acute SEA injection may be important for maintaining elevated levels of corticosterone during periods of prolonged immunologic stress. However, SEA-induced, down-regulation of CRH-R2 mRNA was similar across genotypes and therefore, by itself, could not fully account for the effect of SEA on plasma corticosterone in $ORL_1^{+/+}$ mice, 4 h after SEA administration. More than likely, the ORL_1 gene may modulate both glucocorticoid and CRH receptor mediated feedback processes that together ensure proper HPA axis function in response to stress.

ORL_1 may be Associated with Basal TNFα Cytokine Signaling in the Hypothalamus

Measures were also taken of hypothalamic and amygdaloid, TNFα mRNA expression, since previously in C57BL6/J mice the neural, endocrine and behavioral effects of SEA were shown to be dependent on TNFα (Rossi-George et al, 2005). Systemically, the 129S6 background mice tested in the current study showed normal splenic TNFα (and IL-2) responses to SEA. Most importantly, however, brain TNFα mRNA was also increased in the amygdala, but not hypothalamus, in SEA challenged mice. This occurred for both wildtype and knockout mice, ruling out the possibility that ORL_1 is necessary for modulation of central TNFα mRNA levels by immunologic challenge with SEA. It is of interest to note, however, that CRH has been shown to promote TNFα production in mouse macrophages in vitro and in vivo (Agelaki, Tsatsanis et al. 2002). Considering both the CRH and TNFα mRNA data for the amygdala, it could be hypothesized that amygdaloid CRH may influence TNFα production following a systemic immunologic challenge. Moreover, the source of central TNFα production may actually be of microglial origin. These cells are derived from the same myeloid lineage

as macrophages, perform phagocytic functions in the brain, and produce TNFα (Mott, Ait-Ghezala et al. 2004; Lambertsen, Clausen et al. 2009). Given that CRH is capable of enhancing the production of TNFα from macrophages, it is conceivable that central TNFα production may be stimulated by CRH.

Many of the major neurobiological effects of TNFα, such as HPA axis activation and hypophagia, are mediated via the TNFRI receptor (Rossi-George, Urbach et al. 2005). In spite of the SEA-induced variations in amygdaloid TNFα mRNA, corresponding changes in TNFR1 mRNA were not observed in the amygdala, or in the hypothalamus. However, overall TNFRI mRNA in the hypothalamus was higher in $ORL_1^{-/-}$ mice, suggesting that ORL_1 signaling may be associated with basal TNFα cytokine signaling in the hypothalamus. Interestingly, although there is little information on the relationship between the OFQ/N system and TNFα in the brain, there is in vitro evidence showing that astrocyte activation of the OFQ/N precursor gene is enhanced by TNFα (Buzas, Rosenberger et al. 2002). In view of this evidence, at the very least, the present data support the notion of a potential functional relationship between TNFα, TNFα receptors and the OFQ/N system in the hypothalamus that awaits further investigation.

$ORL_1^{-/-}$ mice Show Enhanced Gustatory Neophobia following Acute SEA Exposure

The current study also investigated the behavioral effects of immunologic challenge with SEA. This involved testing for consummatory effects 2 and 4 h after SEA challenge. It has already been demonstrated in other strains of mice (viz., BALB/c and C57BL/6) that T cell superantigens reduce intake of a novel liquid diet (Kusnecov and

Goldfarb 2005), raising the hypothesis of augmented gustatory neophobia, which was later shown to be TNFα dependent (Rossi-George, Urbach et al. 2005). In the current study, it was notable that after SEA treatment, 129S6 wildtype mice did not show a major reduction in consumption of the novel liquid diet. As noted earlier, this was not due to a failure of mice to respond with TNFα and IL-2 production to SEA. Additional experiments in our lab confirmed that the 129S6 strain differs significantly from C57BL/6 mice in terms of the hypophagic effect of SEA using the Prosobee test. That is, as previously reported (Rossi-George, Urbach et al. 2005; Urbach-Ross, Crowell et al. 2008), 2 h after 5 μg SEA challenge C57BL/6 mice showed 60% reduction in consumption, while 129S6 mice fail to show a significant reduction (unpublished data). Therefore, we are confident that the 129S6 mouse strain is resistant to the anorexic effects of SEA under the given conditions of consumption testing.

Of considerable interest, however, is that ORL_1 deletion produces significant effects on consumption of the liquid diet. For instance, saline-injected $ORL_1^{-/-}$ mice showed higher levels of consumption relative to $ORL_1^{+/+}$ mice similarly injected with saline. Therefore, deletion of the ORL_1 gene appeared to produce an orexigenic effect. This may indeed have conferred greater sensitivity to the anorexic effects of SEA, that otherwise was lacking in $ORL_1^{+/+}$ 129S6 mice. These observations suggest that intact OFQ/N signaling in the 129S6 mouse may confer resistance or reduced sensitivity to anorexic signals generated in response to SEA challenge. Since in the C57BL/6 mouse, deletion of TNFα or *in vivo* neutralization of circulating TNFα abolishes the anorexic response to SEA (Rossi-George, Urbach et al. 2005), it is possible that the anorexigenic effect of endogenous TNFα is weak in 129S6 mice, and that this relies on the presence of

a functional ORL_1 gene. Indeed, it is interesting to note that as discussed above, TNFRI mRNA in the hypothalamus was higher in the ORL_1 knockout mice. Therefore, given that the hypothalamus is central to the regulation of food intake (Cowley 2003; Shioda, Takenoya et al. 2008), it is possible that the presence of the ORL_1 gene in the hypothalamus down-regulates TNFRI expression, and renders 129S6 mice less sensitive to the anorexigenic effects of TNFα.

ORL_1 Modulates Splenic TNFα and IL-2 Release 4 h after Acute Injection

The current study measured concentrations of TNFα and IL-2 in spleens collected 2 and 4 hours after administration of SEA. This was conducted to ensure that SEA challenge in 129S6 mice engaged T cells. As expected, SEA produced significant elevations in both cytokines at these time points. Interestingly, at the 2 hr time point, these elevations were similar in both genotypes, suggesting that ORL_1 deletion was not important in the early production of TNFα and IL-2 induced by SEA. At the 4 h time point, TNFα concentrations had declined substantially, but were still slightly higher in wildtype mice. Although significant, this amounted to only a modest difference unlikely to account for the corticosterone differences between SEA-treated wildtype and knockout mice at the 4 h time point. Interestingly, splenic IL-2 was significantly lower in wildtype mice 4 h after injection suggesting that downregulation of IL-2 production requires ORL_1 signaling.

These cytokine data stand in contrast to our previous finding in mice with a deletion in the precursor for OFQ/N, which had suggested that endogenous OFQ/N levels are necessary to ensure optimal levels of TNFα production 2h after SEA challenge

(Goldfarb et al, 2006). However, in the present study, the failure to see relatively less 2 hr splenic TNFα production in ORL-1$^{-/-}$ mice challenged with SEA might be due to differences in the background genetic strain (129S6 vs. C57BL/6), and is a question requiring further investigation. Moreover, the 4 hr data (a time point not tested by Goldfarb et al, 2006) suggests that endogenous OFQ/N signaling might be necessary for preserving sufficient amounts of TNFα as the rate of synthesis declines. Furthermore, activation of the ORL_1 receptor may also promote down-regulation of IL-2 production, a finding that is consistent with others (Miller and Fulford 2007). Since the balance of TNF and IL-2 production are features evident in neuroimmunopathological conditions (including multiple sclerosis (Shi et al, 2009) it will be important to explore the role of OFQ/N signaling in immunoregulation.

Conclusions and Future Directions

In conclusion, the present findings suggest that the presence of the ORL_1 gene is associated with neuroendocrine changes that exert positive and negative feedback control of the HPA axis during acute, systemic stress. Specifically, activation of the ORL_1 receptor prolonged SEA-induced levels of corticosterone. This finding is important in light of the fact that in response to immunologic stress, corticosterone exerts anti-inflammatory effects that protect the host from chronic inflammation and excessive tissue damage. Therefore, the nociceptin system may exert anti-inflammatory effects by sustaining high levels of corticosterone in response to acute SEA administration. In agreement with this is the fact that both *in vitro* (Miller and Fulford 2007) and in the present study (*in vivo*) activation of the ORL_1 receptor significantly attenuated splenic IL-2 levels in response to T cell activation. In the context of immunologic stress this is important because IL-2 stimulates further T cell proliferation and promotes an inflammatory response.

The findings of the current study also suggest that nociceptin signaling is associated with stress-induced changes in CRH, CRH-R1 and CRH-R2 mRNA expression (Rivest, Laflamme et al. 1995; Imaki, Katsumata et al. 2001). For example, activation of the ORL_1 receptor may be important for limiting the synthesis of CRH during stress and for the re-establishment of homeostasis. This is especially important in the context of chronic stress because repeated stress alters the structure and function of the HPA axis, up-regulating CRH expression in the PVN and CeA (Cook 2004). In this way, chronic stress sensitizes the HPA axis to subsequent acute stress (i.e., increased excitability to novel stress) and increases anxiogenic behavior (Ulrich-Lai and Herman

2009). Moreover, because OFQ/N is essential for behavioral adaptation during acute stress, the nociceptin system may play an equally important role during chronic stress. For example, by exerting an inhibitory effect over extra-hypothalamic CRH production OFQ/N may attenuate anxiogenic behavior. Finally, with respect to its putative anti-inflammatory effects, OFQ/N may be important for behavioral adaptation during chronic inflammation. Specifically, by attenuating the production of proinflammatory cytokines, OFQ/N may confer resistance to cytokine-induced depression (Dantzer, O'Connor et al. 2008). In conclusion, further investigation into the mechanisms by which the nociceptin system modulates the HPA axis in response to systemic immune stress (both acute and chronic) is necessary because when HPA axis feedback is compromised, the neuroendocrine and behavioral changes following stress may be enhanced. This would be of great clinical significance because many disorders that afflict humans, such as anxiety and depression are associated with dysregulation of the HPA axis response to stress.

Region	ORL_1 Expression	ppOFQ/N Expression
Forebrain		
Telencephalon		
Cerebral Cortex	+ +	
Prefrontal		+, + +
Frontal		+, + +
Orbital		+, + +
Piriform		+, + +
Entorhinal		+, + +
Temporal		+
Parietal		+
Basal Ganglia		
Globus pallidus	+ +	*(+ + +) , *(-)
Caudate putamen	-, +	-
Ventral pallidum		+
Limbic Cortex	+ +, + + +	
Hippocampal Formation		+ +, + + +
Dentate gyrus		+, + +
Cornu Ammonis 1, 3	+ +, + + +	
Amygdala		+ + +
Central nucleus		+ + +
Medial nucleus		*(+) , *(-)
Nucleus Accumbens	+ +	+ +
Shell	-, +	+
Core		-
Fornix		+ + +
Septum	+ + +	+ + +
Bed nucleus of stria terminalis	+ + +	+
Olfactory nucleus	+ +	-
Pituitary		
Diencephalon		
Thalamus	+ +	+ + +
Hypothalamus	+ +, + + +	+ + +
anterior-ventral Preoptic area		+ + +
Arcuate nucleus		
Midbrain	+ + +	
Tegmentum		
Substantia nigra		+ + +
Tectum		
Pineal		-
Hindbrain		
Cerebellum	-, +	-

Locus coeruleus		+ +
Corpus Callosum	-, +	
Anterior Commissure	-, +	
Spinal Cord	+ + +	+ + +
Region	**ORL_1 Expression**	**ppOFQ/N Expression**
Periphery		
Intestine	+	-
Skeletal muscle	+	
Vas deferens	+	
Testis		-
Ovary	-	
Stomach		-
Kidney	-	+
Liver	+	-
Lung	-	-
Heart	-	
Retina	-	
Adrenals	+	-
Spleen	+	+
Thymus		+
Pancreas	-	
Peripheral blood leukocytes		+

Table 1. Degree of mRNA expression of the opioid receptor like-1 receptor (ORL_1) and the precursor to its endogenous ligand pre-pro orphaninFQ/nociceptin (ppOFQ/N). Gradations used for mRNA expression are as follows: High density, + + +; Moderate density, + +; Low density, +; Undetectable/Absent, - ; Discrepancy in density of expression, *( ).

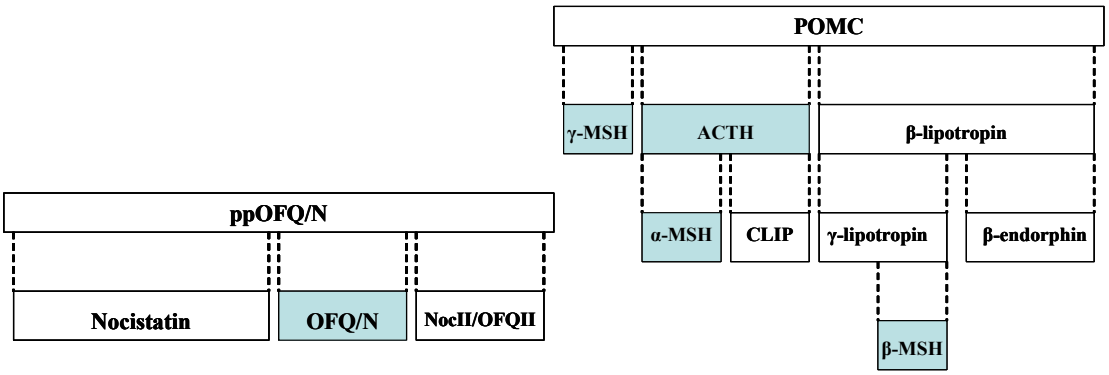

Figure 1. Schematic of similarities between the derivation of traditional opioid peptides and OFQ/N. Abbreviations; ppOFQ/N; pre-pro OrphaninFQ/Nociceptin; OFQ/N; OrphaninFQ/Nociceptin; NocII/OFQII; NociceptinII/OrphaninFQII; POMC; Proopiomelanocortin; γ-MSH; Gamma-melanocyte-stimulating hormone; ACTH; Adrenocorticotropic hormone; β-lipotropin; Beta-lipotropin; α-MSH; Alpha-melanocyte-stimulating hormone; CLIP; Corticotropin-like intermediate peptide; γ-lipotropin; Gamma-lipotropin; β-endorphin; Beta-endorphin; β-MSH; Beta-melanocyte-stimulating hormone.

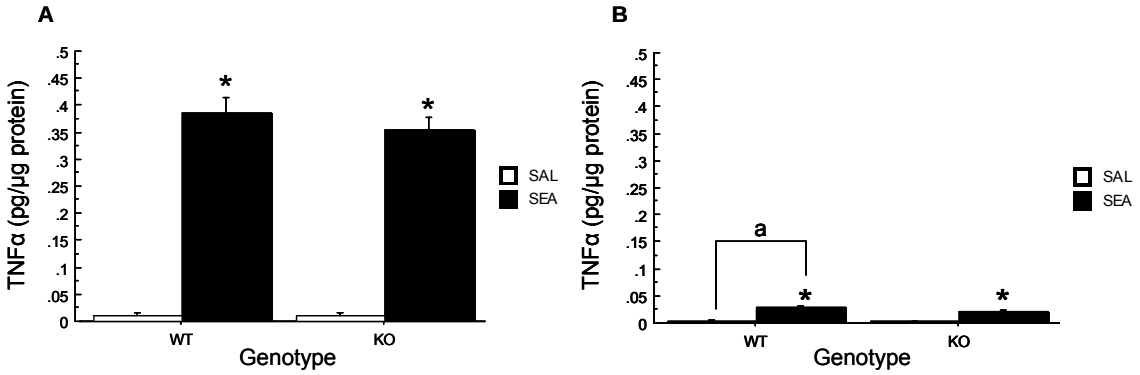

Figure 2. Concentration of splenic TNFα (A) 2 h and (B) 4 h after acute SEA or saline injection in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed as means ± standard error of the means (SEMs). n = 5-6/gp. * $p < .05$ relative to corresponding saline. [a] $p < .05$ relative to $ORL_1^{-/-}$ mice

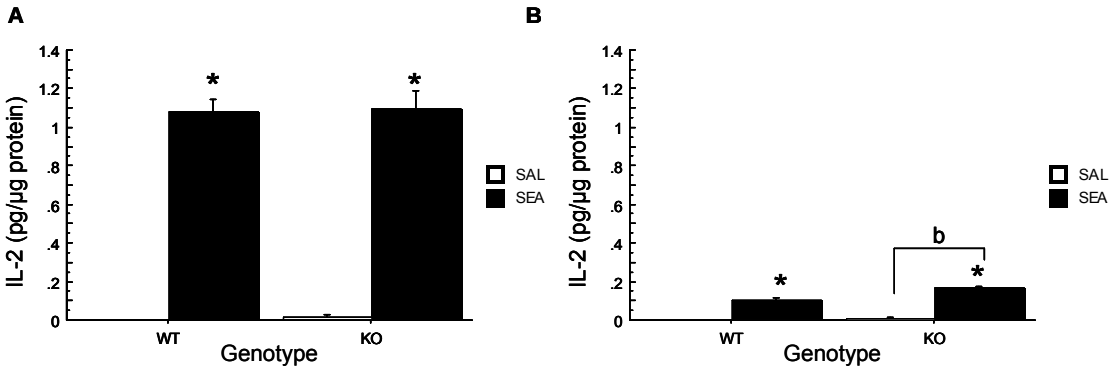

Figure 3. Concentration of splenic IL-2 (A) 2 h and (B) 4 h after acute SEA or saline injection in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed as means ± standard error of the means (SEMs). n = 5-6/gp. * $p < .05$ relative to corresponding saline. ^{b} $p < .05$ relative to $ORL_1^{+/+}$ mice.

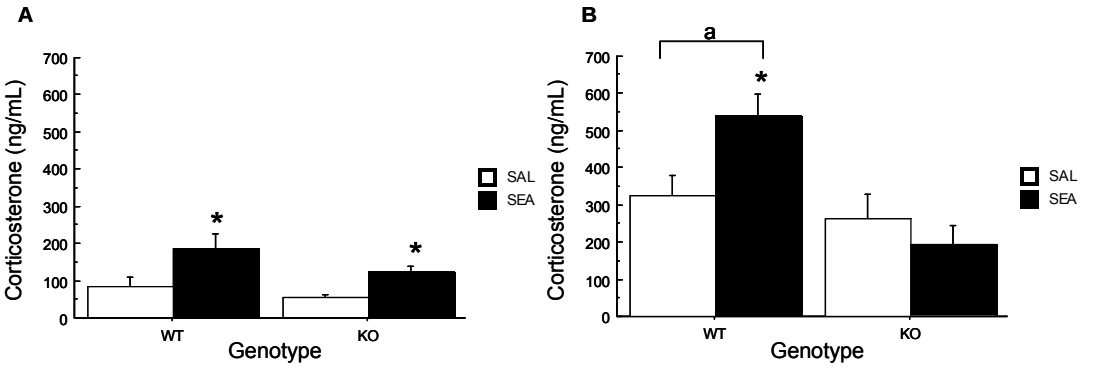

Figure 4. Concentration of plasma corticosterone (A) 2 h and (B) 4 h after SEA or saline injection in $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed as means ± standard error of the means (SEMs). n = 5-6/gp. * $p < .05$ relative to corresponding saline. [a] $p < .05$ relative to $ORL_1^{-/-}$ mice.

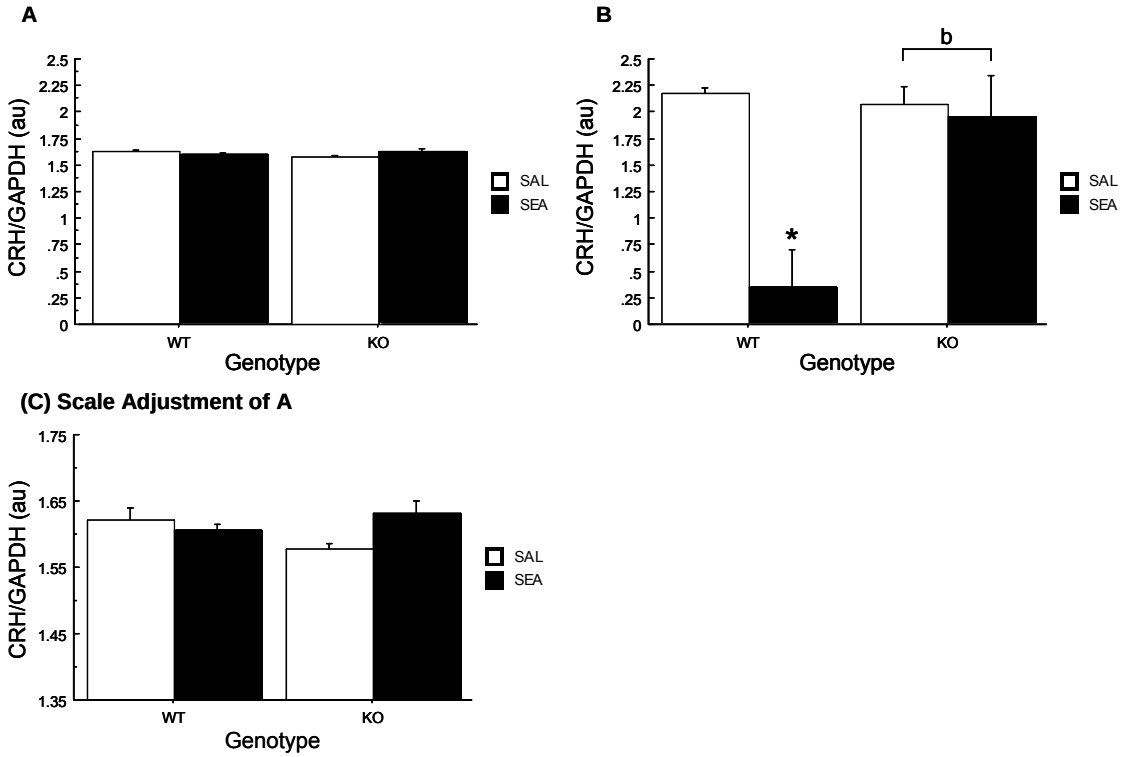

Figure 5. CRH mRNA expression in the hypothalamus (A) 2 h and (B) 4 h after acute SEA administration in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Hypothalamic CRH mRNA expression 2 h after acute SEA; scale adjustment (c). Data are expressed in arbitrary units (au) as means ± SEM. n = 5-6/gp. * $p < .05$ relative to corresponding saline. $^{b}p < .05$ relative to $ORL_1^{+/+}$ mice.

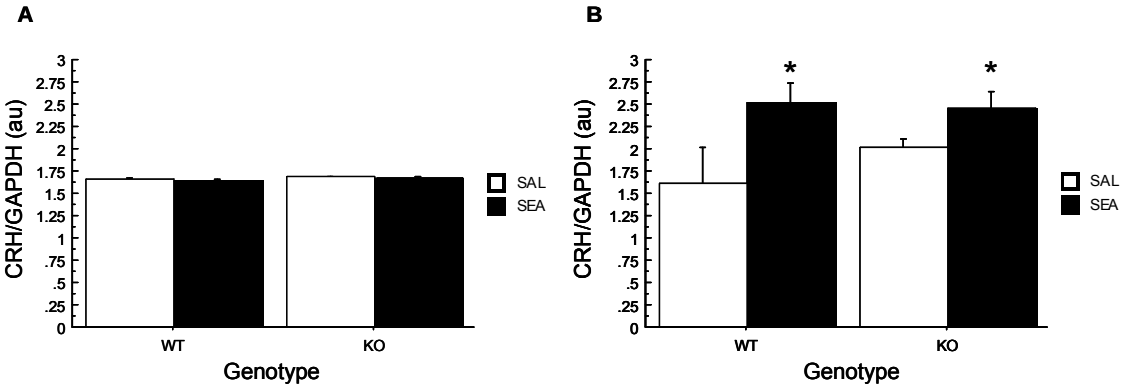

Figure 6. CRH mRNA expression in the amygdala (A) 2 h and (B) 4 h after acute SEA administration in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed in arbitrary units (au), as means ± SEM. n = 5-6/gp. * $p < .05$ relative to corresponding saline.

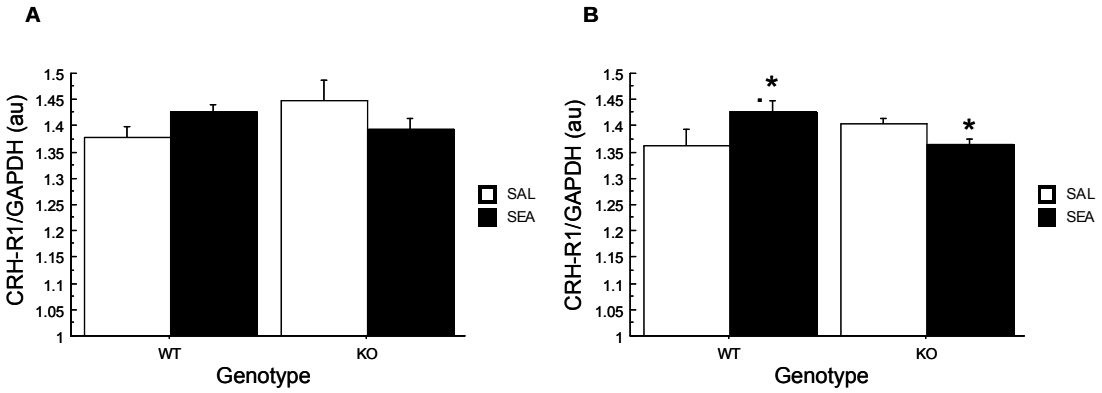

Figure 7. CRH-R1 mRNA expression in the hypothalamus (A) 2 h and (B) 4 h after acute SEA administration in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed in arbitrary units, as means ± SEM. n = 5-6/gp. **Note:** scale begins at 1. * $p < .05$ relative to corresponding saline.

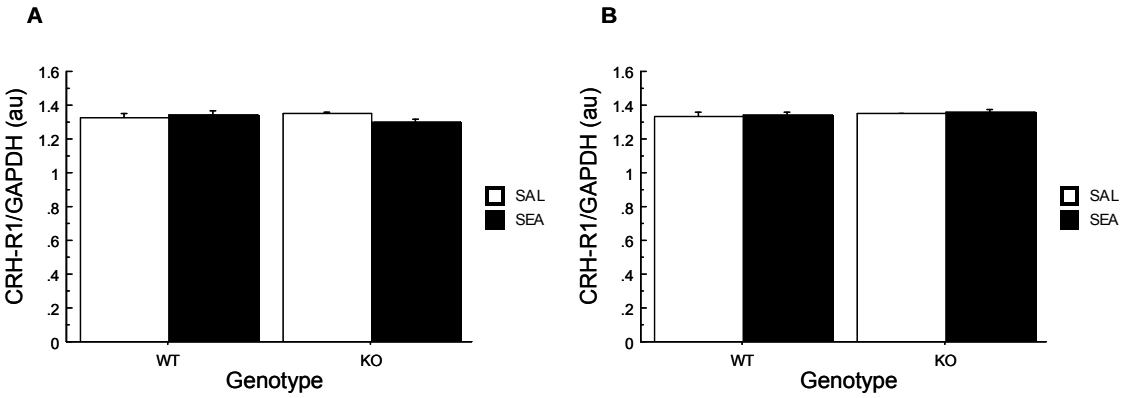

Figure 8. CRH-R1 mRNA expression in the amygdala (A) 2 h and (B) 4 h after acute SEA administration in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed in arbitrary units (au) as means ± SEM. n = 5-6/gp. * $p < .05$ relative to corresponding saline.

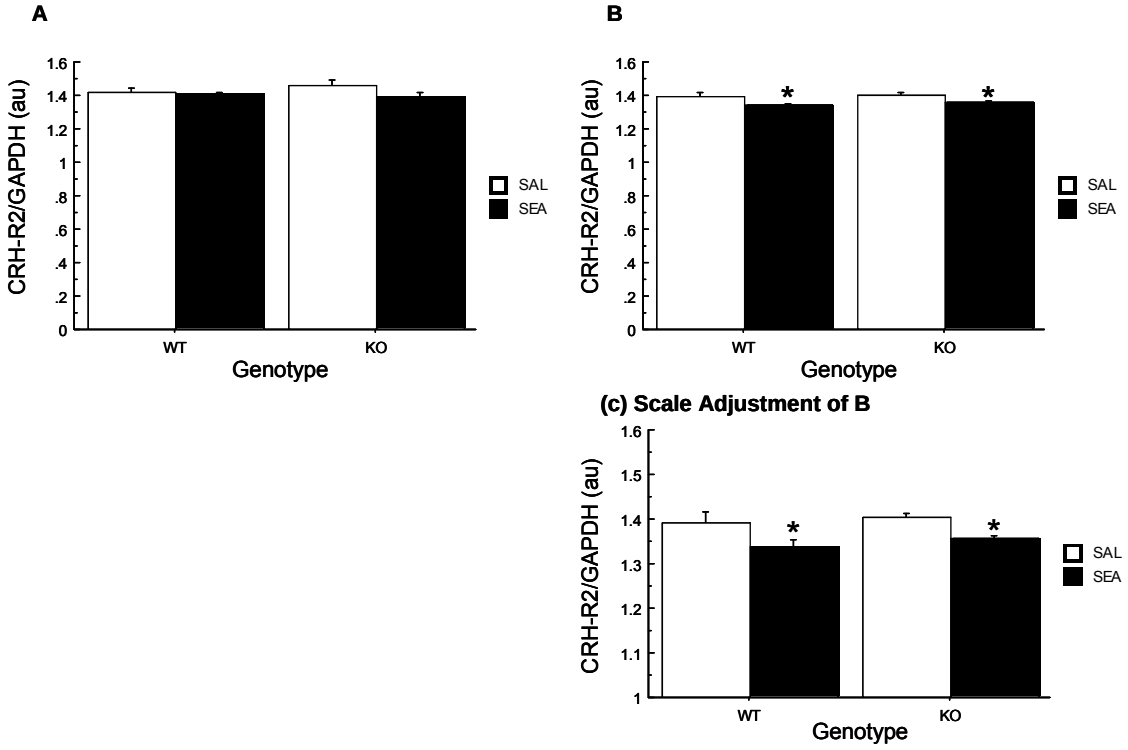

Figure 9. CRH-R2 mRNA expression in the hypothalamus (A) 2 h and (B) 4 h after acute SEA administration in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Hypothalamic CRH-R2 mRNA expression 4 h after acute SEA; scale adjustment (c) Data are expressed in arbitrary units (au) as means ± SEM. n = 5-6/gp. * $p < .05$ relative to corresponding saline.

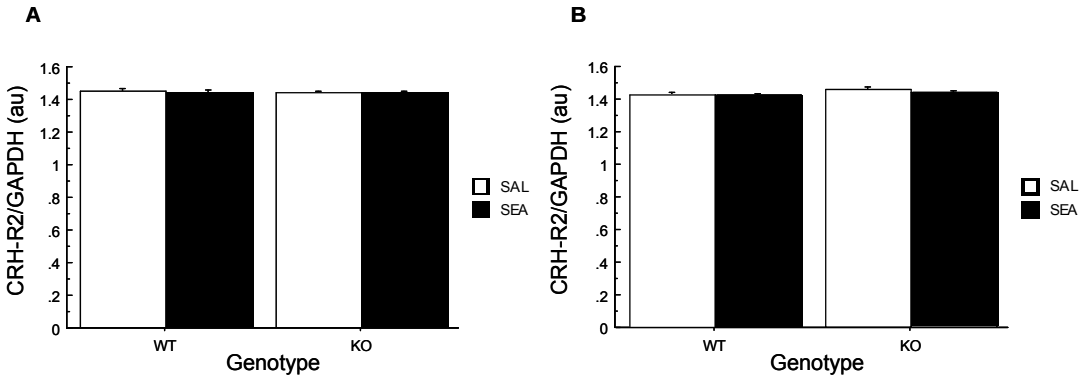

Figure 10. CRH-R2 mRNA expression in the amygdala (A) 2 h and (B) 4 h after acute SEA administration in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed in arbitrary units (au) as means ± SEM. n = 5-6/gp. * $p < .05$ relative to corresponding saline.

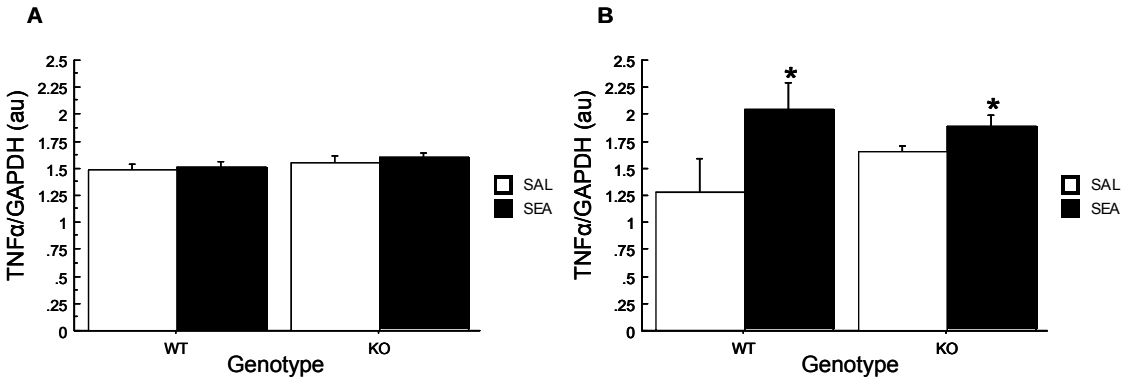

Figure 11. Central TNFα mRNA expression in $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice 4 hours after acute SEA injection. (A) Hypothalamus; (B) Amygdala. Data are expressed in arbitrary units (au) as means ± SEM. n = 5-6/gp. * $p < .05$ relative to corresponding saline.

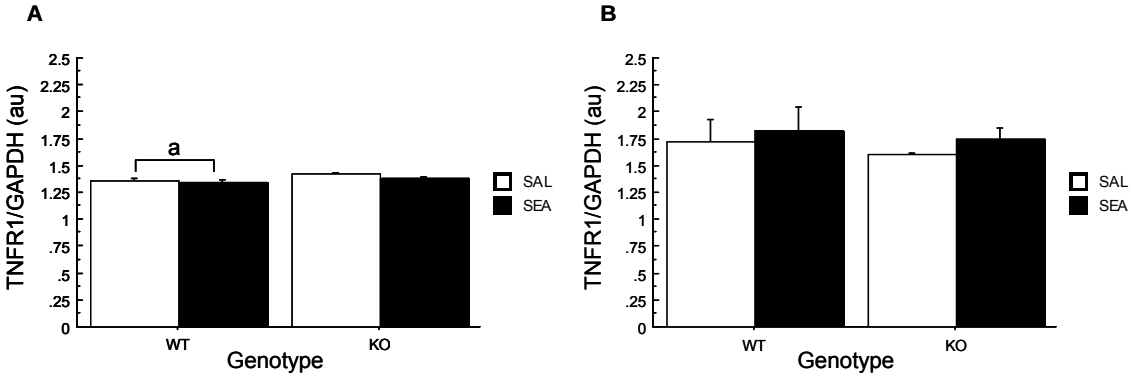

Figure 12. Brain TNFα Receptor I (TNFR1) mRNA expression in $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice 4 hours after acute SEA injection. (A) Hypothalamus; (B) Amygdala. Data are expressed in arbitrary units (au) as means ± SEM. n = 5-6/gp. $^{a}p < .05$ relative to $ORL_1^{-/-}$ mice.

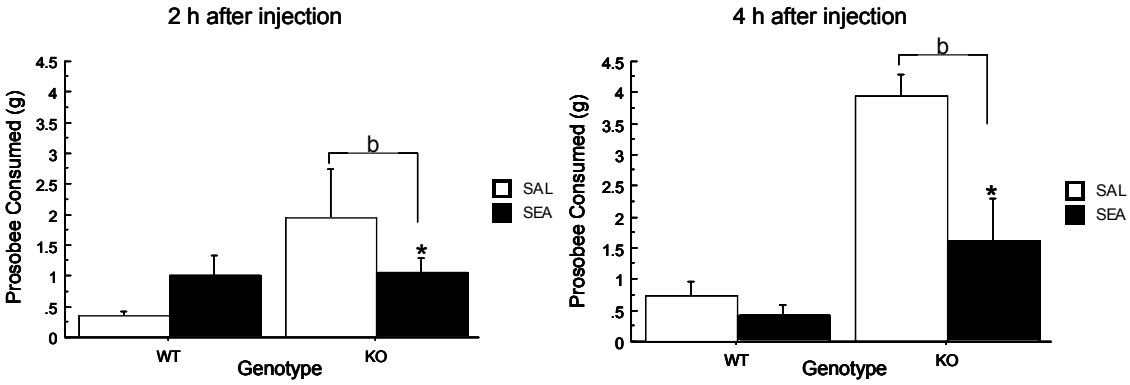

Figure 13. Grams of Prosobee consumed 2 and 4 h after acute SEA or saline injection in 129S6 $ORL_1^{+/+}$ and $ORL_1^{-/-}$ mice. Data are expressed as means ± standard error of the means (SEMs). n = 4-6/gp. * $p < .05$ relative to corresponding saline. ^{b} $p < .05$ relative to $ORL_1^{+/+}$ mice.

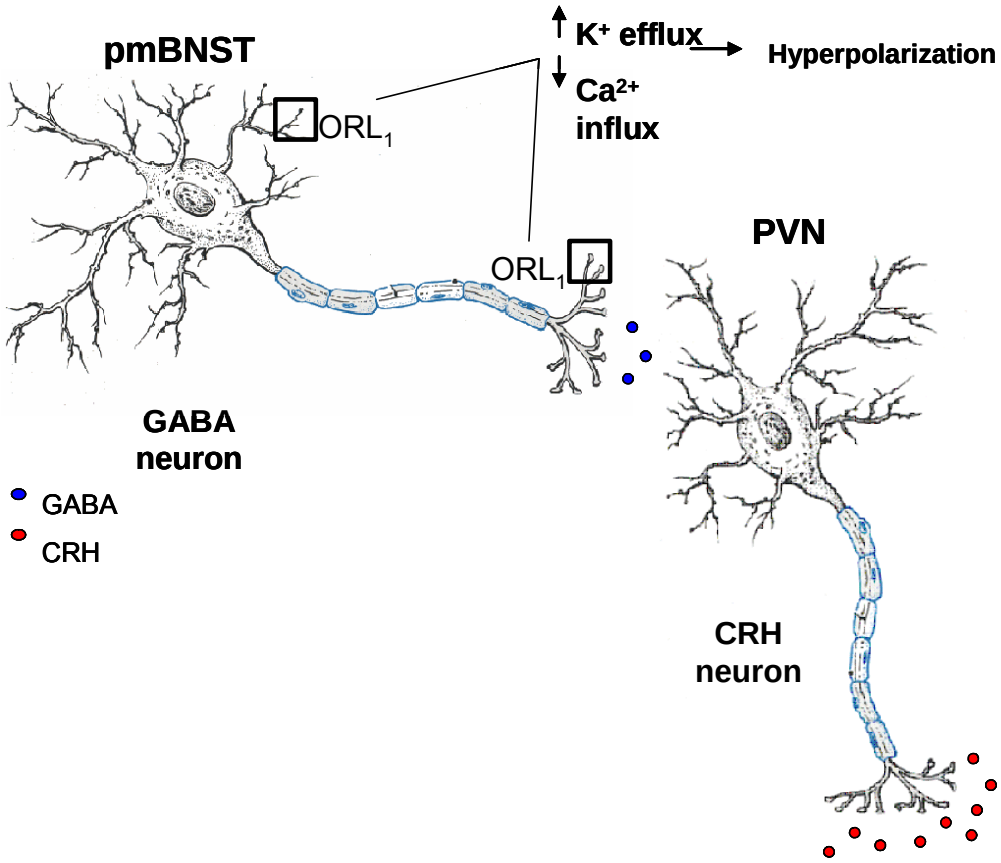

Figure 14. Hypothesized mechanism of ORL_1 mediated disinhibition of the PVN in response to acute SEA challenge. pmBNST; posterior-medial bed nucleus of stria terminalis, PVN; paraventricular nucleus, ORL_1; opioid receptor like-1 receptor, CRH; corticotropin releasing hormone, GABA; γ-Aminobutyric acid, K^+; potassium, Ca^{2+}; calcium.

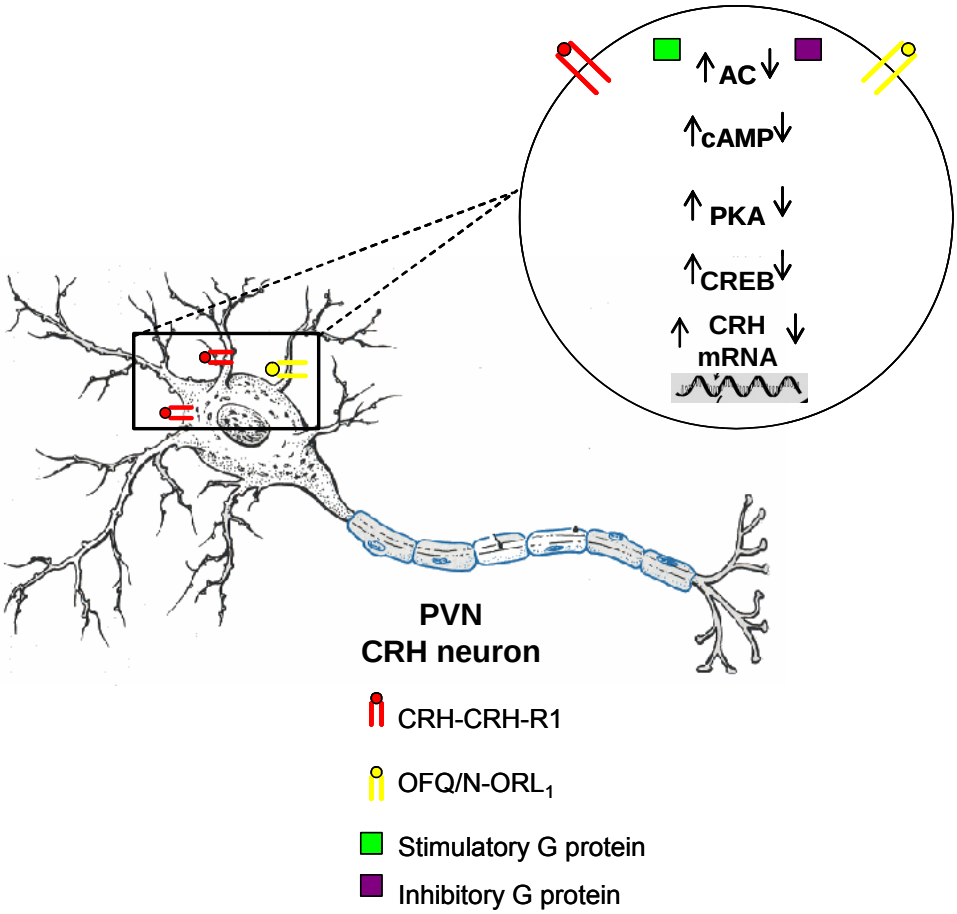

Figure 15. Hypothesized role for ORL_1 in CRH-R1 mediated repression of CRH synthesis. AC; adenylyl cyclase, cAMP; cyclic adenosine monophosphate, PKA; protein kinase A, CREB; cAMP response element binding, PVN; paraventricular nucleus, CRH; corticotropin releasing hormone, CRH-R1; corticotropin releasing hormone receptor 1, OFQ/N; orphaninFQ/nociceptin, ORL_1; opioid receptor like-1 receptor.

References

Agelaki, S., C. Tsatsanis, et al. (2002). "Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages in vitro and in lipopolysaccharide-induced endotoxin shock in mice." Infect Immun **70**(11): 6068-74.

Aguilera, G., A. Kiss, et al. (2007). "Negative regulation of corticotropin releasing factor expression and limitation of stress response." Stress **10**(2): 153-61.

Akashi, K., D. Traver, et al. (2000). "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature **404**(6774): 193-7.

Altier, C. and G. W. Zamponi (2008). "Signaling complexes of voltage-gated calcium channels and G protein-coupled receptors." J Recept Signal Transduct Res **28**(1-2): 71-81.

Assenmacher, M., M. Lohning, et al. (1998). "Sequential production of IL-2, IFN-gamma and IL-10 by individual staphylococcal enterotoxin B-activated T helper lymphocytes." Eur J Immunol **28**(5): 1534-43.

Bretscher, P. and M. Cohn (1970). "A theory of self-nonself discrimination." Science **169**(950): 1042-9.

Bunzow, J. R., C. Saez, et al. (1994). "Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type." FEBS Lett **347**(2-3): 284-8.

Buzas, B., J. Rosenberger, et al. (1998). "Activity and cyclic AMP-dependent regulation of nociceptin/orphanin FQ gene expression in primary neuronal and astrocyte cultures." J Neurochem **71**(2): 556-63.

Buzas, B., J. Rosenberger, et al. (2002). "Inflammatory mediators increase the expression of nociceptin/orphanin FQ in rat astrocytes in culture." Glia **39**(3): 237-46.

Chen, Y., Y. Fan, et al. (1994). "Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family." FEBS Lett **347**(2-3): 279-83.

Ciccocioppo, R., M. Biondini, et al. (2002). "Reversal of stress- and CRF-induced anorexia in rats by the synthetic nociceptin/orphanin FQ receptor agonist, Ro 64-6198." Psychopharmacology (Berl) **161**(2): 113-9.

Ciccocioppo, R., A. Cippitelli, et al. (2004). "Nociceptin/orphanin FQ acts as a functional antagonist of corticotropin-releasing factor to inhibit its anorectic effect." Physiol Behav **82**(1): 63-8.

Ciccocioppo, R., D. Economidou, et al. (2004). "Attenuation of ethanol self-administration and of conditioned reinstatement of alcohol-seeking behaviour by the antiopioid peptide nociceptin/orphanin FQ in alcohol-preferring rats." Psychopharmacology (Berl) **172**(2): 170-8.

Cook, C. J. (2004). "Stress induces CRF release in the paraventricular nucleus, and both CRF and GABA release in the amygdala." Physiol Behav **82**(4): 751-62.

Coste, S. C., K. A. Heldwein, et al. (2001). "IL-1alpha and TNFalpha down-regulate CRH receptor-2 mRNA expression in the mouse heart." Endocrinology **142**(8): 3537-45.

Coste, S. C., R. A. Kesterson, et al. (2000). "Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2." Nat Genet **24**(4): 403-9.

Cowley, M. A. (2003). "Hypothalamic melanocortin neurons integrate signals of energy state." Eur J Pharmacol **480**(1-3): 3-11.

Dantzer, R., J. C. O'Connor, et al. (2008). "From inflammation to sickness and depression: when the immune system subjugates the brain." Nat Rev Neurosci **9**(1): 46-56.

Devine, D. P., S. J. Watson, et al. (2001). "Nociceptin/orphanin FQ regulates neuroendocrine function of the limbic-hypothalamic-pituitary-adrenal axis." Neuroscience **102**(3): 541-53.

Drolet, G. and S. Rivest (2001). "Corticotropin-releasing hormone and its receptors; an evaluation at the transcription level in vivo." Peptides **22**(5): 761-7.

Fernandez, F., M. A. Misilmeri, et al. (2004). "Nociceptin/orphanin FQ increases anxiety-related behavior and circulating levels of corticosterone during neophobic tests of anxiety." Neuropsychopharmacology **29**(1): 59-71.

Finley, M. J., C. M. Happel, et al. (2008). "Opioid and nociceptin receptors regulate cytokine and cytokine receptor expression." Cell Immunol **252**(1-2): 146-54.

Florin, S., C. Suaudeau, et al. (1997). "Orphan neuropeptide NocII, a putative pronociceptin maturation product, stimulates locomotion in mice." Neuroreport **8**(3): 705-7.

Fu, X., Z. H. Zhu, et al. (2007). "Regulation of proinflammatory cytokines gene expression by nociceptin/orphanin FQ in the spinal cord and the cultured astrocytes." Neuroscience **144**(1): 275-85.

Fukuda, K., S. Kato, et al. (1994). "cDNA cloning and regional distribution of a novel member of the opioid receptor family." FEBS Lett **343**(1): 42-6.

Gavioli, E. C., A. Rizzi, et al. (2007). "Altered anxiety-related behavior in nociceptin/orphanin FQ receptor gene knockout mice." Peptides **28**(6): 1229-39.

Germain, R. N. (2002). "T-cell development and the CD4-CD8 lineage decision." Nat Rev Immunol **2**(5): 309-22.

Goda, Y. and M. Mutneja (1998). "Memory mechanisms: the nociceptin connection." Curr Biol **8**(24): R889-91.

Goehler, L. E., R. P. Gaykema, et al. (2001). "Staphylococcal enterotoxin B induces fever, brain c-Fos expression, and serum corticosterone in rats." Am J Physiol Regul Integr Comp Physiol **280**(5): R1434-9.

Goldfarb, Y., R. K. Reinscheid, et al. (2006). "Orphanin FQ/nociceptin interactions with the immune system in vivo: gene expression changes in lymphoid organs and regulation of the cytokine response to staphylococcal enterotoxin A." J Neuroimmunol **176**(1-2): 76-85.

Gonzalo, J. A., A. Gonzalez-Garcia, et al. (1993). "Glucocorticoid-mediated control of the activation and clonal deletion of peripheral T cells in vivo." J Exp Med **177**(5): 1239-46.

Green, M. K., E. V. Barbieri, et al. (2007). "Roles of the bed nucleus of stria terminalis and of the amygdala in N/OFQ-mediated anxiety and HPA axis activation." Neuropeptides **41**(6): 399-410.

Halford, W. P., B. M. Gebhardt, et al. (1995). "Functional role and sequence analysis of a lymphocyte orphan opioid receptor." J Neuroimmunol **59**(1-2): 91-101.

Hawes, B. E., M. P. Graziano, et al. (2000). "Cellular actions of nociceptin: transduction mechanisms." Peptides **21**(7): 961-7.

Heinricher, M. M. (2005). "Nociceptin/orphanin FQ: pain, stress and neural circuits." Life Sci **77**(25): 3127-32.

Herman, J. P. and W. E. Cullinan (1997). "Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis." Trends Neurosci **20**(2): 78-84.

Hong, S. C., G. Waterbury, et al. (1996). "Different superantigens interact with distinct sites in the Vbeta domain of a single T cell receptor." J Exp Med **183**(4): 1437-46.

Houtani, T., M. Nishi, et al. (2000). "Distribution of nociceptin/orphanin FQ precursor protein and receptor in brain and spinal cord: a study using in situ hybridization and X-gal histochemistry in receptor-deficient mice." J Comp Neurol **424**(3): 489-508.

Imaki, T., H. Katsumata, et al. (2001). "Expression of corticotropin-releasing hormone type 1 receptor in paraventricular nucleus after acute stress." Neuroendocrinology **73**(5): 293-301.

Jameson, S. C. and M. J. Bevan (1998). "T-cell selection." Curr Opin Immunol **10**(2): 214-9.

Jenck, F., J. L. Moreau, et al. (1997). "Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress." Proc Natl Acad Sci U S A **94**(26): 14854-8.

Jenck, F., J. Wichmann, et al. (2000). "A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat." Proc Natl Acad Sci U S A **97**(9): 4938-43.

Kaneta, T. and A. W. Kusnecov (2005). "The role of central corticotropin-releasing hormone in the anorexic and endocrine effects of the bacterial T cell superantigen, Staphylococcal enterotoxin A." Brain Behav Immun **19**(2): 138-46.

Kawashima, N., J. Fugate, et al. (2002). "Immunological challenge modulates brain orphanin FQ/nociceptin and nociceptive behavior." Brain Res **949**(1-2): 71-8.

Kawashima, N. and A. W. Kusnecov (2002). "Effects of staphylococcal enterotoxin A on pituitary-adrenal activation and neophobic behavior in the C57BL/6 mouse." J Neuroimmunol **123**(1-2): 41-9.

Kohman, R. A., B. Crowell, et al. (2009). "Influence of age on behavioral, immune and endocrine responses to the T-cell superantigen staphylococcal enterotoxin A." Eur J Neurosci **30**(7): 1329-38.

Kondo, M., I. L. Weissman, et al. (1997). "Identification of clonogenic common lymphoid progenitors in mouse bone marrow." Cell **91**(5): 661-72.

Koster, A., A. Montkowski, et al. (1999). "Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice." Proc Natl Acad Sci U S A **96**(18): 10444-9.

Kumar, S. and R. Jack (2006). "Origin of monocytes and their differentiation to macrophages and dendritic cells." J Endotoxin Res **12**(5): 278-84.

Kusnecov, A. W. and Y. Goldfarb (2005). "Neural and behavioral responses to systemic immunologic stimuli: a consideration of bacterial T cell superantigens." Curr Pharm Des **11**(8): 1039-46.

Kusnecov, A. W., R. Liang, et al. (1999). "T-lymphocyte activation increases hypothalamic and amygdaloid expression of CRH mRNA and emotional reactivity to novelty." J Neurosci **19**(11): 4533-43.

Lachowicz, J. E., Y. Shen, et al. (1995). "Molecular cloning of a novel G protein-coupled receptor related to the opiate receptor family." J Neurochem **64**(1): 34-40.

Lambertsen, K. L., B. H. Clausen, et al. (2009). "Microglia protect neurons against ischemia by synthesis of tumor necrosis factor." J Neurosci **29**(5): 1319-30.

Leggett, J. D., K. L. Dawe, et al. (2009). "Endogenous Nociceptin/Orphanin FQ system involvement in HPA axis responses: relevance to models of inflammation." J Neuroendocrinol.

Leggett, J. D., M. S. Harbuz, et al. (2006). "The nociceptin receptor antagonist [Nphe1,Arg14,Lys15]nociceptin/orphanin FQ-NH2 blocks the stimulatory effects of nociceptin/orphanin FQ on the HPA axis in rats." Neuroscience **141**(4): 2051-7.

Leggett, J. D., D. S. Jessop, et al. (2007). "The nociceptin/orphanin FQ antagonist UFP-101 differentially modulates the glucocorticoid response to restraint stress in rats during the peak and nadir phases of the hypothalamo-pituitary-adrenal axis circadian rhythm." Neuroscience **147**(3): 757-64.

Liu, Y. and C. A. Janeway, Jr. (1992). "Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells." Proc Natl Acad Sci U S A **89**(9): 3845-9.

Lutfy, K., H. Lam, et al. (2008). "Alterations in the level of OFQ/N-IR in rat brain regions by cocaine." Neuropharmacology **55**(2): 198-203.

Makino, S., P. W. Gold, et al. (1994). "Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus." Brain Res **640**(1-2): 105-12.

Mamiya, T., Y. Noda, et al. (1998). "Enhancement of spatial attention in nociceptin/orphanin FQ receptor-knockout mice." Brain Res **783**(2): 236-40.

Marquez, P., A. T. Nguyen, et al. (2008). "The endogenous OFQ/N/ORL-1 receptor system regulates the rewarding effects of acute cocaine." Neuropharmacology **54**(3): 564-8.

Marrack, P. and J. Kappler (1990). "The staphylococcal enterotoxins and their relatives." Science **248**(4956): 705-11.

Meijer, O. C., P. J. Steenbergen, et al. (2000). "Differential expression and regional distribution of steroid receptor coactivators SRC-1 and SRC-2 in brain and pituitary." Endocrinology **141**(6): 2192-9.

Meis, S. and H. C. Pape (2001). "Control of glutamate and GABA release by nociceptin/orphanin FQ in the rat lateral amygdala." J Physiol **532**(Pt 3): 701-12.

Mellman, I. and R. M. Steinman (2001). "Dendritic cells: specialized and regulated antigen processing machines." Cell **106**(3): 255-8.

Meunier, J. C., C. Mollereau, et al. (1995). "Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor." Nature **377**(6549): 532-5.

Miller, T. R. and A. J. Fulford (2007). "Regulation of nociceptin/orphaninFQ secretion by immune cells and functional modulation of interleukin-2." Peptides **28**(11): 2243-52.

Mogil, J. S., J. E. Grisel, et al. (1996). "Orphanin FQ is a functional anti-opioid peptide." Neuroscience **75**(2): 333-7.

Mogil, J. S. and G. W. Pasternak (2001). "The Molecular and Behavioral Pharmacology of the OrphaninFQ/Nociceptin Peptide and Receptor Family." Pharmacological Reviews **53**(3): 381-415.

Mollereau, C., M. Parmentier, et al. (1994). "ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization." FEBS Lett **341**(1): 33-8.

Mollereau, C., M. J. Simons, et al. (1996). "Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene." Proc Natl Acad Sci U S A **93**(16): 8666-70.

Mott, R. T., G. Ait-Ghezala, et al. (2004). "Neuronal expression of CD22: novel mechanism for inhibiting microglial proinflammatory cytokine production." Glia **46**(4): 369-79.

Murphy, K. M. and S. L. Reiner (2002). "The lineage decisions of helper T cells." Nat Rev Immunol **2**(12): 933-44.

Nabeshima, T., Y. Noda, et al. (1999). "The role of nociceptin in cognition." Brain Res **848**(1-2): 167-73.

Neal, C. R., Jr., A. Mansour, et al. (1999). "Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding." J Comp Neurol **412**(4): 563-605.

Neal, C. R., Jr., A. Mansour, et al. (1999). "Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat." J Comp Neurol **406**(4): 503-47.

Nicholson, J. R., H. Akil, et al. (2002). "Orphanin FQ-induced hyperphagia is mediated by corticosterone and central glucocorticoid receptors." Neuroscience **115**(2): 637-43.

Nishi, M., H. Takeshima, et al. (1994). "Structure and chromosomal mapping of genes for the mouse k-opioid receptor and an opioid receptor homoloue (MOR-C)." Biochemical and Biophysical Research Communications **205**: 1353-1357.

Nothacker, H. P., R. K. Reinscheid, et al. (1996). "Primary structure and tissue distribution of the orphanin FQ precursor." Proc Natl Acad Sci U S A **93**(16): 8677-82.

Okuda-Ashitaka, E., T. Minami, et al. (1998). "Nocistatin, a peptide that blocks nociceptin action in pain transmission." Nature **392**(6673): 286-9.

Olianas, M. C., S. Dedoni, et al. (2008). "Activation of nociceptin/orphanin FQ-NOP receptor system inhibits tyrosine hydroxylase phosphorylation, dopamine synthesis, and dopamine D(1) receptor signaling in rat nucleus accumbens and dorsal striatum." J Neurochem **107**(2): 544-56.

Olszewski, P. K., C. J. Billington, et al. (2000). "Fos expression in feeding-related brain areas following intracerebroventricular administration of orphanin FQ in rats." Brain Res **855**(1): 171-5.

Onali, P., A. Ingianni, et al. (2001). "Dual coupling of opioid receptor-like (ORL1) receptors to adenylyl cyclase in the different layers of the rat main olfactory bulb." J Neurochem **77**(6): 1520-30.

Ono, N., J. C. Bedran de Castro, et al. (1985). "Ultrashort-loop positive feedback of corticotropin (ACTH)-releasing factor to enhance ACTH release in stress." Proc Natl Acad Sci U S A **82**(10): 3528-31.

Pampusch, M. S., J. R. Serie, et al. (2000). "Expression of nociceptin/OFQ receptor and prepro-nociceptin/OFQ in lymphoid tissues." Peptides **21**(12): 1865-70.

Pan, Y. X., J. Xu, et al. (1996). "Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor." Biochem J **315 (Pt 1)**: 11-3.

Peluso, J., K. S. LaForge, et al. (1998). "Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells." J Neuroimmunol **81**(1-2): 184-92.

Polidori, C., G. de Caro, et al. (2000). "The hyperphagic effect of nociceptin/orphanin FQ in rats." Peptides **21**(7): 1051-62.

Pomonis, J. D., C. J. Billington, et al. (1996). "Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats." Neuroreport **8**(1): 369-71.

Proft, T. and J. D. Fraser (2003). "Bacterial superantigens." Clin Exp Immunol **133**(3): 299-306.

Reinscheid, R. K., A. Ardati, et al. (1996). "Structure-activity relationship studies on the novel neuropeptide orphanin FQ." J Biol Chem **271**(24): 14163-8.

Reinscheid, R. K., H. Nothacker, et al. (2000). "The orphanin FQ/nociceptin gene: structure, tissue distribution of expression and functional implications obtained from knockout mice." Peptides **21**(7): 901-6.

Reinscheid, R. K., H. P. Nothacker, et al. (1995). "Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor." Science **270**(5237): 792-4.

Reiss, D., J. Wichmann, et al. (2008). "Effects of nociceptin/orphanin FQ receptor (NOP) agonist, Ro64-6198, on reactivity to acute pain in mice: comparison to morphine." Eur J Pharmacol **579**(1-3): 141-8.

Rivest, S., N. Laflamme, et al. (1995). "Immune challenge and immobilization stress induce transcription of the gene encoding the CRF receptor in selective nuclei of the rat hypothalamus." J Neurosci **15**(4): 2680-95.

Rossi-George, A., F. LeBlanc, et al. (2004). "Effects of bacterial superantigens on behavior of mice in the elevated plus maze and light-dark box." Brain Behav Immun **18**(1): 46-54.

Rossi-George, A., D. Urbach, et al. (2005). "Neuronal, endocrine, and anorexic responses to the T-cell superantigen staphylococcal enterotoxin A: dependence on tumor necrosis factor-alpha." J Neurosci **25**(22): 5314-22.

Rossi, G. C., M. Perlmutter, et al. (1998). "Orphanin FQ/nociceptin analgesia in the rat." Brain Res **792**(2): 327-30.

Serhan, C. N., I. M. Fierro, et al. (2001). "Cutting edge: nociceptin stimulates neutrophil chemotaxis and recruitment: inhibition by aspirin-triggered-15-epi-lipoxin A4." J Immunol **166**(6): 3650-4.

Shepard, J. D., Y. Liu, et al. (2005). "Role of glucocorticoids and cAMP-mediated repression in limiting corticotropin-releasing hormone transcription during stress." J Neurosci **25**(16): 4073-81.

Shioda, S., F. Takenoya, et al. (2008). "Neural networks of several novel neuropeptides involved in feeding regulation." Nutrition **24**(9): 848-53.

Shirasaka, T., S. Miyahara, et al. (2001). "Nociceptin/orphanin FQ and [Phe(1)psi(CH2-NH)Gly2]nociceptin(1-13)NH2 modulates the activity of hypothalamic paraventricular nucleus neurons in vitro." Brain Res **890**(1): 147-53.

Shurin, G., N. Shanks, et al. (1997). "Hypothalamic-pituitary-adrenal activation by the bacterial superantigen staphylococcal enterotoxin B: role of macrophages and T cells." Neuroendocrinology **65**(1): 18-28.

Smith, G. W., J. M. Aubry, et al. (1998). "Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development." Neuron **20**(6): 1093-102.

Stratford, T. R., M. R. Holahan, et al. (1997). "Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake." Neuroreport **8**(2): 423-6.

Timpl, P., R. Spanagel, et al. (1998). "Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1." Nat Genet **19**(2): 162-6.

Trombella, S., R. Vergura, et al. (2005). "Nociceptin/orphanin FQ stimulates human monocyte chemotaxis via NOP receptor activation." Peptides **26**(8): 1497-502.

Ulrich-Lai, Y. M. and J. P. Herman (2009). "Neural regulation of endocrine and autonomic stress responses." Nat Rev Neurosci **10**(6): 397-409.

Urbach-Ross, D., B. Crowell, et al. (2008). "Relationship of varying patterns of cytokine production to the anorexic and neuroendocrine effects of repeated Staphylococcal enterotoxin A exposure." J Neuroimmunol **196**(1-2): 49-59.

van der Laan, S., S. B. Lachize, et al. (2008). "Nuclear receptor coregulators differentially modulate induction and glucocorticoid receptor-mediated repression of the corticotropin-releasing hormone gene." Endocrinology **149**(2): 725-32.

van Gaalen, M. M., M. P. Stenzel-Poore, et al. (2002). "Effects of transgenic overproduction of CRH on anxiety-like behaviour." Eur J Neurosci **15**(12): 2007-15.

Vanderah, T. W., R. B. Raffa, et al. (1998). "Orphanin-FQ/nociceptin: lack of anti nociceptive, hyperalgesic or allodynic effects in acute thermal or mechanical tests following intracerebroventricular or intrathecal administration to mice or rats." Eur J Pain **2**(3): 267-78.

Vitale, G., R. Arletti, et al. (2006). "Anxiolytic-like effects of nociceptin/orphanin FQ in the elevated plus maze and in the conditioned defensive burying test in rats." Peptides **27**(9): 2193-200.

Wang, J. B., P. S. Johnson, et al. (1994). "cDNA cloning of an orphan opiate receptor gene family member and its splice variant." FEBS Lett **348**(1): 75-9.

Wang, J. B., P. S. Johnson, et al. (1994). "Human kappa opiate receptor second extracellular loop elevates dynorphin's affinity for human mu/kappa chimeras." J Biol Chem **269**(42): 25966-9.

Wang, X., B. R. Wang, et al. (2004). "Fos expression in the rat brain after intraperitoneal injection of Staphylococcus enterotoxin B and the effect of vagotomy." Neurochem Res **29**(9): 1667-74.

Westphal, N. J., R. T. Evans, et al. (2009). "Novel expression of type 1 corticotropin-releasing hormone receptor in multiple endocrine cell types in the murine anterior pituitary." Endocrinology **150**(1): 260-7.

Wick, M. J., S. R. Minnerath, et al. (1994). "Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors." Brain Res Mol Brain Res **27**(1): 37-44.

Xu, Y., T. A. Day, et al. (1999). "The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta administration." Neuroscience **94**(1): 175-83.

Xue, J. C., C. Chen, et al. (1994). "Differential binding domains of peptide and non-peptide ligands in the cloned rat kappa opioid receptor." J Biol Chem **269**(48): 30195-9.

Zalcman, S. S. (2001). "Interleukin-2 potentiates novelty- and GBR 12909-induced exploratory activity." Brain Res **899**(1-2): 1-9.

Zhao, H., H. W. Huang, et al. (2002). "Effect of orphanin FQ on interleukin-1beta mRNA transcripts in the rat CNS." Neuroscience **114**(4): 1019-31.