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IMMUNE STRESS AND REPRODUCTION: INSIGHTS INTO THE ROLE OF NOREPINEPHRINE AND GABA

By

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ABSTRACT

IMMUNE STRESS AND REPRODUCTION: INSIGHTS INTO THE ROLE OF NOREPINEPHRINE AND GABA

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Immune stress compromises reproduction. The cytokine, interleukin-1β (IL-1β), an immune stressor has been has been shown to activate the stress axis and compromise the reproductive axis, resulting in loss of ovulation. The mechanisms behind these effects are still under investigation. The present work addresses the role of stimulatory neurotransmitters like norepinephrine (NE) and inhibitory ones like GABA in mediating IL-1β's effects on reproduction. We hypothesized that systemic IL-1β modulates the levels of NE either at the hypothalamus or brainstem and that GABA too might play a role in this phenomenon. The results of these studies revealed important and novel insights into neuroendocrine changes during a systemic immune challenge: 1) IL-1β decreases NE input at the level of hypothalamus to inhibit LH, which could be reversed by NE precursor, L-dopa 2) This decrease in NE was accompanied by increase in GABA and GABA-B antagonist could reverse the inhibitory effect of IL-1β 3)Simultaneous activation of the stress and reproductive axes in female rats by IL-1β involves changes in NE levels in hypothalamus 4)This could be due to changes in IL-1β signaling at the cell bodies in the brainstem NE neurons. Together these findings provide strong evidence substantiating the role of NE in mediating the effects of a systemic immune challenge on reproduction and opens up exciting future research possibilities.

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LIST OF ABBREVIATIONS

AN	Arcuate Nucleus
BNST	Bed nucleus of the stria terminalis
CeA	Central amygdala
CRH	Corticotrophin releasing hormone
COX-2	Cyclo oxygenase-2
DA	Dopamine
DBB	Diagonal Band of Broca
DBH	Dopamine Beta Hydroxylase
DHBA	Dihydroxy Benzylamine
DOPAC	Dihydroxy phenylacetic acid
FSH	Follicle Stimulating Hormone
GABA	Gamma Amino Butyric Acid
GnRH	Gonadotropin Releasing Hormone
5-HIAA	5-hydroxy Indole Acetic acid
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High Performance Liquid Chromatography
5-HT	5-Hydroxy Tryptamine or Serotonin
IL-1β	Interleukin-1 beta
LH	Luteinizing Hormone
LPS	lipopolysaccharide

MBH	Medial Basal Hypothalamus
ME	Median Eminence
MPA or MPOA	Medial Preoptic Area
NE	Norepinephrine
OVLT	Organum vasculosum lamina terminalis
OVX	Ovariectomy
PCR	Polymerase chain reaction
PE	Proestrus
PSTS	Preoptico suprachiasmatic tuberoinfundibular system
RIA	Radio Immunoassay
SCN	Suprachiasmatic Nucleus
TH	Tyrosine Hydroxylase

CHAPTER 1. GENERAL INTRODUCTION

A. STATEMENT OF PURPOSE:

Organisms survive by maintaining a dynamic equilibrium with the environment around them. Stress can be described as a threat to this equilibrium and adaptation to stress involves regaining homeostasis at molecular, cellular and physiological levels. In the present study, we used peripheral cytokine administration as an immune stress paradigm and examined numerous homeostatic disturbances in terms of neuroendocrine alterations. In the model used in this study, the effects of systemic IL-1β on the reproductive mechanisms were investigated with emphasis on how central noradrenergic systems might contribute to those effects.

To be able to develop strategies to safe guard against any potential deleterious consequences of stress on reproduction, it is essential to first understand the mechanisms by which suppression of reproductive axis takes place. Immune stress is a part of the intangible stressors which are encountered during the course of an infection. We propose to use an immune stress model to understand how stress compromises reproductive function.

Cytokines are components of immune stress and affect various functions of body systems which include suppression of the hypothalamic-pituitary-gonadal (HPG) axis. Among various pro-inflammatory cytokines, IL-1β has been shown to be the most potent cytokine to suppress reproduction. IL-1β, a paradigm for immune stress is capable of bringing about a plethora of changes in the immune, endocrine and the nervous systems (Dinarello, 1994). It is one of the important mediators of the central effects of

lipopolysaccharide (LPS), a hallmark product of bacterial infections (Watanobe & Hayakawa, 2003). The effects of IL-1β on reproductive axis include reduction in LH, anovulation, persistent corpora lutea and pseudo-pregnant like state. One of the manifestations of disease induced stress is hypogonadism, which could involve IL-1 (Baker, 1998). Non-infectious uterine pathological conditions like endometriosis also have been associated with elevated serum levels of IL-1β (Kondera-Anasz et al., 2005). Apart from this, several stressor paradigms like immobilization have been shown to increase hypothalamic levels of IL-1β (Minami et al., 1991; Shintani et al., 1995). So not only in disease conditions, but in other stressful conditions also, elevations in IL-1β can compromise reproductive function. This aspect is not limited to human beings but can be applied to breeding of wild animals in captivity that is constantly challenged by various stressors. Elevated stress levels have been reported in some captive species (Baker et al., 1998; Terio et al., 2004) and reproductive failure in some species has been attributed to lower LH and disrupted ovarian cyclicity (Faulkes et al., 1990). Hence it is important to understand the effect of IL-1β on the reproductive axis. The present work aims to understand the mechanisms by which IL-1β produces these effects on the reproductive axis and results from this study would probably provide insights into strategies to counter this inhibitory effect.

B. ORGANIZATION OF THE HYPOTHALAMIC-PITUITARY GONADAL AXIS:

Reproduction is an intricately coordinated complex event which is essential to the survival and progeneration of species. In higher mammals, the circuitry responsible for this complex phenomenon is described to be compartmentalized into three levels of regulation – the hypothalamus, anterior pituitary and the gonads – ovary in the female and testis in the male. The gonadotrophin-releasing hormone (GnRH) which is produced from the hypothalamus is released from the neurovascular terminals of the median eminence and conveyed via the hypophysial portal circulation to reach the anterior pituitary gland. It binds to the receptors in the gonadotrophs and effectuates the synthesis and release of two hormones – follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH enter circulation and act on the ovary to cause maturation of the follicles and ovulation respectively.

C.ESTROUS CYCLE IN RATS:

Rats are described as spontaneously ovulating, polyestrous and non-seasonal breeders. This is because they do not require an overt nervous stimulation for ovulation and hence are spontaneous ovulators, their ovarian cycle continues throughout the year, hence non-seasonal and ovulation takes place once every 4-5 days, hence polyestrous.

The estrous cycle in rats is divided into four stages: estrus, diestrus I (also referred to as metestrus), diestrus II and proestrus. During these four stages of the estrous cycle, dynamic alterations in all the components of the reproductive axis take place. Changes in the hypothalamic release of GnRH will eventuate in changes in the pituitary release of

FSH and LH. These gonadotrophins influence various changes in the ovarian steroid milieu and the ovarian steroids, estrogen and progesterone in turn influence the other components of the reproductive tract. The mean length of the estrous cycle is described to be 4.5 days (Freeman 2004). So it can be either 4 or 5 days long with each stage lasting a day.

During diestrus, the ovarian follicles would range from small to medium sized ones and produce only a small amount of estrogen. As a result, there would be low level of negative feedback on the HPG axis. The pulsatility of LH and FSH start to increase following increasing inputs from the GnRH system. The FSH that is released into the bloodstream acts on the ovarian follicles to produce maturational changes in the follicles and stimulate the production of estrogen from the follicles. As the levels of estrogen increase, they would in turn provide negative feedback to the hypothalamic-pituitary unit resulting in decreased pulsatility of FSH and LH. However, as the follicle matures, increasing production of estrogen during early hours of proestrus leads to a positive feedback on the hypothalamic GnRH system (Brown-Grant et al. 1970; Butcher et al. 1974). This triggers a GnRH surge, which culminates in the central event of reproductive cycle, the preovulatory LH surge that is essential for ovulation (Gay et al. 1970; Kalra 1975). During proestrus, a peak in the levels of estrogen as well as progesterone produced by the granulosa cells of the mature follicle (Hashimoto *et al.* 1968). Estrus is the stage when the rat is sexually receptive and this is time window when sexual receptivity and ovulation are synchronized. During estrus, the levels of LH, estrogen return to basal levels. Reorganization of the cells of the ovarian follicle leads to the formation of corpus luteum which produces progesterone and hence a second, smaller peak of progesterone

during the course of the estrous cycle (Hashimoto and Wiest 1969). This marks the early stages of diestrus.

Paralleling these fascinating changes in the hormonal profile, tissue rearrangement takes place in other components of the reproductive tract like the vagina and the uterus. Vaginal epithelial cells have been shown to exhibit trypsin-like activity (Havran and Oster 1977) which could account for the exfoliative changes in the vaginal epithelium during the various stages of the estrous cycle. The stage of estrous cycle in rats can be determined by a non-invasive procedure by studying changes in the exfoliated vaginal epithelium, the details of which are described in the Methods section.

The circulating levels of estrogen elicit a complex pattern of differentiating events in the vaginal epithelium. In ovariectomized rodents, the vaginal epithelium is only 2-3 cell layers thick, similar to that observed during diestrus. In response to estrogen, basal epithelial cells proliferate and this leads to the formation of highly stratified epithelium. These rapid proliferative changes in the epithelium are attributed to shortening of the S-phase of the cell cycle in pre-existing G_1 cells (Thrasher et al. 1967; Galand et al. 1971). Levels of estrogen have also been correlated with mucification of the vaginal epithelium (Parlanti and Monis 1980). Hence there would be an increase in epithelial cell clusters and mucus production on proestrus paralleling the increasing levels of estrogen. The non-mitotic epithelial cells undergo a differentiative sequence as they move up thorugh the epithelium and become enlarged and cornified (Buchanan *et al.* 1998). Hence, the presence of large numbers of anucleated, keratinized cells during estrus. It has been shown that the microflora and immune cells composition of the vagina undergoes changes during the estrous cycle (Larsen *et al.* 1977). As levels of estrogen

drop and progesterone increase during diestrus, leukocytic infiltration of the vaginal epithelium is observed, probably assisting in the phagocytosis of the anuclear cell debris. As diestrus advances, the leukocytic population diminishes and proliferative changes for the next proestrus take place.

D.GnRH NEURONAL NETWORK:

GnRH neurons are unique in several aspects. Though they are principally distributed in the hypothalamus in the adult mammal, embryonically they originate in the medial olfactory placode of the developing nose, migrate across the nasal septum and enter the forebrain arching into the septal-preoptic area and hypothalamus (Schwanzel-Fukuda and Pfaff 1989). Unlike other neuropeptidergic systems of the hypothalamus that are organized as a compact cluster, GnRH neurons are scattered and distributed over a number of areas in the brain. The distribution of GnRH neurons in the rat brain is described as an inverted 'V' pattern surrounding the organum vasculosum of the lamina terminalis (OVLT) (Hiatt *et al.* 1992). It is believed that GnRH neurons in the hypothalamus are organized as the preopticosuprachiasmatic tuberoinfundibular system (PSTS) with perikarya in the medial preoptic area (MPA), suprachiasmatic nucleus and the arcuate nucleus and terminals in the median eminence (Barraclough and Wise 1984). The distribution of GnRH cell bodies is also referred to as a scattered "continuum" along their migratory pathway from olfactory bulbs to the median septal nuclei, diagonal band of Broca, and the medial preoptic area through to the medial basal hypothalamus (MBH)(Herbison 2006). Based on the stage of estrous cycle, the release of GnRH under physiological conditions takes place in two modes – a pulsatile, low amplitude secretion

and a high amplitude surge. However the mechanism by which these modes of secretion take place has lacked consensus. The different theories that explain the two modes of secretion can be summed up as those that consider the notion of distinct anatomical compartments that regulate these patterns and those that would not.

One of the explanations for this phenomenon is the presence of anatomically distinct neuronal groups of GnRH neurons that might be responsible for this pulsatile and surge secretions. The neuronal groups responsible for pulsatile release constitute the 'pulse generator' which might be located in the mediobasal hypothalamus (MBH) and the groups that produce the surge constitute the 'surge generator' located in the OVLT-MPA region(Kimura and Funabashi 1998). However modern understanding of the phenomenon discounts this notion of the existence of exclusive and segregated components. GnRH neurons are capable of intrinsic pulsatility as demonstrated in single cell recordings from immortalized GnRH neuronal cell line, GT-1(Wetsel *et al.* 1992). Several explanations involving Ca^{2+}, cAMP and circadian genes of the Period, Clock and Cryptochrome have been suggested to be responsible for firing a high frequency volley of action potentials periodically, eventuating in the generation of a GnRH pulse (Khadra and Li 2006). But how does synchronization of these individual GnRH neuronal pulses take place is another aspect which has not been well understood. One of the models proposed involves synchronization due to extensive interconnections between GnRH neurons which allows for reciprocal activation (Herbison 2006).

A surge like pattern of GnRH secretion is responsible for the generation of preovulatory LH surge in female mammals. Using c-fos as a marker of neuronal activation, it has been shown that a sub-population of GnRH neurons in the OVLT-MPA

region is activated most during the proestrus LH surge (Lee *et al.* 1990) suggesting that surge generation could be confined to a specialized subset of GnRH neurons. In a recent model proposed by Levine, the initiation of GnRH surge is conceived to be an integration of signals from circadian input and estrogen's positive feedback mechanisms (Levine 1997). This is supposed to ensure coordination of two major components of ovulation-ovarian readiness and sexual behavior. The dependence of surge on estrogen ensures that ovulation takes place only after necessary maturational changes in the follicles and dependence on neural circadian signal restricts its occurrence to a certain circadian time. This enables that sexual behavior and ovulation both occur in the same window of time.

The regulation of GnRH neurons during their pulsatile and surge secretion involves a multitude of neurotransmitters. In fact, it might not be exaggeration if it were to be assumed that almost every class of known neurotransmitter has been shown to influence the secretion of GnRH. This only underscores the complexity of signal integration and reciprocal connectivity among members of the GnRH network. A detailed analysis of the neurotransmitters involved in regulation of GnRH is beyond the scope of this section. However, the details about the role of norepinephrine and GABA would be discussed in this section while a table of the major effects by other neuropeptides and neurochemicals would be summarized in Table 1.

E. ROLE OF NOREPINEPHRINE (NE) IN THE REGULATION OF LUTEINIZING HORMONE (LH):

The suggestion that NE might be involved in the regulation of LH dates back to 1947, based on a classic study by Sawyer et al. which demonstrated that blockade of

alpha-adrenergic receptors by dibenamine could inhibit ovulation in rabbits (Sawyer *et al.* 1947). There has been a multitude of studies in all these fifty years from then which have not only strengthened the argument, but have also muddled the waters. Barraclough and Wise in their exemplary review (Barraclough and Wise 1984) on the role of catecholamines in the regulation of LH to remark that "*more often than not, the vast amounts of confusing literature on this subject have bewildered the novice and discouraged the faint of heart and, as such, research into the complexities of this system have waxed and waned*" summing up the state of research in this area. This section is intended to provide an overview of the most influential studies in this field, with emphasis on current understanding of the concept.

Several experimental paradigms have been employed to dissect out the role of NE in the regulation of LH. As mentioned in the earlier sections, there are two distinct components in the release of LH – a tonic, pulsatile secretion and an exaggerated surge on the day of proestrous in rats. Some of the inconsistency in the literature has been due to the discrepancy in the experimental models used- some of which have observed effects on the pulsatile secretion and some on the surge mechanism. It would be prudent to exclusively interpret these studies on the basis of whether the experimental strategy studies the effect on pulsatile LH secretion or LH surge. However, more often than not, this has not been the case and lot of misinterpretation has been due to drawing inappropriate conclusions by generalizing the mode of LH secretion.

Most of the initial experiments that studied the role of NE in LH secretion were pharmacological interventions. However the lack of specificity in the drugs used has contributed in part to the confusing inferences derived from them. Broadly, the strategies

used were: blockade of postsynaptic adrenergic receptors, using drugs that deplete NE from the presynaptic terminals, inhibiting the enzymes involved in the synthesis of NE, or using drugs that prevent the metabolism of NE.

The observation that dibenamine, an alpha-adrenergic blocker that inhibited ovulation in rabbits was subsequently extended to rats also. Dibenamine and SKF-501 which is 3-10 times more potent than dibenamine were shown to inhibit ovulation in cycling rats (Sawyer *et al.* 1950). Another study which used a different alpha-adrenergic antagonist, Chlorpromazine too concurred with the previous results that LH surge as well as ovulation were blocked (Barraclough and Sawyer 1957). However, in ovariectomized, steroid primed models, alpha adrenergic blocker, phenoxybenzamine blocked the progesterone induced LH surge but a beta adrenergic blocker, propanolol failed to show any effect (Kalra et al. 1972; Weick 1978). Even in a recent study in ewes, beta adrenergic blocker, doxasin failed to produce any effect on the LH (Clarke *et al.* 2006) surge reiterating the claim that modulation of GnRH neurons by NE is through alpha adrenergic mechanisms.

Pharmacological studies in the 1970s led to the description of alpha adrenergic subtypes (Civantos Calzada and Aleixandre de Artinano 2001) and subsequent studies in LH regulation attempted to pin down which receptor subtype might be responsible for transducing the stimulatory signal onto GnRH neurons. Selective alpha-1 adrenergic receptor blocker, Prazosin was shown to inhibit LH surge while alpha-2 selective blockers did not show this effect (Drouva et al. 1982; Le et al. 1997a). Alpha-1 adrenergic mechanisms were also implicated in the regulation of pulsatile secretion of LH(Gearing and Terasawa 1991; Le et al. 1997a) as well as regulation of GnRH mRNA

in the hypothalamus (Weesner *et al.* 1993). Later, immunohistochemical studies were also able to demonstrate the localization of alpha-1 adrenergic receptor on the GnRH neurons (Hosny and Jennes 1998). Recent studies point that stimulation of GnRH neurons by NE through alpha-1B subtype of adrenergic receptors involving downstream activation of phospholipase-C and cytosolic phospholipase-A2 (Kreda *et al.* 2001).

Apart from receptor blockade, one of the other strategies used to implicate the role of NE in LH regulation was the use of drugs which deplete NE from the presynaptic terminals. Reserpine depletes NE by constant release from the storage granules and inhibiting granular reuptake (Kopin and Gordon 1962). This drug has been shown to block LH surge and ovulation (Barraclough and Sawyer 1957; Coppola et al. 1966). This blockade could be reversed by pargyline, a drug which inhibits the enzymes involved in rapid NE breakdown (Meyerson and Sawyer 1968; France 1970). Deprenyl, an inhibitor of monoamine oxidase has also been shown to potentiate LH release from the pituitary(MohanKumar *et al.* 1997a). This further reinforces the direct involvement of NE on GnRH neurons.

NE is synthesized from dietary amino acid L-tyrosine by enzyme-mediated catalysis (Nagatsu 1991). The first step involves conversion of L-tyrosine to L-dopa by tyrosine hydroxylase (TH) and this has been shown to be the rate-limiting step in NE biosynthesis (Nagatsu *et al.* 1964). Specific inhibitors of the enzymes involved in the biosynthesis of NE have been demonstrated to be capable of affecting LH surge. Specifically, alpha-methyl paratyrosine (α-MPT), an inhibitor of TH has been shown to block LH surge and ovulation (Kalra and McCann 1974; Brann and Mahesh 1991). Pesticides like DDT, fungicides like Sodium N-methyldithiocarbamate which can inhibit

dopamine β-hydroxylase have also been shown to decrease NE synthesis and subsequently block LH surge (Coen and Coombs 1983; Goldman et al. 1994). The inhibitory effect of α-MPT or DDC on ovulation could be reversed by NE precursors, DOPS or L-DOPA which effectively restored the LH surge (Donoso et al. 1971; Kalra et al. 1972).

One of the strategies to implicate the role of NE in the regulation of LH surge has been using neurotoxic drugs or lesions of the noradrenergic system on LH release. 6-hydroxydopamine (6-OHDA) is capable of destroying terminal endings of NE neurons and cause long lasting noradrenergic depletion. Injections of 6-OHDA into the preoptic area or the median forebrain bundle showed lowering of LH secretion for 24hrs (Kitchen *et al.* 1974) and injections into the midbrain ventral noradrenergic tract was shown to block LH surge (Martinovic and McCann 1977). However, a subsequent study using 6-OHDA injections into the caudal ventral pons found decrease in NE concentrations in the hypothalamus after 16-23 days, no changes in the serum LH were observed (Nicholson *et al.* 1978). This could be attributed to the time gap between the injection of 6-OHDA and measurement of NE and LH. When 6-OHDA was directly infused into the medial preoptic area, disruption in estrous cyclicity as well decrease in the height of the LH surge was demonstrated (Hancke and Wuttke 1979). In ovariectomized, steroid-primed models, LH surge was blocked by 6-OHDA lesions in the ME (Simpkins *et al.* 1979) or medial basal hypothalamus (Simpkins and Kalra 1979). *N*-(2-chloroethyl)-*N*-ethyl 2-bromobenzylamine (DSP-4) is a neurotoxin capable of inducing selective denervation of NE terminals. In contrast to 6-OHDA, DSP-4 can pass the blood–brain barrier and appears to have a preferential, long-lasting neurodegenerative action on NE terminal

projections. Intraperitoneal administration of DSP-4 has been shown to selectively decrease NE levels and block progesterone-induced LH surge in ovariectomized rats while the levels of dopamine were unaffected (Kang *et al.* 1998).

An alternative strategy to study noradrenergic influence on the LH surge has been to mechanically or electrolytically destroy certain components of the NE system and study the effects on LH surge. Complete deafferentiation of the medial basal hypothalamus lead to complete depletion of NE and the animals exhibited persistent diestrus and low LH concentrations (Weiner *et al.* 1972). Studies on the enzymatic activity of TH after bilateral lesions of the ventral noradrenergic bundle showed no effect on the activity in median eminence. After transections of ascending noradrenergic pathway, chronic depletion of NE for 40 days did not seem to affect the estrous cyclicity or LH surge (Clifton and Sawyer 1979) and this shows the possibility for remarkable plasticity of hypothalamic neuroendocrine systems after the loss of a major neurotransmitter input. The results from this study and other similar studies where hypothalamic NE was chronically depleted suggest that alternative systems may be brought into play to subserve reproductive mechanisms when hypothalamic NE is chronically depleted. Bilateral electrolytic lesions of the locus coeruleus showed a decrease in NE in the MBH resulting in a subsequent loss in cyclicity and blocked LH surge (Franci and Antunes-Rodrigues 1985; Anselmo-Franci et al. 1997).

Several studies have used NE turnover rate as an index of noradrenergic activity in the hypothalamic areas and have shown an increase in NE turnover in these areas in proestrous preceeding the LH surge (Honma and Wuttke 1980; Rance et al. 1981; Wise et al. 1981).

Direct intra-cerebroventricular infusions of NE have only yielded equivocal and confusing results which have been a subject of unfortunate misinterpretation. When NE was infused at a rate of 0.3nM in 4μl/min (2pg/min) in Nembutal-blocked proestrus rats after electrochemical stimulation, an augmentation in LH secretion was observed. However, when higher concentrations of 30nM and 3000nM (200pg/min and 20,000pg/min respectively) were used, it was shown that LH secretion was blocked(Cramer and Barraclough 1978). Using ovariectomized rats, it was showed that 0.3-0.6μg/h (5000-10,000 pg/min) had no effect on LH secretion in the absence of steroids but increased LH upon treatment with estrogen and progesterone. However, higher concentrations of 5.5-11μg/h (92,000-184,000 pg/min) were shown to block LH secretion irrespective of the steroid status (Gallo and Drouva 1979). Similar results were obtained with respect to pulsatile release of LH where 0.3-1.8μg/h (5000-30,000pg/min) were used (Gallo 1984). Recent advances in the use of sensitive methods of direct determination of NE in the areas rich in GnRH neurons has richly contributed to our understanding of the role of noradrenergic systems in the regulation of LH surge. Measurement of NE by push-pull perfusion or microdialysis followed by HPLC-EC has provided invaluable insights into the dynamics of NE release and how it coordinates with LH surge. It has been demonstrated that levels of NE rhythmically increase in the medial preoptic area on the day of proestrus to 2-6 pg/min as measured by push-pull perfusion followed by HPLC-EC and this correlates very well with the timing of the LH surge (Mohankumar et al. 1994; Szawka et al. 2007) in female rats (Mohankumar et al. 1994). These results were recently replicated in a different study using microdialysis (Szawka et al. 2007). In the light of these recent observations, it could be argued that using 1000-fold

higher concentrations than physiological doses in earlier studies could have lead to intriguing results obtained. Moreover, in continuous infusion studies, the rhythmic release of NE which occurs physiologically could not be recapitulated. This could be another reason why they could not observe a stimulatory role of NE on LH.

Table 1-1 Influence of various neurochemicals/neuropeptides on GnRH/LH secretion

Neuropeptide/ Neurochemical	Effect On GnRH/LH-Immuno-Colocalization, Receptor Expression On GnRH Neurons	References
Neuropeptide -Y	Stimulates GnRH release from median eminence in vitro	(Sabatino *et al.* 1989, Urban *et al.* 1996)
	Expression of hypothalamic NPY increases prior to LH surge	(Sahu *et al.* 1995)
	Direct connection between NPY-GnRH neurons	(Li *et al.* 1999)
Vasoactive intestinal peptide (VIP)	Inhibitory to pulsatile LH secretion	(Alexander *et al.* 1985, Stobie & Weick 1990)
	Acts on GnRH	(Vijayan *et al.* 1979)
	Stimulates GnRH release from median eminence in vitro	(Samson *et al.* 1981)
	Localization of VIP2 receptor on GnRH neurons	(Smith *et al.* 2000)
Neurotensin	Stimulatory to GnRH	(Kalra 1993)
	Direct infusion increases LH and receptors localized on GnRH neurons	(Rostene & Alexander 1997)
Orexin	Stimulatory and inhibitory actions based on central site of injection	(Small *et al.* 2003)
	Stimulatory or inhibitory action of orexin-A, B –ovarian steroid dependent	(Furuta *et al.* 2002, Pu *et al.* 1998)
	Direct connections between Orexin A and GnRH neurons	(Campbell *et al.* 2003, Small *et al.* 2003)
Dynorphin	Suppressive effect on LH	(Kinoshita *et al.* 1982, Leadem & Kalra 1985)

Table 1-1 continued.

	Direct infusion blocks LH and immuno-neutralization reverses	(Zhang & Gallo 2002, Zhang *et al.* 2002)
Substance P	Decreases LH	(Battmann *et al.* 1991, Duval *et al.* 1996)
	Stimulates GnRH from MBH in vitro	(Ohtsuka *et al.* 1987)
	Synaptic contact with GnRH neurons	(Dudas & Merchenthaler 2002a, Tsuruo *et al.* 1991)
Corticotrophin-releasing hormone (CRH)	Inhibitory to LH	(Ono *et al.* 1984, Petraglia *et al.* 1987, Rivest & Rivier 1995)
	Direct synaptic contacts with GnRH neurons	(Dudas & Merchenthaler 2002b, MacLusky *et al.* 1988)
Arginine-Vasopressin (AVP)	Vasopressin antagonists decrease LH surge	(Funabashi *et al.* 1999)
	High doses of AVP suppresses LH surge	(Cates *et al.* 1999)
	Time dependent stimulatory effect of AVP on LH surge	(Palm *et al.* 2001)
Oxytocin	Stimulatory effect on LH surge	(Johnston *et al.* 1992, Selvage & Johnston 2001)
	Stimulates GnRH release in vitro	(Rettori *et al.* 1997)
Kisspeptin	Stimulatory to LH	(Navarro *et al.* 2004, Navarro *et al.* 2005)
	Hypothalamic expression of kisspeptins	(Roa *et al.* 2006)
	Direct effect on GnRH via receptor mediated mechanisms	(Messager *et al.* 2005)

Table 1-1 continued.

Neuromedin-S,U	Stimulatory to LH	(Oppido *et al.* 2006, Vigo *et al.* 2007)
Leptin	Stimulatory to LH	(Carbone *et al.* 2005, Shebl 2002, Watanobe *et al.* 1999)
Glutamate	Stimulatory to LH	(Brann & Mahesh 1997, Terasawa 2001)
	NMDA and non-NMDA receptors involved	(Lopez *et al.* 1992)
	Immuno-colocalization with GnRH neurons	(Kocsis *et al.* 2003)
	VGLUT1,VGLUT2 present on GnRH neurons	(Giri & Kaufman 1995, Lopez et al. 1992)
	Increased glutamate levels in the MPA corresponding to LH	(Jarry *et al.* 1992)
Serotonin	Stimulates GnRH release in vitro	(Meyer 1989)
	Immuno-colocalization with GnRH neurons	(Kiss & Halasz 1985)
Acetylcholine	Inhibitory effect on LH	(Piva *et al.* 1980)
	Stimulatory effect on LH in ovariectomized rats	(Vijayan & McCann 1980)
	Atropine blocks LH	(Libertun & McCann 1973)
	Receptor antagonists inhibit LH	(Billiar *et al.* 1988, Kalash *et al.* 1989)
Cannabinoids	Inhibitory to LH	(Ayalon *et al.* 1977, Wenger *et al.* 1987)
	Endogenous cannabinoid, anandamide also inhibitory to LH	(Wenger *et al.* 1995)

Table 1-1 continued.

Dopamine	Stimulatory/Modulatory	(Honma & Wuttke 1980, ThyagaRajan *et al.* 1995)
	Synaptic connections	(Jennes *et al.* 1983)

F. ORGANIZATION OF NORADRENERGIC PATHWAYS TO THE GnRH NEURONAL SYSTEM:

The noradrenergic input to the MPA is provided by catecholaminergic cell bodies in the brainstem (Mueller and Nistico 1989b). By using a combined horseradish peroxidase/catecholamine fluorescence study, it was demonstrated that the principal noradrenergic innervation to medial preoptic area comes from brain stem ventrolateral medulla(A1) and nucleus of solitary tract (A2) cell groups (Day *et al.* 1980). Using Fluoro-gold combined with immunohistochemistry, it was found that retrogradely labeled neurons were observed in the A1, A2 noradrenergic cell groups and locus coeruleus (A6). Among these neurons, the highest concentrations of DBH-positive double-labeled neurons were found in the A2 and A1 cell groups (Wright and Jennes 1993). Electrophysiological stimulation of A1 cell group has been shown to have a stimulatory input on the medial preoptic area (Kaba et al. 1983; Kim et al. 1987). During the various stages of estrous cycle, the levels of activation of brainstem neurons in A1 and A2 cell groups have been shown to be different. The highest levels of neuronal activation as measured by the expression of c-fos were found on proestrus (Jennes et al. 1992; Conde et al. 1995). The functional changes in the expression of TH and *c-fos* in the brainstem noradrenergic nuclei during estradiol induced LH surge has also been demonstrated (Conde et al. 1995; Temel et al. 2002). Also, stimulation of A1 noradrenergic neurons resulted in temporal changes in TH mRNA expression in A1, A2 and A6 regions suggesting the dynamic interaction between the noradrenergic cell groups coordinating a stimulatory input onto GnRH neurons (Liaw *et al.* 1992). To delineate the relative contribution of the various noradrenergic cell groups, neuronal stimulation followed by

measurement of NE discharge by microdialysis was performed. It was found that A1-evoked NE release was much higher than that observed after A2 stimulation (Fernandez-Galaz *et al.* 1994). Surprisingly, a more recent study by the same group using conditional viral tract tracing with Cre-dependent pseudorabies virus has demonstrated that the preoptic GnRH neurons receive principal noradrenergic innervation from A2 and A6 cell groups (Campbell and Herbison 2007).

Lesioning of the locus coeruleus has been shown to decrease GnRH release as well as block LH surge in rats (Anselmo-Franci et al. 1997; Helena et al. 2002; Martins-Afferri et al. 2003). However, it is not clear if A6 region is directly involved by providing NE input to the GnRH neurons or involved in feedback regulatory activity.

The synaptic connections between noradrenergic terminals and GnRH neurons were demonstrated by electron microscopic double immunostaining procedures (Ajika 1979; Leranth et al. 1988). Also, localization of alpha-1B receptors on GnRH neurons has been demonstrated (Hosny and Jennes 1998). Recent studies in immortalized GnRH neurons (GT-1) have shown the involvement of alpha-2A and beta-1 adrenergic receptors in the regulation of GnRH neurons and receptor activation mediated release of GnRH (Martinez de la Escalera et al. 1992; Lee et al. 1995). Though there have been several lines of evidence to indicate the role of brainstem noradrenergic neurons in modulating GnRH release, there are other lines of thought which contemplate that NE mediated stimulation of GnRH neurons could be by indirect means, say through other neurotransmitters like GABA or NPY (Herbison 1997) .

G. ROLE OF GAMMA AMINO BUTYRIC ACID (GABA) IN THE REGULATION OF LH:

The amino acid neurotransmitter, GABA is considered to be the principal inhibitory neurotransmitter of the adult nervous system. GABA neurons are considered to play a major role in the regulation of GnRH. A direct synaptic connection between GABA neurons on GnRH neurons has been established in the medial preoptic area (Leranth *et al.* 1985). The functional expression of GABA-A receptors in GnRH neurons has been demonstrated in a number of models. Using double immunohistofluorescence, it has been shown that GnRH neurons express alpha 1, alpha 2, beta 2/3, and gamma 2 GABA-A receptor subunits (Jung *et al.* 1998). Using GFP-tagged GnRH in transgenic mice, the electrophysiological properties of GABA-A receptors has been elucidated (Spergel *et al.* 1999).

GABAergic neurons are considered to be major part of the GnRH network and have shown to influence the pulsatile as well as the surge release of GnRH (Adler and Crowley 1986; Jarry et al. 1988). Endogenous GABA has been shown exert an inhibitory effect on the firing of GnRH neurons (Han *et al.* 2004). It has been demonstrated by push-pull perfusion technique that levels of GABA are low during the afternoon of proestrus when LH levels are found to increase (Demling et al. 1985; Jarry et al. 1992).These low levels of GABA are believed to increase the responsiveness of GnRH neurons which facilitates LH surge (Lamberts et al. 1983; Fuchs et al. 1984). GABA agonists inhibit LH (Feleder et al. 1999b), while application of GABA receptor antagonists increase GnRH responsiveness to NE (Hartman *et al.* 1990). Hence existing

evidence suggests a temporally reciprocal relationship between GABA and NE in the regulation of LH.

H. INTERLEUKIN-1β – MOLECULAR BIOLOGY AND SIGNALING:

During systemic infections, a complex interaction takes place between the immune, endocrine and the nervous systems. Recent advances in the research in this area have given us insights not only into the macro systems, but into the intricate molecular cross talk between these various components of the homeostatic machinery. One of the immediate humoral events following and immune challenge is the release of cytokines by activated macrophages. Among the cytokines released, interleukin-1 (IL-1) is considered to be an extremely potent pro-inflammatory cytokine. IL-1 has been shown to be involved in interactions between immune, reproductive, stress and homeostatic regulatory systems (Dinarello 1994; Cannon 1998; Licinio and Frost 2000).

The role of IL-1 in acute phase response and as an endogenous pyrogen was first described in 1977 (Merriman *et al.* 1977). Ever since its discovery, the several members of its gene family have been described. The classical members of the IL-1 gene family are: IL- lα, IL-1β, and IL-1 receptor antagonist (IL-1ra). IL-lα and IL-lβ are agonists and IL-1ra is a specific receptor antagonist (Dinarello 1996). The presence of IL-1ra is considered to be a unique cytokine control mechanism because it can bind to the receptor without inducing signal transduction. Studies that have compared of the intron-exon organization of these three genes have implied the role of gene duplication as an early event in the evolution of this gene family (Eisenberg *et al.* 1991). However other members like the surface receptor, IL-1RI, soluble IL-1 receptor (sIL-1R or IL-1RII) and

IL-1R accessory protein (IL-1RAcP), IL-18, another pro-inflammatory cytokine have been described (Boraschi and Tagliabue 2006). Both IL-1RI and IL-1RII bind to IL-1 but IL-1RII is not capable to initiating a signal transduction event and hence described as a decoy receptor (Colotta *et al.* 1993), another unique aspect of interleukin-1 biology.

The two isoforms, IL-1α and IL-1β have a sequence similarity of 22% in humans and are present in the same chromosome (Chromosome 2 in humans and Chromosome 3 in rats) and possess similar biological activities. However, IL-1β is considered to be a more potent form, as it is the principal secreted form whereas IL-1α is a cell-associated molecule. IL-1β is initially synthesized as a precursor molecule (31KDa) without a signal peptide which is then cleaved into the mature peptide (17.5kDa) by the action of IL-1β-converting enzyme (ICE) which belongs to a family of intracellular cysteine proteases called caspases (Dinarello 2001). The primary sources of circulating IL-1β are monocytes, macrophage and dendritic cells; however other sources of IL-1β like the CNS have also been described (Deak *et al.* 2005). IL-1β deficient mouse has been described and it has not shown any phenotypical or developmental abnormalities suggesting the minimal physiological involvement of IL-1β and the potential redundancies among cytokine networks. However, this model has been shown to be resistant to fever induction, absence of acute phase response and anorexia upon immune stimulation underlining the role of IL-1β in mediating these events (Zheng *et al.* 1995).

The various biological activities of IL-1 are brought about by the sequential signal transduction events that take place after IL-1 binds to its cognate receptor, IL-1RI. These receptors, IL-1RI and IL-1RII as well as IL-1RAcP which belong to the immunoglobulin superfamily of proteins, contain three immunoglobulin-like domains which are critical

for ligand binding. IL-1RI has a single transmembrane segment and a cytoplasmic domain, which differs greatly with the cytoplasmic domain of IL-1RII which is short and inactive (Colotta et al. 1993). IL-1RAcP helps in forming a high affinity complex upon IL-1's binding with its receptor, IL-1RI. Interestingly, IL-1RI has been shown to share homology with an evolutionarily conserved set of proteins referred to as Toll-like proteins (Gay and Keith 1991). IL-1Rs and Toll-like receptors (TLR) have a conserved cytoplasmic domain known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions referred to as boxes 1, 2 and 3(Slack *et al.* 2000).

Comprehensive details about each of the signaling molecules involved in this pathway are beyond the scope of this section; however an outline of the signaling events that take place in the signaling cascade initiated by IL-1β is provided. After ligand binding, IL-1RI dimerizes and undergoes conformational change required for the recruitment of downstream signaling molecules. IL-1RAcP also binds to the IL-1RI and this complex recruits and adapter molecule, myeloid differentiation primary-response protein-88 (Myd88) to the cytoplasmic TIR domain. This facilitates association of IL-1R-associated kinase-4 (IRAK-4) with the receptor complex through dimer-dimer interaction. This association leads to phosphorylation of another kinase, IRAK-1 which is associated with Toll-like interacting protein (Tollip). Phosphorylation of IRAK-1 leads to its dissociation and autophosphorylation due to its kinase activity. This hyperphosphorylated IRAK-1 enables another protein, TNF receptor-associated factor -6 (TRAF-6) to bind to this complex. Upon binding of TRAF-6, the IRAK-1-TRAF-6 complex disengages and phosphorylates another preformed complex of proteins,

comprising of transforming growth factor-β activated kinase (TAK 1) and its protein partners. Phosphorylation of TAK-1 can lead to activation of divergent outcomes – activation of NF-κB pathway or JNK-MAPK pathway or other transcription factors like AP-1(Akira and Takeda 2004; Subramaniam et al. 2004; Miggin and O'Neill 2006). It has been shown that IL-1β is capable of cell type specific activation of downstream pathways. In hippocampal cultures, IL-1β could activate MAPK pathway in neurons whereas NF-κB pathway was shown to be activated in the astrocyte cultures (Srinivasan *et al.* 2004). Activation of NF-κB pathway involves phosphorylation of the inhibitor of NF-κB (IκB) which leads to degradation of IκB by polyubiquitylation and proteasome-mediated degradation. This allows release of NF-κB, so that it gets translocates to the nucleus and initiates transcription of the target genes. An alternative pathway for signal transduction of IL-1RI involving 1,2-diacylglycerol, generated by a phosphatidylcholine-specific phospholipase C, and ceramide, generated by sphingomyelinase leading to activation of the mitogen-activated protein (MAP) kinase cascade and NF-κB translocation (Schutze *et al.* 1994).

I. NEUROIMMUNE INTERFACE: IL-1β'S SIGNALING FROM PERIPHERY TO THE CNS:

How do peripheral immune signals communicate with the brain is the central question in the field of neuroimmunology. The advances in our understanding of how neuro, endocrine and immune systems talk to each other has provided us critical breakthroughs in our approach to various pathological conditions. The mode by which cytokines rapidly relay immune signals from the periphery to the brain has perplexed

researchers for many years. According to the present understanding many routes of communication have been proposed: neural route involving the vagus, humoral route involving transport via the blood-brain barrier (BBB), circumventricular organs, secretion of cytokines by the BBB in response to systemic inflammatory stimuli as well as relay of information via second messengers across the BBB into the brain (Quan and Banks 2007).

LPS was shown to induce sickness behavior by a vagally mediated mechanism; this was the first evidence to imply the role of vagus in relaying peripheral immune stimulus to the brain (Bluthe *et al.* 1994). Subsequently an exodus of experiments (Watkins *et al.* 1994) that followed attempted to mechanistically imply the role of vagus by using a technique called sub-diaphragmatic vagotomy did not yield unequivocal results, unfortunately. For example, one study shows that sub-diaphragmatic vagotomy does not block fever during intraperitoneal LPS administration (Hansen *et al.* 2000) while another study states it does block fever but does not affect cytokine production (Gaykema *et al.* 2000). Those discrepancies apart, results from a different set of studies provides some evidence that vagal route indeed is involved in relaying the immune signal from periphery to the brain. Vagal paraganglia are capable of binding to IL-1RA and levels of IL-1 increase in the abdominal vagus as early as 45 min after LPS administration (Goehler *et al.* 1997). Also, dendritic cells and macrophages associated with abdominal vagus have been shown to be express IL-1 immunoreactivity within 60 min after intraperitoneal injection (Goehler *et al.* 1999). Support for this 'hard-wired' neural route of communication keeps building due to the fact that this presents an attractive

hypothesis to explain the speed with which signals travel to the brain and how specific targets of the brain are activated without the interference of the BBB.

IL-1 can also enter the brain by crossing the BBB. A saturable transport mechanism for IL-1 to cross the BBB by using transporter has been described (Banks *et al.* 1991). IL-1 by itself is capable of modifying the permeability of BBB and this could also account for easy access of IL-1 to the brain (Blamire *et al.* 2000). The cellular elements of the BBB by themselves have been shown to be capable of producing IL-1 in response to immune stimuli. In that context, IL-1 is also described as a neurotransmitter across the BBB (Quan and Banks 2007). Some studies have described the leakage of IL-1 into the brain, through the circumventricular organs (CVO) which are outside the BBB (Breder et al. 1988; Komaki et al. 1992). After intravenous injection of IL-1, it has been estimated that 0.08% of that dose would reach the brain by using kinetics of iodinated-IL-1(Banks and Kastin 1991). Also using similar technique, it was shown that saturable transport through the BBB accounts for the major mode of transport while CVO mediated extracellular transport accounted only for 5% of the total transported IL-1 (Plotkin *et al.* 1996).

J. EFFECTS OF IL-1β ON THE REPRODUCTIVE AXIS:

Immune stress compromises reproduction. During an infection or an inflammatory process, release of cytokines mediates numerous changes in the neuroendocrine systems. One of the principal cytokine that has been extensively studied with regard to its effects on the reproductive axis is IL-1. It is beyond any doubt that IL-1 is inhibitory to the HPG axis; however there are a few discrepancies between various

studies with regard to nature and extent of its effects. It would be very helpful to appreciate the animal model used– whether it is male/female, intact/gonadectomized or if the treatment was acute/chronic or peripheral/central in drawing conclusions from the studies in question.

The effect of cytokines on LH secretion was first shown in castrated male rats (Rivier and Vale 1989). In this study, it was shown that centrally injected IL-1α interfered with the HPG axis at hypothalamic level and not at the pituitary level to affect LH secretion. Later studies in this model showed that when equimolar doses of IL-1α and IL-1β were administered, the latter had more pronounced inhibitory effect on LH secretion and that probably glutamate or hypothalamic opiate pathways might be involved in mediating this inhibition (Bonavera et al. 1993b, a). During an endotoxin challenge, by using neutralizing antibodies to downstream cytokines, the role of IL-1β was implicated in the suppression of LH (Ebisui *et al.* 1992). The release of GnRH into the median eminence was decreased by IL-1β and the involvement of prostaglandins was postulated (Rivest and Rivier 1993a). Central administration of IL-1β was able to decrease GnRH mRNA, translational efficiency of GnRH as well as hypothalamic levels of GnRH receptor (Kang *et al.* 2000).

In female rats, the prevoulatory LH surge on proestrus and ovulation was shown to be blocked by central administration of 40ng of IL-1β or 400ng of IL-1α (Rivier and Vale 1990). This block was similar to that produced by 10 μg of GnRH antagonist. However in this experiment, iv administration of 500ng of IL-1β failed to produce this effect. Interestingly systemic injection of the same dose of IL-1β produced changes in body temperature similar to the ones observed with central administration. Apart from

these effects on LH, central administration of IL-1β a time dependent block on the induction of c-fos, a neuronal marker of activation in GnRH neurons on proestrus (Rivest and Rivier 1993c). 50ng of icv IL-1β given at 1200h significantly reduced percentage of GnRH neurons expressing c-fos, but no effect was observed when given at 0830 or at later time points of 1430h, 1700h. This raises the possibility that there is a narrow window of time during which the GnRH system is most susceptible to immune stress. Also GnRH release from the median eminence in proestrus females also was decreased upon treatment with IL-1β. Taken together, IL-1β is capable of perturbing the GnRH neuronal system at the level of mechanisms controlling, transcription, translation and/or release. Among the areas of GnRH neuronal network, MPA appears to be most susceptible to IL-1β, as direct infusions into this area produced effects similar to those with icv administration whereas infusions into arcuate nucleus did not yield the same results (Rivier and Rivest 1993).

In ovariectomized-steroid-primed model of inducing LH surge, central administration of 30ng of IL-1β completely blocked LH while 1μg intravenous dose was reported to be ineffective (Kalra et al. 1990a). In the same model, immobilization stress was able to abrogate the increase in LH induced by steroid administration (Kam *et al.* 2000).

In in-vitro studies, direct application of IL-1β on MBH-preoptic area significantly reduced GnRH production while application on the median eminence did not produce such effect (Kalra et al. 1990a).

During a sub-chronic icv infusion of 4ng/h IL-1β for 4-6 days, it was observed that estrous cyclicity was disrupted and the animals remained in diestrus. The levels of

LH in circulation as well as GnRH mRNA in the MPA were decreased by IL-1β (Rivest *et al.* 1993). This effect was similar to to that obtained by daily s.c injections of GnRH antagonist. At the gonadal level, an increase in progesterone levels and induction of pseudopregnant-like corpora lutea were observed (Rivier and Erickson 1993). However, this effect was not observed by the treatment with GnRH antagonist. From these experiments, it might be inferred that IL-1β primarily affects the HPG axis at the level of hypothalamus but has inhibitory effects on the ovary too. Exposure to cold stress daily for 3-9 days could disrupt estrous cyclicity, decrease plasma LH, GnRH mRNA and increase hypothalamic levels of IL-1β (Tanebe *et al.* 2000) indicating that chronic stress induced reproductive dysfunction could be mediated through the effects of endogenous cytokines.

Peripheral administration of IL-1β has also been shown to affect sexual behavior and partner preference. Female rats injected with 5μg/kg IL-1β showed decreased proceptive behavior, lordosis (Yirmiya *et al.* 1995) and during partner preference, males preferred saline-treated rats over IL-1β-treated rats (Avitsur *et al.* 1997). This indicates that during an immune challenge, apart from the endocrine disturbances of the HPG axis, behavioral alterations that decrease chances of conception are manifested.

K. EFFECTS OF IL-1β ON THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS:

The hypothalamo-pituitary-adrenocortical (HPA) axis is the primary modulator of the stress response. Activation of the HPA axis takes place in response to a variety of homeostatic perturbations. This involves activation of corticotrophin-releasing

hormone (CRH) neurons in a discrete hypothalamic area, the paraventricular nucleus (PVN). CRH is secreted into the portal circulation to influence the release of adrenocorticotrophic hormone (ACTH) from the pituitary which enters into the circulation to act on the adrenals to secrete corticosteroids. The level of corticosterone in the circulation is often used as measure of the activation of the HPA axis. The PVN receives numerous direct inputs from the brainstem noradrenergic neurons, the bed nucleus of the stria terminalis and the dorsal raphe, which modulate the secretion of CRH from this area (Champagne *et al.* 1998). Apart from these direct inputs, it also receives numerous indirect inputs from areas like the hippocampus, central amygdala and the lateral septum (Herman *et al.* 2003).

The activation of the HPA axis by IL-1 is one of the most studied phenomena in neuro-immune interactions. Besedovsky et al., demonstrated for the first time that peripheral immune challenges stimulate the production of glucocorticoids (Besedovsky *et al.* 1986), thereby providing evidence for interactions of the immune system with neuroendocrine systems. Subsequently the involvement of CRH in this phenomenon was described by two independent groups, interestingly at the same time (Berkenbosch et al. 1987; Sapolsky et al. 1987). The increase in ACTH as well as corticosterone could be blocked by immunoneuralization of CRH whereas incubation of pituitary cultures with IL-1 did not produce any effect. Hence it was inferred that cytokine-mediated inhibition of HPA axis is at the hypothalamic level and not by direct interactions at either the pituitary or adrenal levels. Among the hypothalamic areas, direct infusion of IL-1β into the PVN alone showed responses comparable to i.c.v or i.v administration highlighting

the role of this area in mediating the HPA responses to immune challenges (Barbanel et al. 1990; Watanobe and Takebe 1993).

One of the principal stimulatory inputs to the CRH neurons in the PVN is derived from the noradrenergic system (Szafarczyk *et al.* 1987). The central as well as peripheral administration of IL-1β resulted in increased ACTH and this could be blocked by using noradrenergic receptor antagonists (Rivier *et al.* 1989). Also, lesioning the noradrenergic innervation of PVN using 6-OHDA prevented IL-1β-induced increase in HPA activity, underlining the involvement of NE in process (Matta et al. 1990; Chuluyan et al. 1992). The metabolism of norepinephrine was shown to be affected by injection of IL-1 providing further evidence for the relevance of NE in neuro-immune interactions (Kabiersch *et al.* 1988).

Sub-chronic administration of IL-1β for showed a centrally mediated adaptive response by the HPA axis. Repeated administration of IL-1β by central and peripheral routes for three days showed increases in ACTH and corticosterone. However, by the third day, the responses were comparable to baseline values (van der Meer *et al.* 1996). The levels of NE in the PVN, a marker of CRH activation as well as corticosterone levels which increased after acute administration of IL-1β, failed to show such effect upon repeated injections for five days (MohanKumar *et al.* 2003). Chronic stress paradigms have been shown to induce IL-1 in the brain and the expression of glucocorticoid receptor has been shown to decrease by such treatment (Bartolomucci *et al.* 2003).

Most of the above cited studies had used the male rat as their experimental model. The effect of immune challenges on the HPA axis in female rats has not been well studied. In female rats, the response of the HPA axis has shown to be dependent on the

stage of estrous cycle. During an acute restraint stress paradigm, it was found ACTH and corticosterone responses were more pronounced on proestrus compared to estrus or diestrus. Also, in ovariectomized rats, administration of estrogen and progesterone accentuated the HPA responses compared to oil-treated controls indicating the role of gonadal steroids in mediating these responses (Viau and Meaney 1991).

HPA responses to immune challenges have been shown to be sexually dimorphic (Spinedi et al. 1992; Da Silva et al. 1993; Shanks et al. 1994; Lee and Rivier 1996). In immature, 40d old and 70d old rats, administration of 0.5-2 μg/kg i.v showed an exaggerated ACTH, corticosterone responses in female rats when compared to their male counterparts which was negated upon gonadectomy (Rivier 1994). In similar studies, the HPA responses of females have consistently been higher than the males (Frederic et al. 1993; Spinedi et al. 1997). However, the intact females in these experiments were used at random stages of estrous cycle. Given the fact that HPA responses differ with respect to the stage of the estrous cycle, it is hard to draw meaningful conclusions from such results.

L.THESIS OBJECTIVE:

The mechanisms underlying IL-1β's action on the HPG axis are still rather unclear. Several possible candidates have been investigated-among them are neurotransmitters like NE, GABA, opioids, CRH, tachykinins (Kalra et al. 1990b; Kalra et al. 1998; MohanKumar and MohanKumar 2002; Akema et al. 2005). The medial preoptic area (MPA) of the hypothalamus houses a large number of GnRH cell bodies. We have shown that peripheral administration of IL-1β drastically disrupts rhythmic

release of NE in the MPA and thereby blocks LH surge in steroid-primed ovariectomized rat model (MohanKumar and MohanKumar 2002). The logical extension of this idea would be to explore how this decrease in NE is brought about by IL-1β and at what level of the noradrenergic system does IL-1β act to produce these effects.

The regulation of GnRH neurons in the MPA involves a complex interplay between stimulatory neurotransmitters like NE and inhibitory ones like GABA. NE dynamics include the changes in the release and reuptake patterns in the axon terminals as well as the changes in the biosynthesis at the level of cell bodies. The cell bodies of the NE neurons that project to the MPA are located in the brainstem. IL-1β could affect locally at the MPA or rather more globally at the level of brainstem. However the questions of how IL-1β interacts with this complex network of neurotransmitters and would GABA come into this equation remains unexplored.

We hypothesize that IL-1β could affect the HPG axis by acting at the level of noradrenergic terminals and/or the cell bodies in the brainstem and GABA would play a role in this process. To test this hypothesis, the present study is designed with the following specific aims: 1. To determine if systemic IL-1β is capable of affecting NE biosynthesis to suppress LH and if this can be reversed by using a NE precursor, L-dopa. 2. To determine if systemic IL-1β acts on GABA inter neurons in the noradrenergic terminals to suppress LH and if GABA antagonists can reverse this phenomenon. 3. To determine if systemic IL-1β differentially affects the levels of NE in the hypothalamic areas associated with the HPA and HPG axes, 4. To determine if this differential action of systemic IL-1β is due to its action at the level of brainstem involving changes in the

expression of the key enzyme for NE biosynthesis, tyrosine hydroxylase (TH) and other genes in the IL-1β signaling pathway.

CHAPTER 2. MATERIALS AND METHODS

A.Animals:

Three-to-four-month-old female Sprague Dawley rats weighing approximately 250g were obtained from Harlan Inc. (Indianapolis, IN). They were housed in air-conditioned (23 ± 2 °C) and light controlled (lights on from 0500 to 1900 h) animal rooms and provided with rat chow and water ad libitum. Animals were used in the experiments in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee at MSU.

B.Cytology:

Vaginal Cytology:

To assess the stage of estrous cycle, vaginal lavage was performed daily at 0800-0900 h for all the rats. Smears were made by lavage with lukewarm, autoclaved nanopure water of the vagina using a medicine dropper and thinly spread on a pre-numbered clean glass slide. The smears were allowed to dry for 15 minutes at 60°C and stained with 0.5% Methylene blue (Sigma) using a staining rack. The stained slides were rinsed in clean tap water and then allowed to dry. The smears were examined under light microscope using 10x objective. The stage of the estrous cycle was determined using the criteria described in previous studies (Everett 1989; Mohankumar et al. 1994; Goldman et al. 2007). Rats in proestrus are identified by presence of clusters of round, nucleated epithelial cells, which often have a granular appearance under the microscope with few to no cornified cells.

Proestrus lasts for one day and is followed by estrus, routinely identifiable by the presence of large numbers of cornified cells or more rounded cells with jagged edges. The following day diestrus I is characterized by the presence of a combination of leukocytes and cornified and rounded epithelial cells. The concentration of leukocytes can vary, and the smear can often be almost exclusively leukocytic. The second day of diestrus (diestrus II) may also show a few small clumps of nucleated epithelial cells, leukocytes and a few cornified cells; but the absolute cell numbers are decreased. This stage is followed by the next proestrus.

C. Surgical procedures:

Jugular vein catherization and Blood sample collection

The rats were maintained under anesthesia using Isoflurane (2%) and oxygen mixture during the procedure. The catheter was prepared with Silastic lab tubing (0.64mm ID x 1.19 mm OD, Dow Corning, Midland, MI) and sterilized with ethanol prior to surgical implantation. With the rat resting in supine position, the skin caudal to the base of the neck was sterilized and a small incision made. The vein was carefully isolated and freed from adhering membranous tissue. A small incision was made on the vein using an 18 ga needle and the catheter introduced. The catheter was secured to the vein by black braided 3-0 silk thread (Ethicon, Somerville, NJ).The catheter was exteriorized at the nape using a trochar. The catheter was flushed with heparinized saline and then a blunt piece of a 22 ga needle was used to plug the line. The surgical incision was secured by wound clips. The rat was allowed to recover in a clean cage over a temperature-regulated heating pad.

During the experiment, blood samples were collected every hour. Samples were centrifuged at about 800xg and plasma collected and frozen at -20°C until they were analyzed for LH by RIA.

Ovariectomy

After a two-week acclimatization period, all the animals were weighed and randomly divided into six groups. All animals were bilaterally ovariectomized and were implanted with a push–pull cannula in the MPA and an icv cannula as described before. Briefly, the animals were given Atropine sulphate (2.2 mg/kg, subcutaneous (s.c)) as a preanesthetic anti-sialagogue and were anesthetized using sodium pentobarbital (35 mg/kg, i.p.). The ovaries were removed through a midventral incision after ligation of the uterine horns and the ovarian vasculature. The abdominal muscles were sutured using sterile chromic gut 2-0 sutures and the skin incision was closed using sterile wound clips (Beckton Dickinson, Sparks, MD).

Intracerebroventricular cannula implantation

For the intracerebroventricular injections, the rats were implanted with a stainless steel cannula (22-G) in the lateral ventricle stereotaxically(Kopf, Tujunga, CA), using the following coordinates: 1.8 mm posterior, 2.6 mm lateral, 3.6 mm ventral to bregma. After the cannula was secured with dental cement, a stylet made of 29-G stainless steel tubing was used to plug the guide cannula to avoid blockage. At the time of the experiment, the stylet was removed and a 30G inner cannula connected to a Hamilton syringe with polyethylene tubing was used to deliver the drugs used.

Push – pull cannula implantation

In Chapter 3, intact cycling rats and in chapter 4, ovariectomized rats were implanted with push-pull cannulae in the MPA as described before (MohanKumar and MohanKumar 2005). The rats were given atropine sulphate (0.1mg/kg) and anesthetized with a combination of xylazine (7mg/kg) and ketamine (55mg/kg) i.p. After the rat was secured with a stereotaxic apparatus (Kopf, Tujunga, CA), it was implanted with a push-pull cannula in the MPA with the coordinates: 8.5 mm ventral, 0.3 mm posterior, and 0.3 mm lateral to the bregma. The cannula was constructed as described before (Mohankumar et al. 1994). It consisted of an 8.5mm-long outer cannula made from a 22-ga-aluminium hub hypodermic needle (Jeffers Inc, Dothan, AL). The cannula was secured in place with screws and dental cement. After implantation, a 29-ga stainless steel stylet was introduced to prevent clogging of the outer cannula. The rats were allowed to recover and have free access to food and water during the rest period of two weeks.

Push-pull perfusion procedure

The push–pull perfusion procedure has been described in detail earlier (Mohankumar et al. 1994; MohanKumar and MohanKumar 2004, 2005). On the day of push–pull perfusion, the stylet was replaced with an inner cannula assembly, which consisted of two 29-ga stainless steel tubes of unequal lengths. The longer tube (3.5 cm), which protruded 0.5 mm beyond the outer cannula, was used to introduce (push) the perfusion medium at the implantation site. The shorter tube (2.0 cm) was used to collect (pull) perfusate from

the implantation site. The two tubes were kept together in a 2 mm-long piece of Silastic tubing which was mounted with epoxy resin in the lower part of a tuberculin syringe cut at the 0.05 ml mark. The push and pull tubes were connected to two identically calibrated peristaltic pumps (Pharmacia, Uppsala, Sweden). Before starting the perfusion, it was ensured that the pumps were perfectly balanced. Artificial cerebrospinal fluid (ACSF) was used as the perfusion medium. It consisted of $CaCl_2$ (0.087 g/l), NaCl (7.188 g/l), KCl (0.358 g/l), $MgSO_4$ (0.296 g/l), and Na_2HPO_4 (1.703 g/l) and had a pH of 7.3. Pump speeds were adjusted to achieve a flow rate of 10 µl/min. The rats were introduced into the perfusion cages at 1000 h and perfusion was started at 1200 h. Push–pull perfusates were collected in both groups from 1300 to 1800 h/1900h at 30-min intervals at the rate of 10 µl/min. Perfusates were mixed with 0.5 M $HClO_4$ at the rate of 25:1 v/v and stored at −70°C until HPLC analysis. Venous blood samples were collected through the jugular catheter at 1-h intervals from 1300 to 1800h/1900h. Blood samples were centrifuged at 3000rpm for 15 min to separate the serum. Serum samples were stored in −20°C until they were used for LH-RIA.

D.Histology

Brain Histology

The animals used for perfusion study were sacrificed at 1830h/1930h and their brains quickly removed, frozen and stored at -70°C until further processing. Coronal sections (60µm thick) of the brain were obtained using a cryostat maintained at -10°C. The sections were arranged on precleaned Superfrost slides (Fisher, Pittsburgh,PA) and stained with FD Cresyl violet (FD Neurotech, Elliot city, MD) as per the manufacturer's

instructions. The stained sections mounted with Permount medium (Fisher) and examined under light microscope to verify the cannula implantation site.

Ovarian Histology

Adult, cycling female rats were randomly subjected to one of the four treatments: control (PBS-1.0% BSA; n=4), IL-1β (5μg i.p; n=4), L-dopa (50mg/kg BW i.p; Sigma, St. Louis, MO; n=4) or IL-1β + L-dopa (n=4) at 1300 h on proestrus. They were sacrificed between 1400-1500 h on the following day (estrus) and the ovaries were collected in neutral buffered formalin and processed for sectioning. The sections were stained with hemotoxylin and eosin for detailed histological examination.

The histological sections of the ovary stained by hematoxylin-eosin were analyzed for the presence of fresh corpora lutea (FCL), indicated by the presence of increased vascularity within the CL. The numbers of old corpora lutea (OCL) as well as mature follicles (MF) were also enumerated. Fresh corpora lutea (FCL) were identified by the histological presence of a hemorrhage and the transition from a follicular appearance to luteal phenotype, old corpora lutea (OCL) by the presence of densely packed luteal cells while the mature follicles (MF) were identified by the presence of prominent antral space (E, 100x magnification).

E. Microdissection

Palkovits microdissection of discrete brain areas

Serial coronal sections (300 μm thick) of the brainstem were obtained using a cryostat (Slee Mainz, London, UK) maintained at −10°C. The sections were transferred to

precleaned microscopic slides placed on a cold stage at −10°C. The brain areas – organum vasculosum lamina terminalis (OVLT), diagonal band of Broca (DBB), medial preoptic area (MPA), suprachiasmatic nucleus (SCN), arcuate nucleus (AN), paraventricular nucleus (PVN), central amygdala (CeA), bed nucleus of the stria terminalis (BNST) and the brain stem areas - catecholaminergic nuclei A1, A2 and A6 were microdissected by the Palkovits's micropunch technique using a 500-μm-diameter punch with the rat brain stereotaxic atlas as a reference. Tissue samples were obtained bilaterally, and all the subdivisions of the nuclei were included. For the microdissection of brainstem areas, precleaned microscopic slides were wiped with RNAseZap (Sigma-Aldrich Co., St. Louis, MO) prior to mounting sections for inactivation of potential RNAases. The samples were kept in dry ice before they were used for neurotransmitter detection by HPLC-EC or RNA extraction.

F.HPLC

HPLC – EC for detection of Norepinephrine:

The HPLC-EC procedure for determination of NE has been described previously. (MohanKumar and MohanKumar 2004, 2005).Briefly, the HPLC-EC apparatus consisted of an LC-10 AT/VP pump (Shimadzu, Columbia, MD), a phase II 5-μm ODS reverse-phase C_{18} column (Phenomenex, Torrance, CA), a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN), a model CTO-10 AT/VP column oven (Shimadzu, Columbia, MD) maintained at 37°C, and an LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN). The data were integrated using a computer with the Class-VP chromatography Laboratory Automated Software system (ver 7.3 SP1,Shimadzu).

The mobile phase was made with pyrogen-free water and contained monochloroacetic acid (14.14 g/L), sodium hydroxide (4.675 g/L), octanesulfonic acid disodium salt (0.3 g/L), EDTA (0.25 g/L), and acetonitrile (2.8%). The mobile phase was then filtered and degassed through a Milli-Q purification system (Millipore Co., Bedford, MA). Tetrahydrofuran (0.7%) was added to the mobile phase and pumped at a flow rate of 1.8 mL/min. The range of the detector was 1 nA full scale, and the potential of the working electrode was 0.65 V. The sensitivity of the system was less than 1 pg.

Analysis of perfusates: At the time of analysis, the samples were thawed at room temperature and 120 µl of perfusate and 25 µl of the internal standard (0.05 M dihydroxy benzyl amine; Sigma Chemical Co., St. Louis, MO) were loaded on to the autosampler model SIL-10AF (Shimadzu, Columbia, MD) with a sample cooler maintaining samples at 4°C.

Analysis of microdissected brain areas: At the time of HPLC analysis, microdissected brain tissue samples were homogenized in 150 µl of 0.1 M $HClO_4$ using a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ) and centrifuged at 12,000 x g for 10 min. 60 µl of the supernatant was mixed with 30 µl of the internal standard (0.05 M dihydroxybenzylamine) and 75 µl of this mixture was injected into the HPLC system.

HPLC determination of GABA:

The HPLC apparatus consisted of an ESA model 584 pump (ESA, Chelmsford,MA), a Waters XTerra MS C-18 2.5 µm 3x50mm column (Waters, Milford, MA), a coulometric electrode model 5014B, microdialysis cell (ESA), a column oven maintained at 35°C. The data were integrated using a computer with ESA's Coularray for Windows

automated software system (ver 2.0, ESA). The mobile phase was made with pyrogen-free water and contained 100mM disodium hydirgen phosphate pH 6.7 with phosphoric acid, 20% methanol, 3.5% acetonitrile. The mobile phase was then filtered and degassed through a Milli-Q purification system (Millipore Co., Bedford, MA) and pumped at a flow rate of 0.5 mL/min. There was no gain on this system and the potential of the working electrode was 550mV. At the time of analysis, the samples were thawed at room temperature and 19µl of perfusate and 1 µl of the internal standard (Homoserine at 1ng/µl; Sigma Chemical Co., St. Louis, MO) were loaded on to the autosampler model 542 (ESA, Chelmsford, MA) with a sample cooler maintaining samples at 4°C. The autosampler performed derivitization of the samples before injection as follows: addition of 30 µl of working reagent (2.5ml of derivitization reagent and 7.5ml of 0.1M tertraborate buffer) to the perfusates, mixing four times, incubation for one minute and injecting 20µl. The working reagent was made fresh daily. The derivitization reagent consisted of 0.2 M O-phthalaldehyde in 1ml methanol, 0.05% β-mercaptoethanol, 9ml 0.1M tertaborate buffer pH 9.3. The sensitivity of the system was less than 1 pg/µl.

G.Immunoassays:

LH-RIA:

Double antibody RIA was used to determine LH levels in the serum samples as described before (Mohankumar et al. 1994; MohanKumar and MohanKumar 2002). The reference preparation for LH was NIDDK rLH-RP-3. The primary antibody used was anti rLH-S11. LH standards, iodination quality LH protein and LH primary antibody were obtained from Dr A.F. Parlow, NIDDK. LH was iodinated by Peptide Radioiodination

Service, University of Mississippi. Serum samples (50-75µl) were assayed in duplicate. The assay had a sensitivity of <10 pg. The inter-assay variability was 10.4±6.7% and the intra-assay variability was 3.8±2.16%.

Corticosterone –RIA

Double antibody RIA was used to measure corticosterone levels in the serum as described previously (Francis *et al.* 2000) . Corticosterone standards and the I125 labeled corticosterone were obtained from Diagnostic Products Inc. (Los Angeles, CA). The primary and secondary antibodies were raised in our laboratory and were used at dilutions of 1:17500 and 1:11000 respectively. The sensitivity of the corticosterone assay was 0.2 ng/ml.

Serum IL-1β-ELISA:

Serum IL-1β levels were measured in duplicate using a commercial ELISA kit (TiterZyme Kits, Assay Design, Ann Arbor, MI). The assay was carried out according to the manufacturers' instructions. The sensitivity of the kit was <12 pg/ml. All data are expressed as ng cytokine per ml serum.

H.Protein determination

Protein concentrations in the brain tissue homogenates (10 µl) were used in duplicates for protein quantification using the micro-Bicinchoninic acid (micro BCA) assay (Pierce, Rockford, IL). Bovine serum albumin was used as a reference and the absorbance values were obtained at 562 nm using an ELX 800 microplate reader (Biotek

Instruments, Winooski, VT). The neurotransmitter concentrations were expressed as pg/μg protein.

I.Quantitative RT-PCR:

RNA extraction:

The RNA was extracted from the brainstem punches using MELT Total Nucleic Acid Isolation System (Ambion Inc, Austin, TX) according to the manufacturer's protocol. Using the Multi-Enzymatic Liquefaction of Tissue (MELT) mix provided in the kit, the tissue was digested. After on-bead Turbo DNAse digestion (Ambion), the RNA was eluted in a volume of 500 μl. First strand cDNA was synthesized by reverse transcribing 250ng of total RNA using Superscript III First strand synthesis Supermix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

qRT-PCR:

The real-time quantitative PCR mix consisted of 1x Platinum SYBR Green PCR Masterr Mix-(Applied Biosystems, Foster city, CA) and 300 nM of forward and reverse primers. 2.5 μl (equivalent of 50ng of single-stranded cDNA) of the RT reaction was used for quantization of β-actin mRNA, TH mRNA. A 115-bp product for rat tyrosine hydroxylase (Gen Bank accession no. NM 012740) was amplified using the following primers: forward, 5'-CTACCAGCCTGTGTACTTTGTGTC-3'; reverse, 5'-CAGTGTGTACGGGTCAAACTTC-3. Similarly, a 146-bp product of rat ß-actin (GenBank accession no. NM 031144) was amplified using the following primer set: forward, 5'-ATCATGAAGTGTGACGTTGACAT-3'; reverse, 5'-ATGATCTTGATCTTCATGGTGCTA-3'. The reactions were performed in The Applied

Biosystems 7500 Real-Time PCR System (Applied Biosystems) with the following settings: 50 C for 2 min, 95 C for 2 min, followed by 40 cycles of 95 C for 15 sec, 60 C for 60 sec, and 72 C for 35 sec. At the end of amplification, a melting curve analysis was done by heating the PCR products to 65–95 C and held for 15 sec at increments of 0.2 C, and the fluorescence was detected to confirm the presence of a single amplification product. Each sample was run in duplicate to obtain average C_T values for TH mRNA, and ß-actin mRNA. For negative controls, No-RT controls were used as template in place of single-stranded cDNA in the real-time quantitative PCR. TH mRNA, quantity were expressed as a proportion of ß-actin mRNA quantity following the standard curve method for converting log-linear C_T values to RNA values. The relative amounts of TH mRNA in various brain stem areas were then compared.

qPCR array

Quantitative mRNA expression analysis of 84 genes related to IL-1β signaling pathway, chemokines and their receptors in brainstem regions – A1, A2 and A6 were performed with the rat TLR RT^2 profiler PCR array system-Pathway-Focused gene expression profiling using real-time PCR (SuperArray Bioscience Corporation, Frederick, MD). Total RNA was isolated from brainstem areas of individual rats with vehicle and IL-1β-treated groups (n=4) using the MELT Total Nucleic Acid Isolation System (Ambion Inc, Austin, TX). 400ng of RNA from each sample were then converted to cDNA using RT^2 First strand kit (SuperArray Bioscience Corporation). cDNAs from each experimental condition were pooled, and PCR array analysis was performed according to the manufacturer's protocol with the RT^2 Real-Time SYBR Green PCR Master Mix (SuperArray Bioscience Corporation). PCRs were performed using RT^2 profiler PCR

array PARN-018A (rat TLR) on the Applied Biosystems 7500 system. The total volume of the PCR was 25 µl. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. mRNA expression of each gene was normalized using the expression of multiple house keeping genes – Beta actin, Lactate dehydrogenase and Ribosomal protein L13A and compared with the data obtained from with the control group (vehicle treated) according to the $2^{-\Delta\Delta CT}$ method. The data analysis was performed using the Excel macro provided with the kit and Ingenuity Pathway analysis software (Ingenuity Systems Inc, Redwood City, CA).

J.Statistical analysis:

All statistical procedures were performed using SAS software (Cary, NC) unless specified otherwise. Changes in NE, GABA and LH profiles were analyzed by repeated measures ANOVA followed by Fisher's LSD. The average values of NE and LH were compared using one way ANOVA followed by post-hoc Fisher's LSD. Changes in ovarian histology were analyzed by Kruskal-Wallis test followed by post-hoc Bonferroni-Dunn test. Changes in NE concentrations in brain areas at various time points were analyzed by two way ANOVA followed by Fisher's LSD. The differences in levels of corticosterone, LH and IL-1β were also analyzed by two-way ANOVA followed by Fisher's LSD. The differences in the expression of TH mRNA were analyzed by two-way ANOVA followed by Fisher's LSD. The differences in the gene expression in the PCR array were performed by t-test which was in-built in the Ingenuity pathway analysis software.

CHAPTER 3. SYSTEMIC IL-1β-INDUCED SUPPRESSION OF LH SURGE – EFFECT OF NORADRENERGIC AGONISTS AND L-DOPA

INTRODUCTION:

The cytokine interleukin-1β (IL-1β) produces profound effects on the neuroendocrine system (Neveu and Liege 2000),(Rivier and Rivest 1993). These effects affect the immune, endocrine and nervous systems (Licinio and Frost 2000). For example, IL-1β stimulates the hypothalamic-pituitary-adrenal (HPA) axis (Rivier et al. 1989; Smagin et al. 1996), (Tsagarakis *et al.* 1989), which in turn affects the immune and endocrine systems. It also inhibits the hypothalamic-pituitary-gonadal (HPG) axis, which results in loss of reproductive function (Rivier and Vale 1989; Rivier et al. 1989; Kalra et al. 1990b; Kalra et al. 1990a; Rivier and Vale 1990). This is marked by a reduction in luteinizing hormone (LH) levels and failure of ovulation, which could translate into a number of reproductive problems such as hypothalamic amenorrhea, anovulation and infertility (Bulun and Adashi 2008)

The mechanism by which IL-1β affects the HPG axis to suppress reproductive functions is still unclear. Since LH secretion is under the direct stimulatory control of gonadotropin releasing hormone (GnRH) neurons of the hypothalamus, this has been suggested to be a possible site of action (Rivier and Vale 1990). There are at least two sets of GnRH neurons in the female rat: one that regulates pulsatile secretion of LH throughout the reproductive cycle, and another that regulates the surge in GnRH that results in a peak in LH levels during the afternoon of proestrus (Sarkar et al. 1976; Gallo 1980; Barraclough and Wise 1982, 1984; Lee et al. 1990). GnRH neurons that regulate

the LH surge are organized as the preopticosuprachiasmatic tuberoinfundibular system in the hypothalamus (Barraclough and Wise 1982) and the cell bodies of the neurons belonging to this system are located in the suprachiasmatic nucleus, medial preoptic area (MPA), and the arcuate nucleus. Of these, the MPA houses a large number of GnRH perikarya (Ibata et al. 1979; Witkin et al. 1982) and plays a major role in the generation of the LH surge. The terminals of these neurons extend to the median eminence. GnRH that is released from these terminals enters the portal circulation and reaches the anterior pituitary to release LH, which in turn causes the growth and eventually ovulation of the follicles in the ovary (Mueller and Nistico 1989a).

Regulation of GnRH secretion is highly complex and a large number of neurotransmitters and neuropeptides are believed to be involved in this process (Mueller and Nistico 1989a; Barraclough 1992; Kalra and Crowley 1992; Pau and Spies 1997). The earliest to be identified were the catecholamines, and several studies support their role in LH regulation (Kalra 1977) (Barraclough 1983) reviewed in (Barraclough and Wise 1982). Techniques such as measuring turnover, concentration and release have been used to demonstrate a stimulatory role for NE in the preovulatory LH surge (Wise 1984; Mohankumar et al. 1994, 1995). Moreover, lesioning of specific noradrenergic nuclei that innervate the hypothalamus, were also capable of suppressing LH levels (Anselmo-Franci *et al.* 1997) indicating a positive role for norepinephrine in the LH surge. Thus, it is possible that IL-1β-induced suppression of LH secretion could be mediated through reductions in hypothalamic NE. To study this, we used an intact female rat model. We hypothesized that IL-1β could decrease NE levels in the hypothalamus by decreasing biosynthesis and/or release of NE from the terminals. To

investigate this possibility, we attempted to reverse the effects of IL-1β by using NE agonists and a noradrenergic precursor, L-dihydroxy phenylalanine (L-dopa). We also attempted to reverse the effects of IL-1β on plasma LH and ovulation by using L-dopa. This could provide possible treatment options in cases of reproductive failure associated with chronic inflammatory conditions.

EXPERIMENTAL DESIGN:

Experiment 1: *Effects of systemic administration of IL-1β on serum LH in intact cycling animals:* Estrous cyclicity was monitored by daily vaginal cytology and rats that showed regular estrous cycles were selected and implanted with indwelling jugular catheters on the day of diestrus. The next day (proestrus), after collecting a pretreatment sample at 1300 h, they were randomly injected i.p. with either 250µl of PBS-1.0% BSA (control; n=7) or 5µg of recombinant rat IL-1β (Abazyme, Needham, MA; n=6). Blood samples (0.5 ml) were collected at hourly intervals from 1300-1800 h. Serum was separated by centrifugation at 3000 rpm and stored at -20 °C until further analysis for LH levels by RIA.

Effects of adrenergic agonists on IL-1β-induced suppression of LH: Rats that had regular estrous cycles were implanted with a jugular catheter on the day of diestrus. The next day (proestrus), after collecting a pretreatment blood sample at 1300 h, they were injected i.p. with either 250µl of the vehicle for IL-1β (PBS-1.0% BSA; control; n=7) or 5µg of recombinant rat IL-1β (Abazyme, Needham, MA; n=6). Other groups of rats were treated i.p. with either an α_1 adrenergic agonist, cirazoline (CZ; 0.6mg/kg BW; n=6), an α_2 adrenergic agonist, clonidine (CLON; 0.3 mg/kg BW; n=8), or a β-adrenergic agonist, isoproterenol (ISO; 0.2 mg/kg BW; n=5) alone or in combination with 5µg of IL-1β (n=6 each for IL+CZ, IL+CLON, and IL+ISO) at 1300 h. Adrenergic agonists were obtained from Sigma, St. Louis, MO. The doses of the agonists were based on a previous study conducted in the lab (Clark *et al.* 2008). Blood samples were collected at hourly intervals from 1300-1800 h. Serum was separated and stored at -20 °C until analysis for LH levels by RIA.

Effect of L-dopa on IL-1β-induced suppression of NE in the MPA and serum LH :

Rats were implanted with a push-pull cannula in the MPA as described earlier. Two weeks post–surgery, regularly cycling rats were implanted with a jugular catheter on the day of diestrus. On the day of proestrus, after collecting a pretreatment perfusate sample from the MPA and a blood sample, they were randomly subjected to one of the following four treatments: control (PBS-1.0% BSA; n=6), IL-1β (5μg i.p; n=6), L-dihydroxy phenylalanine (L-dopa; 50mg/kg BW i.p; Sigma, St. Louis, MO; n=4) or IL-1β + L-dopa (n=9) at 1300 h. Perfusate samples were collected every 30 minutes and blood samples at hourly intervals from 1300-1800 h. Perfusates were stored at –70°C until HPLC-EC analysis for NE. Serum was separated and stored at -20 °C until analysis for LH levels by RIA. At the end of the experiment the animals were sacrificed and the brains were removed, sectioned and stained with cresyl violet to verify cannula location. Only those animals that had a cannula in the MPA were included in the analysis.

Effects of L-dopa on IL-1β-induced suppression of ovulation in rats: Adult, cycling female rats were randomly subjected to one of the four treatments: control (PBS-1.0% BSA; n=4), IL-1β (5μg i.p; n=4), L-dopa (50mg/kg BW i.p; Sigma, St. Louis, MO; n=4) or IL-1β + L-dopa (n=4) at 1300 h on proestrus. They were sacrificed between 1400-1500 h on the following day (estrus) and the ovaries were collected in neutral buffered formalin and processed for sectioning. The sections were stained with hemotoxylin and eosin for detailed histological examination.

RESULTS:

Effect of i.p. administration of IL-1 on serum LH in intact cycling animals:

The patterns of serum LH (ng/ml; mean ±SE) in control and IL-treated animals are shown in Fig 3-1A,B. In control animals, LH levels at 1300 h were (0.19±0.06) and increased gradually to 2.42 ± 0.89 at 1500 h and 4.37 ± 1.19 at 1600 h before reaching a peak at 1700 h (6.28 ± 1.67, $p<0.05$; Fig. 3A). In contrast, treatment with IL-1β completely blocked this surge (Fig. 1a). In these animals, LH levels were 0.13±0.07 at 1300 h and remained at about the same level throughout the entire period of observation. Similarly, average serum LH levels (Fig. 3B) during the observation period in control animals were 3.86±0.49 and these levels were significantly higher compared to that in IL-treated animals (0.15±0.49; $p<0.05$).

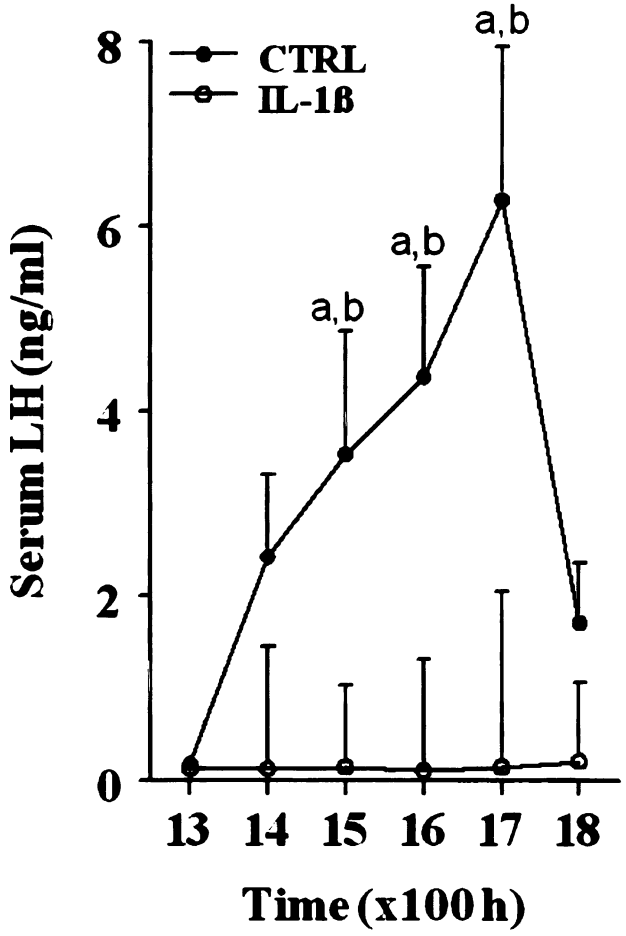

Figure 3-1A: Intact female Sprague Dawley rats (n=6/7 per group) were treated with PBS-1%BSA (Control), or 5μg of IL-1β at 1300h on the afternoon of proestrus. Blood samples were collected at hourly intervals and the serum was analyzed for LH levels by RIA. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h and 'b' indicates difference ($p<0.05$) from levels in IL-1β treated animals. Note that the peak levels of LH in controls were at 5pm and this surge in LH was blocked by treatment with IL-1β.

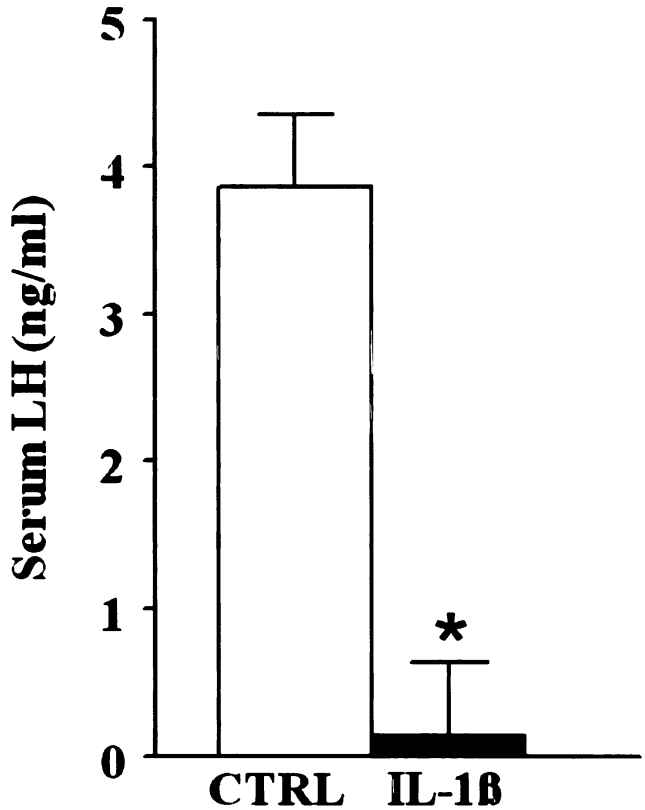

Figure 3-1B: Average LH during the entire period of observation in the control and IL-1β treated animals (*$p<0.05$). Note that treatment with IL-1β significantly reduced the levels of serum LH measured during the entire treatment period.

Effects of adrenergic agonists on IL-1β-induced suppression of LH:

The patterns of serum LH (ng/ml; mean ± SE) in rats treated with alpha and beta noradrenergic agonists alone or in combination with IL-1β are shown in Fig. 3-2.

The effects of alpha and beta noradrenergic agonists on IL-1β induced suppression of LH are shown in Fig. 3-2 A,B and C. The effect of treatment [$F(5,139) = 4.39$] was found to be significant ($p<0.05$). However, the effect of either time [$F(6,32)=1.85$] as well as the interaction between treatment and time [$F(26,32)=1.08$] were not significant. Effects of CZ (an α_1 adrenergic agonist) and CLON (an α_2 adrenergic agonist) are shown in Fig. 3C and 3D respectively. The effect of the β-adrenergic agonist, ISO is shown in Fig. 3E. Administration of either clonidine or isoproteronol along with IL-1β failed to reverse the IL-induced suppression of LH. Treatment with the adrenergic agonists alone failed to increase the LH surge. Adrenergic agonists in combination with IL-1β failed to prevent the suppression of LH by IL-1β.

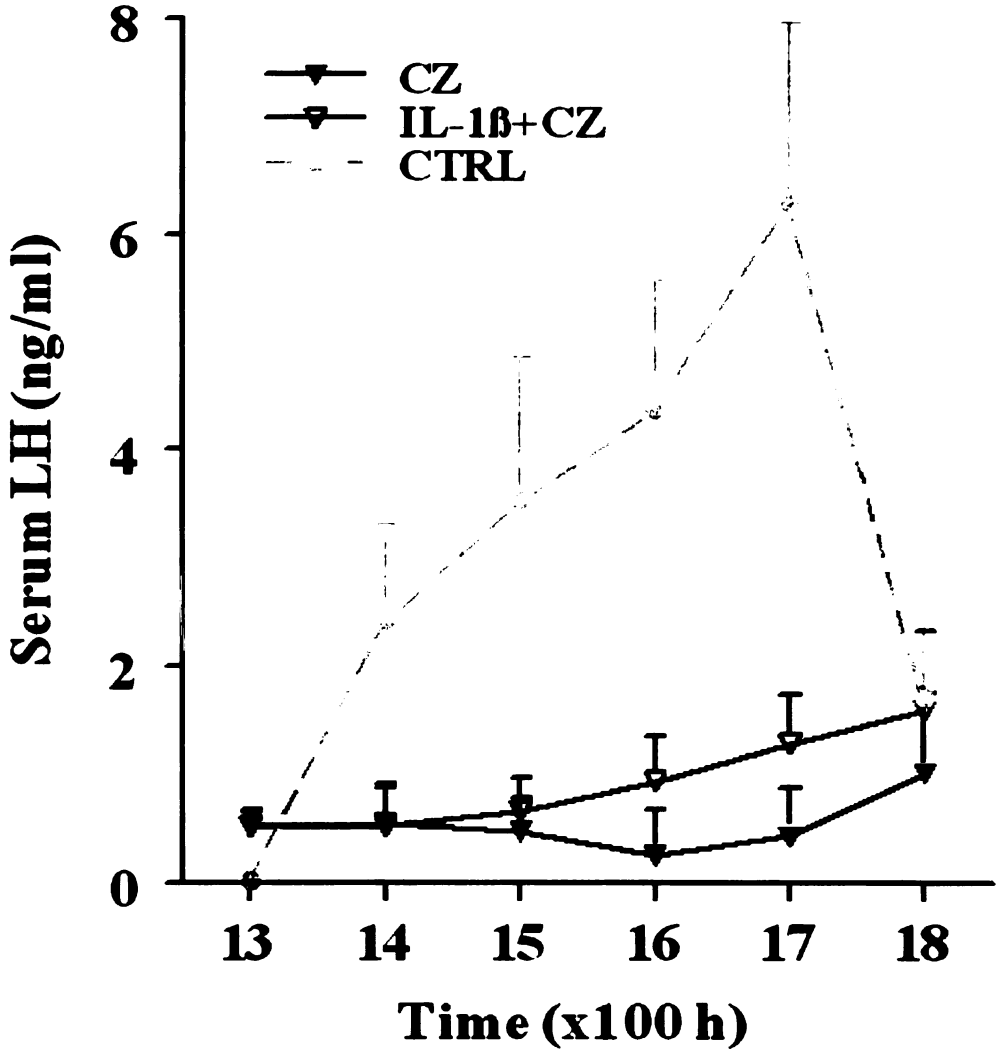

Figure 3-2A: Intact female Sprague Dawley rats (n=6-7 per group) were treated with 0.6mg/kg BW of Cirazoline (CZ) i.p or a combination of 5μg of IL-1β and CZ at 1300h on the afternoon of proestrus. Blood samples were collected at hourly intervals and plasma analyzed for LH levels. Profile of LH levels in control animals is provided in gray for comparison purposes. Note that LH surge present in the control animals could not be reproduced in the animals treated with CZ or IL-1β+CZ.

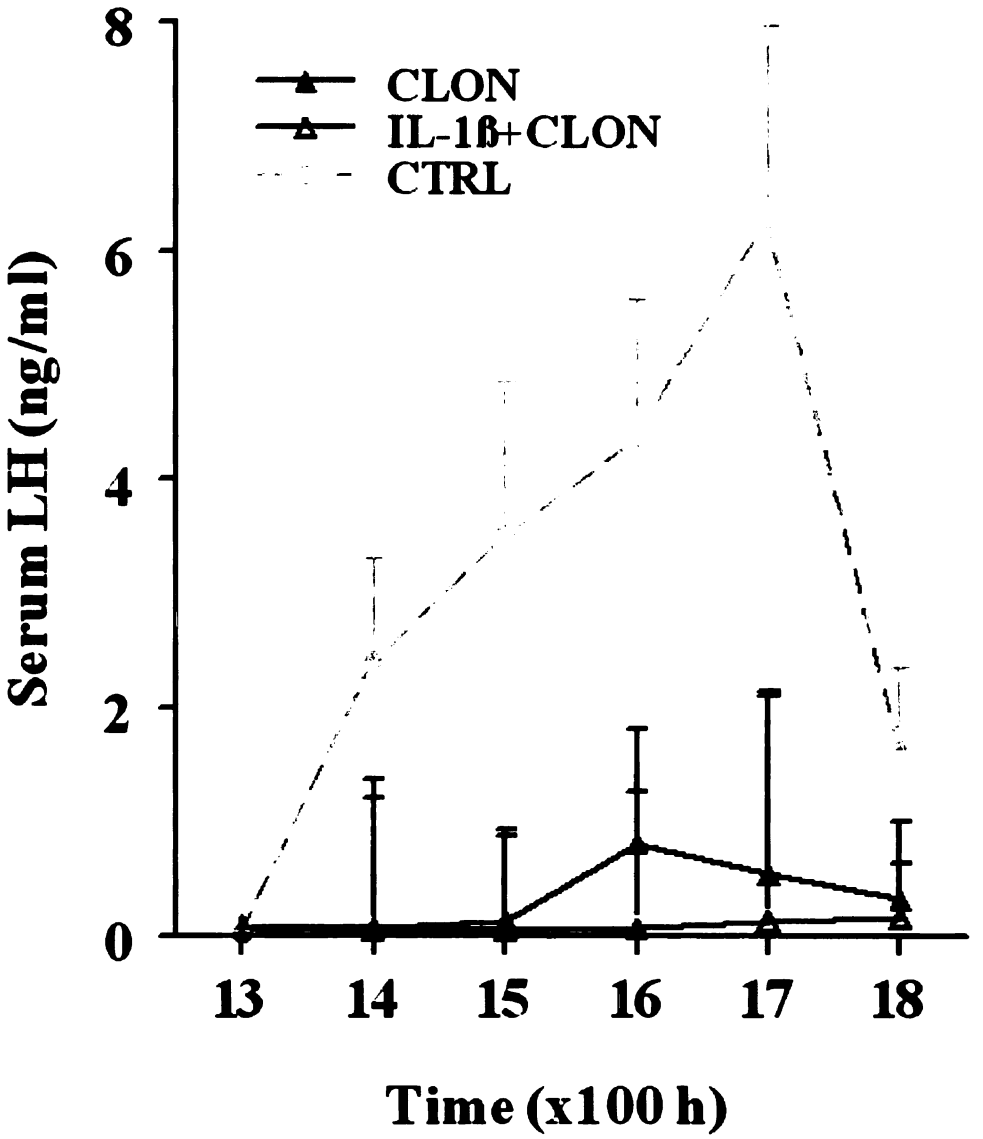

Figure 3-2B: Intact female Sprague Dawley rats (n=6-7 per group) were treated with 0.3mg/kg BW of Clonidine (CLON) or a combination of 5μg of IL-1β and CLON at 1300h on the afternoon of proestrus. Blood samples were collected at hourly intervals and plasma analyzed for LH levels. Profile of LH levels in control animals is provided in gray for comparison purposes. Note that LH surge present in the control animals could not be reproduced in the animals treated with CLON or IL-1β+CLON.

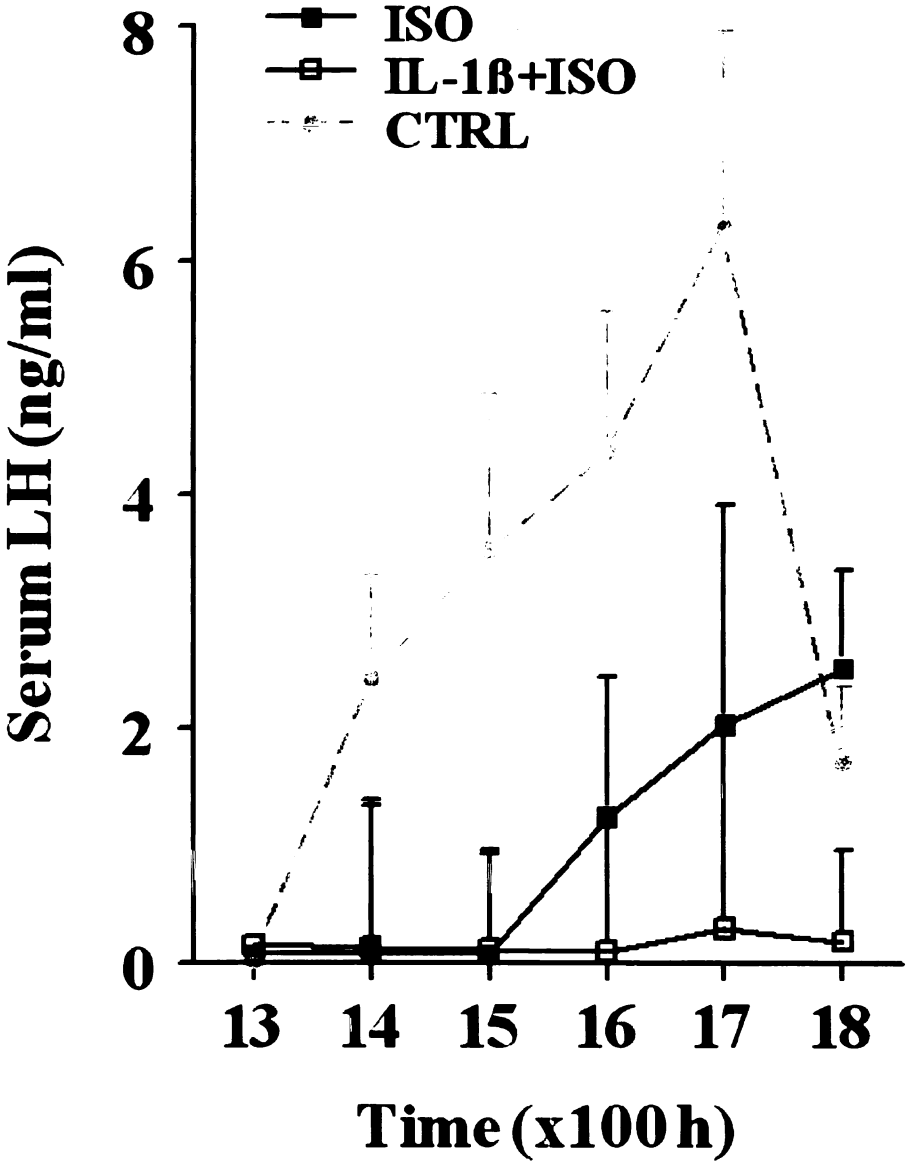

Figure 3-2C: Intact female Sprague Dawley rats (n=6-7 per group) were treated with 0.2mg/kg BW of Isoproterenol (ISO) i.p or a combination of 5μg of IL-1β and ISO at 1300h on the afternoon of proestrus. Blood samples were collected at hourly intervals and plasma analyzed for LH levels. Profile of LH levels in control animals is provided in gray for comparison purposes. Note that LH surge present in the control animals could not be reproduced in the animals treated with ISO or IL-1β+ISO

NE release in the MPA:

The location of the push-pull cannulae in animals subjected to perfusion are shown in Fig 3-3. The profiles of NE release in the MPA in rats treated with IL-1β, L-dopa or IL-1β in combination with L-dopa are shown in Fig 3-4A. The effect of treatment [$F(3,93)= 43.33$], time [$F(11,53)=3.46$] as well as the interaction between treatment and time [$F(33,53)=1.73$] were found to be statistically significant ($p<0.05$). In control animals, NE levels (pg/min, Mean±SE) increased gradually from 1300 h (7.63±1.15), reached a peak at 1530 h (21.53±1.6; $p<0.05$), were 16.94±1.8 at 1600 h and declined to 12.69±1.28 at 1700 h. Similary, in the group treated with L-dopa alone, NE levels increased from 6.77±1.94 at 1300h, by more than two-fold to 17.79±1.16 at 1530 hrs ($p<0.05$), were 15.06±3.34 and 15.15±1.71 at 1600 h and 1700 h respectively. They were not different from the levels in control group. In contrast, treatment with IL-1β suppressed the rise in NE levels. NE levels in this group were 2.34±1.59 at 1300 h and remained at that level for the remaining period of observation. However, treatment with L-dopa in combination with IL-1β partially reversed the suppressive effect of IL-1β on NE. The levels of NE in these animals increased significantly from 5.86±1.99 at 1300 h to 12.2±2.6 at 1600h ($p<0.01$). These levels were not different from the group treated with L-dopa alone at 1600 h (15.06±3.5) but was significantly higher compared to the IL-1β-treated group (3.6±2.2; $p<0.05$) at this time point. The average levels of NE over the entire period of observation are shown in Fig 3-4B. NE levels in control animals (12.26±1.06), animals treated with L-dopa alone (10.24±1.3) and in animals treated with IL-1β+L-dopa (7.67±1.51) were significantly higher than those in animals treated with IL-1β alone (3.01±1.30; $p<0.05$).

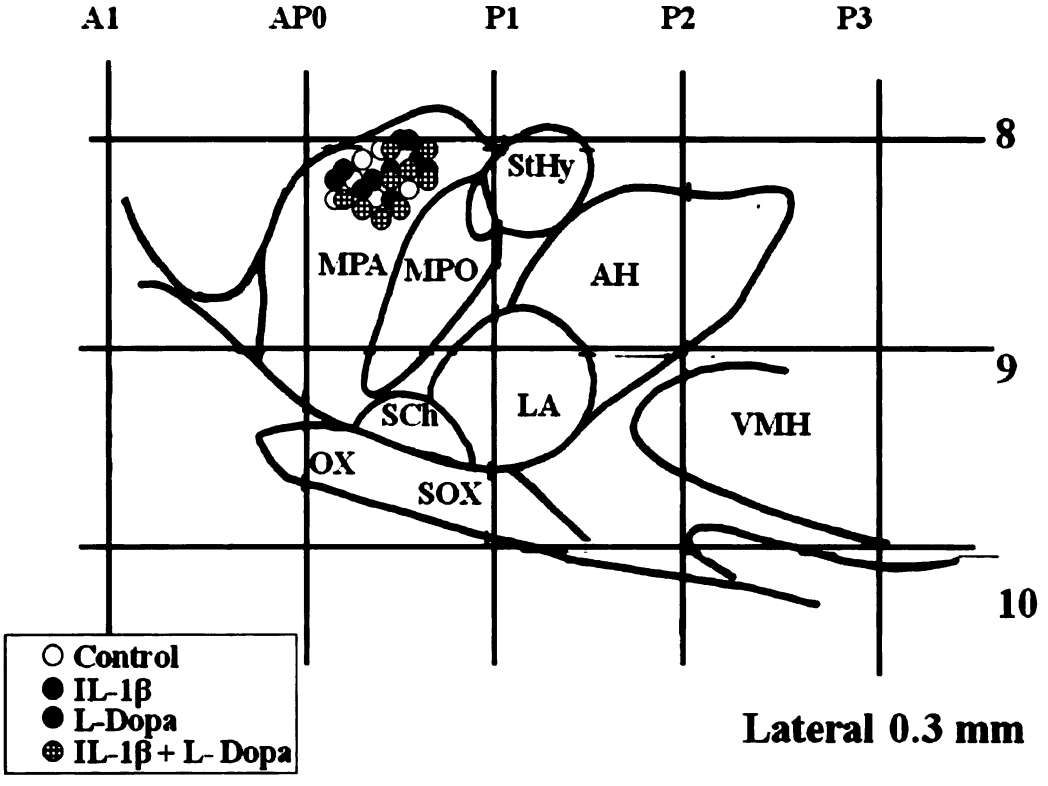

Figure 3-3:

Schematic representation of the sagittal section of a rat brain indicating the locations of the push–pull cannulae in control (PBS-1%BSA-treated group, *n*=6), IL-1β treated (n=6), L-dopa treated (n=6) and the group treated with both IL-1β and L-dopa (n=9). The numbers A1–P3 represent coronal plates extending 1 mm anterior (A1) to 3 mm posterior (P3) to the bregma (AP0). MPA=medial preoptic area, SCh=suprachiasmatic nucleus, AH=anterior hypothalamus, LA=lateroanterior hypothalamic nucleus, MPO=median preoptic nucleus, StHy=striohypothalamic nucleus, VMH=ventromedial hypothalamus, OX=optic chiasm, and SOX=supraoptic decussation. The location of the push–pull cannulae in individual animals was determined by examining stained serial brain sections under a light microscope. Note the location of the cannulae predominantly in the medial preoptic area.

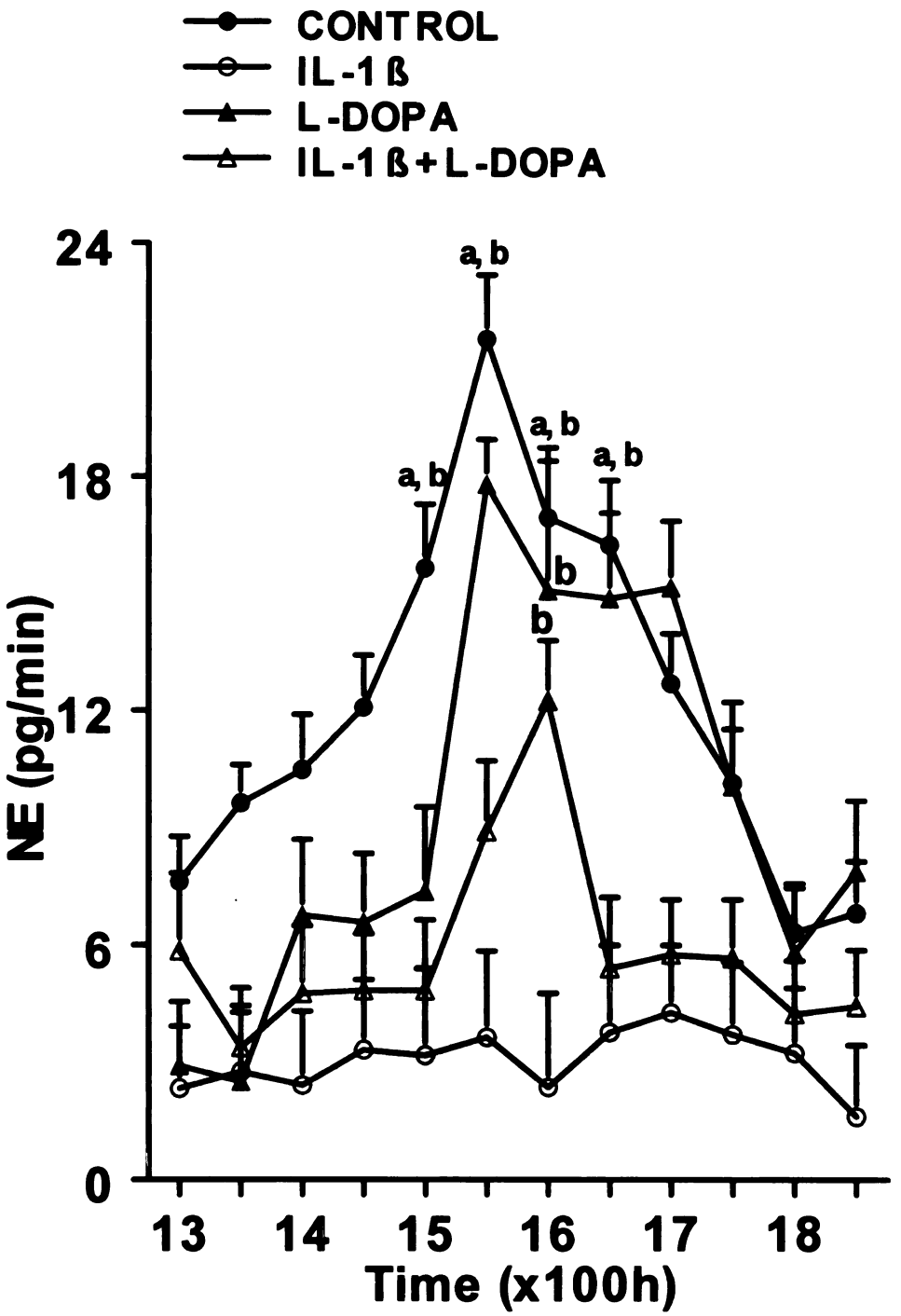

Figure 3-4A: NE release profiles in intact female Sprague Dawley rats (n=6-7 per group) treated with PBS-1%BSA (Control), 5μg of IL-1β, 50 mg/kg BW of L-dopa or IL-1β in combination with L-dopa at 1300h on the afternoon of proestrus. Rats were subjected to push-pull perfusion of the MPA. Perfusates were collected at 30 min intervals and analyzed for NE levels by HPLC-EC. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h and 'b' indicates difference ($p<0.05$) from levels in IL-1β treated animals.

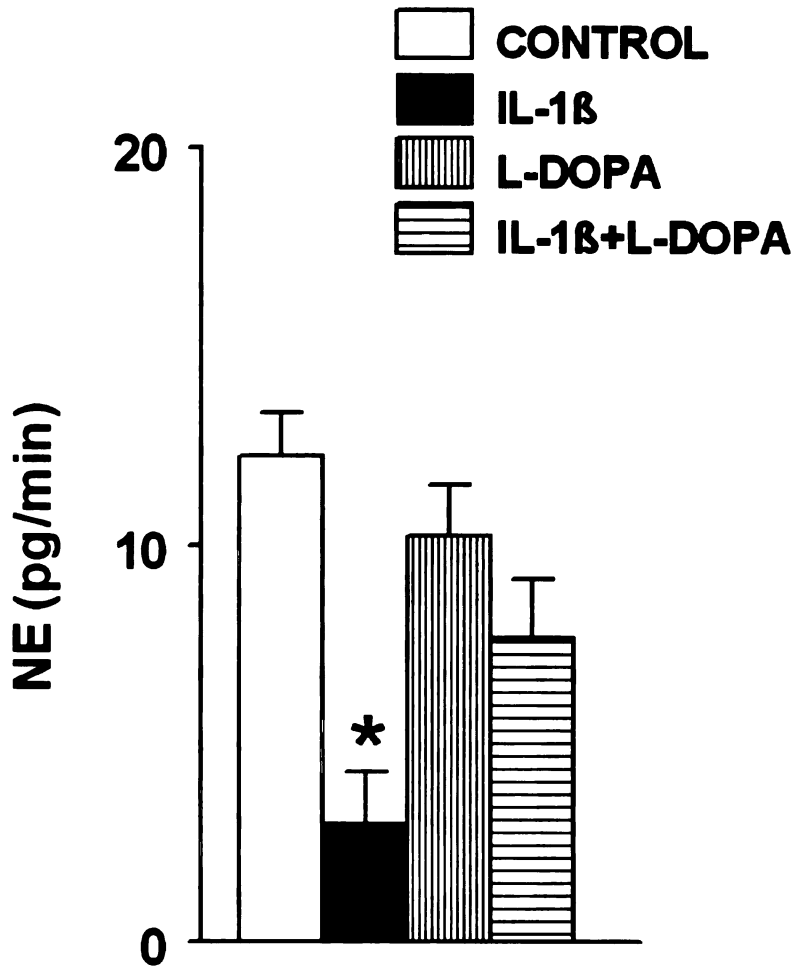

Figure 3-4B. Average levels of NE during the entire period of observation in the different treatment groups. *$p<0.05$ when compared to the rest of the groups.

Plasma LH levels:

Concurrent changes in plasma LH levels in the animals subjected to push-pull perfusion are shown in Fig 3-5. The effects of treatment [F (3, 58) =8.57], and time [F (5, 10) =3.34] were found to be significant ($p<0.05$). In control animals, LH levels (ng/ml, mean±SE) were 0.5 ± 0.2 at 1300 h and gradually increased to peak concentrations of 4.2 ± 0.8 at 1700 h ($p<0.05$), closely following the increase in NE levels in the MPA that occurred at 1530 and 1600 h. In the group treated with L-dopa alone, LH levels increased from 0.55±0.31 at 1300h to 3.29±0.93 at 1700h ($p<0.05$). These levels were not different from those in the control animals. In contrast, in IL-1β treated animals, LH levels were 0.05±0.3 at 1300 h and remained at that level during the rest of the observation period, parallel to the low levels of NE observed in the MPA. The suppressive effect of IL-1β on LH was partially reversed by co-treatment with L-dopa. In this group, LH levels at 1700 h (1.99±0.49) were significantly higher than the levels observed in the IL-1β-treated group (0.4±0.7; $p<0.01$). Average LH levels over the entire period of observation in control animals and those treated with L-dopa alone were 1.91±0.39 and 1.6±0.4 respectively. IL-1β treatment decreased average LH levels significantly to 0.28±0.31 ($p<0.05$). However, the average LH levels in IL-1β+L-dopa-treated animals (0.97±0.23) were in between the control and IL-1β treated groups and were not different from either group (Fig. 3-5B). This suggests that L-dopa partially reversed the IL-1β-induced suppression of the LH surge.

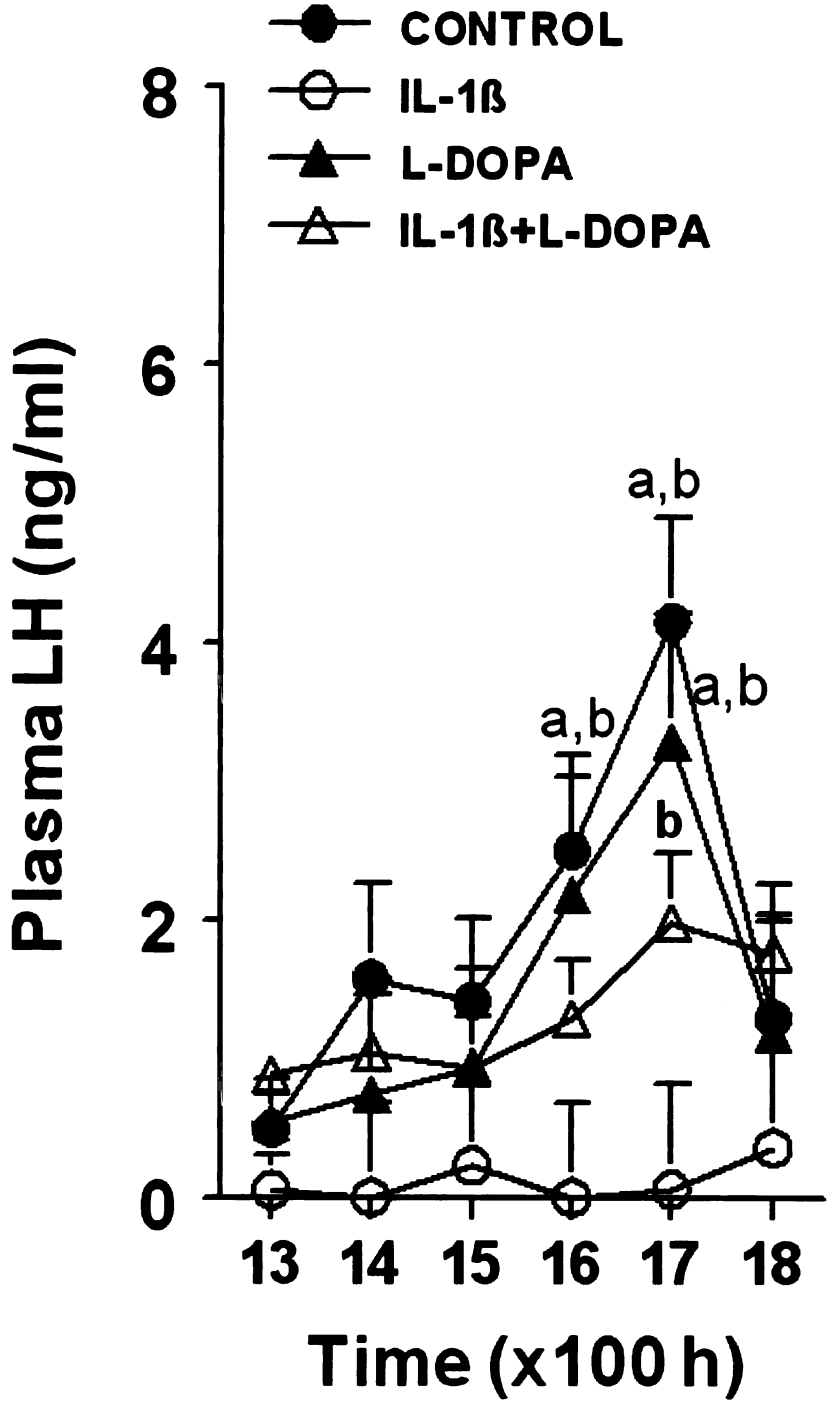

Figure 3-5A. Plasma LH levels in intact female Sprague Dawley rats (n=6-7 per group) treated with PBS-1%BSA (Control), 5μg of IL-1β, 50 mg/kg of BW of L-dopa or IL-1β in combination with L-dopa at 1300h on the afternoon of proestrus. Animals were implanted with jugular catheters for concurrent blood sampling at hourly intervals. Plasma samples were analyzed for LH levels by RIA. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h and 'b' indicates difference ($p<0.05$) from levels in IL-1ß treated animals.

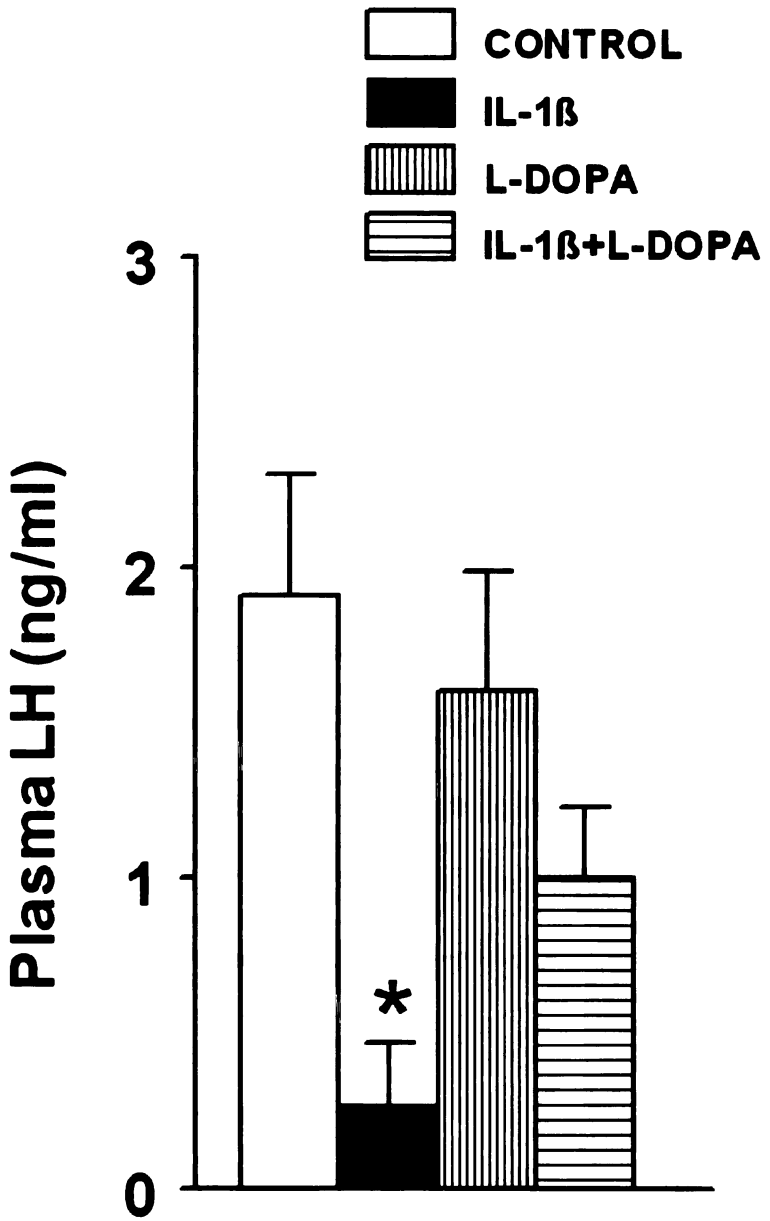

Figure 3-5B. Average levels of LH over the entire observation period in the different treatment groups. *$p<0.05$ when compared to the rest of the groups.

Ovarian histology:

Representative sections of one ovary from each of the treatment groups are depicted in Fig 3-6A. The histological sections of the ovary were analyzed double-blind, for the presence of post-ovulatory fresh corpora lutea (FCL), indicated by the presence of increased vascularity within the CL. The numbers of old corpora lutea (OCL) as well as mature follicles (MF) were also enumerated (Fig 3-6B). The numbers of FCL (Mean±SE) in the control and L-dopa treated groups were 7.5±0.96 and 5.75±0.85 respectively. Treatment with IL-1β significantly decreased the FCL numbers to 1.25±0.48 ($p<0.05$). In contrast, treatment with IL-1β + L-dopa increased the number of FCL to 6.5±0.5, which was significantly higher than that in the IL-1β treated group ($p<0.05$).

Besides producing a significant reduction in FCL, IL-1β treatment was also associated with higher numbers of OCL. The number of OCL in the IL-1β treated group (6.0± 0.57) was significantly higher ($p<0.05$) compared to control (2.25±0.63), L-dopa (2.25±0.24) and IL-1β+L-dopa treated (2.23±0.15) groups. The numbers of mature follicles were not significantly different between the different treatments.

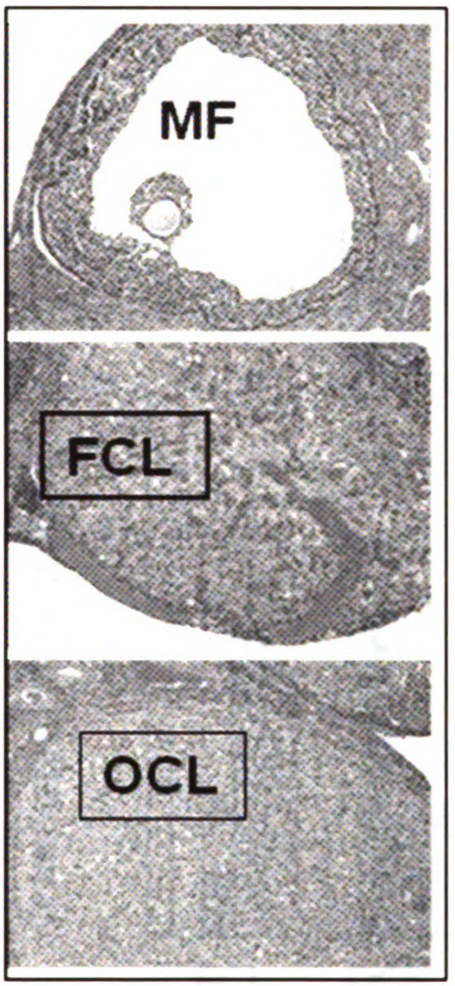

Figure 3-6A. Representative histological sections of ovaries (40x magnification) from female Sprague-Dawley rats (n=4) treated with PBS-1%BSA (Control) (A), 5μg of IL-1β (B), 50 mg/kg of BW of L-dopa (C) or IL-1β in combination with L-dopa (D) at 1300h on the afternoon of proestrus and sacrificed between 1400-1500h on the following day. Postovulatory, fresh corpora lutea (FCL) were identified by the histological presence of a hemorrhage and the transition from a follicular appearance to luteal phenotype, old corpora lutea (OCL) by the presence of densely packed luteal cells while the mature follicles (MF) were identified by the presence of prominent antral space and follicular fluid (E, 100x magnification).

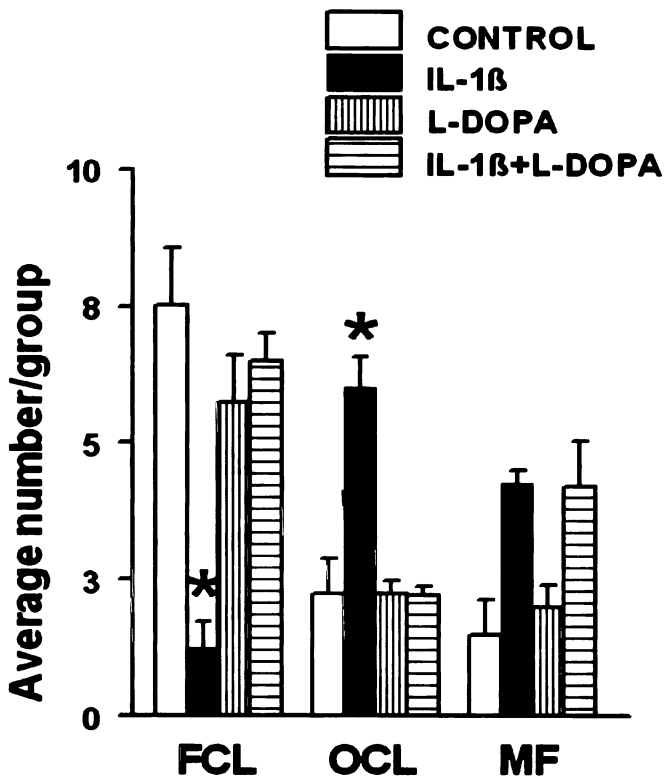

Figure 3-6B. Average numbers (Mean±SE) of post-ovulatory fresh corpora lutea (FCL), old corpora lutea (OCL) and mature follicles (MF) in the different treatment groups are shown. Female Sprague-Dawley rats (n=4 per group) treated with PBS-1% BSA (Control), 5μg of IL-1β, 50 mg/kg BW of L-dopa or IL-1β in combination with L-dopa at 1300h on the afternoon of proestrus and sacrificed between 1400-1500h on the next day. *$p<0.05$ when compared to the rest of the groups.

DISCUSSION:

Results from this study indicate that systemic administration of IL-1β blocks the preovulatory LH surge in intact cycling animals and confirms earlier findings that IL-1β is capable of suppressing reproductive functions. This was accompanied by a reduction in NE release in the MPA indicating a role for NE in this effect. Further, treatment with the noradrenergic precursor, L-dopa was able to block the effect of IL-1β and partially restore NE levels in the MPA. This suggests that the IL-1β most probably affects the biosynthesis of NE to produce its effect. Moreover, L-dopa treatment was also capable of partially reversing the effect of IL-1β on the LH surge and this was sufficient to cause ovulation in IL-1β-treated animals. This can therefore be considered as a viable treatment option in patients who have ovulation failure due to chronic illnesses that are associated with elevated IL-1β levels.

IL-1's effects on the HPG axis became apparent when it was first demonstrated that IL-1α could decrease LH secretion in male rats (Rivier and Vale 1989). Subsequently this finding was extended to cycling female rats in which the more potent isoform, IL-1β not only blocked the preovulatory LH surge but also inhibited ovulation (Rivier and Vale 1990). A similar reduction in LH levels was also observed after IL-1β treatment in ovariectomized, steroid-primed rats (Kalra et al. 1990a). Interestingly, these effects on LH secretion and ovulation were observed only when IL-1β was administered i.c.v and not when given systemically (Kalra et al. 1990a; Rivier and Vale 1990). This could be attributed to the doses of IL-1β that were used in these studies. Rivier and Vale (1990) used 40 ng i.c.v and 25 ng i.v., while Kalra et al (1990) used 30 ng i.c.v. and 1μg i.p.

Earlier studies demonstrated that lower i.c.v. doses of IL-1β (3 ng) can elicit neuroendocrine responses, but much higher doses (300 ng) are required when it is given iv (Katsuura *et al.* 1988). We have previously shown that a dose-dependent change in neurotransmitter levels occurs in response to i.p. administration of 1, 2.5 and 5 μg of IL-1β (MohanKumar and Quadri 1993; MohanKumar et al. 1998). Based on previous findings with iodinated IL-1(Banks *et al.* 1991), we estimate that only 4 ng of IL-1β (0.08%) would have crossed the blood brain barrier after administration of 5 μg i.p. The present study demonstrates that this dose could suppress the HPG axis effectively. This is supported by our previous study in ovariectomized steroid-primed rats (MohanKumar and MohanKumar 2002).

The mechanism by which IL-1β produces its effect on the LH surge is not clear. Any of the multitude of neurochemicals that are implicated in LH regulation may be involved in this phenomenon (Smith and Jennes 2001). Results from the present study indicate that IL-1β acts by decreasing NE levels. There are several lines of evidence to indicate that NE is stimulatory to GnRH neurons and LH secretion. NE concentration, turnover and release are known to increase parallel to the LH surge (Wise 1984; Mohankumar et al. 1994, 1995). Further, adrenergic antagonists (Le *et al.* 1997b) and inhibition of NE synthesis (Brann and Mahesh 1991), both block the LH surge. Moreover, the MPA is richly innervated by brainstem noradrenergic nuclei and lesioning these areas suppresses the preovulatory LH surge (Anselmo-Franci et al. 1997). Therefore, it is possible that IL-1β inhibits the LH surge by decreasing NE levels in the MPA. The decrease in NE levels in the MPA produced by IL-1β treatment in the present study is in agreement with this notion.

To understand the possible mechanisms that are involved in the IL-1β-induced decrease in NE release, we treated animals with several types of adrenergic agonists in combination with IL-1β. However, the NE agonists were unable to block the effect of IL-1β on the LH surge. Several reasons could have contributed to this effect. CLON, for example, has an inherent property of inhibiting NE by acting at presynaptic sites (Mueller and Nistico 1989b). This can account for the lack of the LH surge with CLON treatment and the more severe inhibition of LH secretion seen with IL-1β+CLON treatment. It is possible that a bolus injection of NE agonists as given in the present study could not mimic the profile of NE release in the MPA that is known to be important for the initiation of the LH surge (Mohankumar et al. 1994). Nevertheless, results from this study indicate that systemic bolus administration of NE agonists is ineffective in blocking the IL-1β-induced suppression of the LH surge.

L-dopa, on the other hand was able to partially reverse the effect of IL-1β on NE levels in the MPA and the LH surge. L-dopa is the product of the enzymatic action of tyrosine hydroxylase (TH) on L-tyrosine. This also happens to be the rate limiting step in NE biosynthesis (Nagatsu 1995) and therefore TH is critically involved in LH regulation. TH activity increases significantly in the MPA during the afternoon of proestrus along with the LH surge (Mohankumar *et al.* 1997b). Also, decreases in TH activity has been implicated in the age-related loss of LH surges in rats (Mohankumar et al. 1997b). This suggests the possibility that IL-1β can decrease TH activity to suppress the LH surge. To test this, we used L-dopa to by-pass TH and provided the substrate required for NE biosynthesis. L-dopa crosses the blood-brain barrier easily and has been used to reverse acyclicity and increase LH levels in old animals that have low hypothalamic NE levels

(Huang et al. 1976; Forman et al. 1980). The results from the present study indicate that administration of L-dopa along with IL-1β can indeed partially reverse the IL-1β-induced suppression of the LH surge. This was accompanied by a partial but significant restoration of NE levels in the MPA. The partial rather than complete reversal suggests that IL-1β may affect enzymes downstream of TH such as dopa decarboxylase or dopamine β-hydroxylase that are involved in NE synthesis. This needs further investigation.

Further support for L-dopa's ability to reverse the effect of IL-1β on the HPG axis comes from the histological examination of the ovary. Ovaries of animals treated with IL-1β alone had less numbers of fresh CL compared to the rest of the groups suggesting suppressed ovulation. In contrast, control rats and the rats treated with L-dopa alone or IL-1β+L-dopa had larger numbers of freshly formed CL suggesting that ovulation had occurred in these groups. Moreover, the numbers of old CL were higher in the IL-1β treated animals. This supports an earlier study that observed retention of CL with chronic IL-1β treatment (Rivier and Erickson 1993). The effect of IL-1β treatment on the rat ovaries in vivo has not been studied in detail. An endogenous IL-1 system appears to exist in the ovary and is essential for ovulation (Simon *et al.* 1994). Exogenous administration of IL-1 appears to have the opposite effect. A previous study reported failure of ovulation after central administration of IL-1β (Rivier and Vale 1990). Results from the present study indicate a failure of ovulation in IL-1β-treated rats. In contrast, control rats had larger numbers of freshly formed CL suggesting that ovulation had occurred in this group (Figs 4 and 5). Moreover, the numbers of old CL were higher in the IL-1β treated animals suggesting lack of luteolysis. Although the reasons for this are

not clear, it is possible that systemic IL-1β could block prostaglandin $F_{2\alpha}$ production which is necessary for luteolysis and progression of the oestrous cycle (Dauffenbach *et al.* 2003). Results from the present study also indicate that L-dopa was effective in reversing IL-1β's actions on the ovary. Since L-dopa also reversed the effects of IL-1β on hypothalamic NE and plasma LH levels, it is possible that the effects observed on the ovary are mediated through LH rather than through a direct action on the ovary. Since the minimum levels of LH required for successful ovulation are only a fraction of the peak levels (Gosden *et al.* 1976), it is possible that the modest increase in the LH levels produced by L-dopa was sufficient to cause ovulation in IL-1β treated animals.

The exact route by which IL-1β affects noradrenergic neurons or their terminals is unclear. IL-1β receptors have been identified in several parts of the brain and brain stem (Wong and Licinio 1994; Ericsson et al. 1995; Gayle et al. 1997) and have also been localized in para abdominal ganglia of the vagus nerve (Goehler *et al.* 1997). Since the vagus projects to the brainstem nuclei, systemic IL-1β could act through the vagus to affect central noradrenergic neurons. On the other hand, systemic IL-1β can also cross the blood-brain-barrier to affect the hypothalamus and other brain areas such as the area postrema that lie in close proximity to the brainstem NE neurons (Brady *et al.* 1994). Regardless of the route, results from this study indicate that systemic IL-1β is capable of inhibiting the HPG axis most probably by decreasing NE levels in the hypothalamus. This effect could be partially reversed by treatment with L-dopa indicating that this could be a viable treatment for reproductive failure, especially in the face of chronic infections or inflammatory conditions.

CHAPTER 4. SYSTEMIC INTERLEUKIN-1β BLOCKS STERIOD-INDUCED LH SURGE – INTERACTION BETWEEN GABA AND NOREPINEPHRINE

INTRODUCTION:

Immune stress has a negative impact on the reproductive axis. We have used systemic administration of a potent cytokine, IL-1β, to model immune stress and study its effects on the female reproductive axis. As demonstrated in Chapter 3, IL-1β suppresses the pre-ovulatory LH surge and inhibits ovulation in female rats. We have also demonstrated that this suppression involves a decrease in the stimulatory input of NE on the GnRH system. IL-1β could induce this decrease in NE as a direct action on noradrenergic neurons or it could be an indirect effect involving other neurotransmitters like GABA.

The involvement of GABA release in the hypothalamus in regulating LH secretion has been previously demonstrated (Mansky et al. 1982; Adler and Crowley 1986). GABA has been shown to have a reciprocal relationship with NE in the MPA. During the LH surge on proestrus in female rats, NE levels increase in the MPA while GABA levels decrease gradually (Demling et al. 1985; Akema et al. 1990). Further, administration of GABA receptor agonists has been shown to inhibit LH secretion as well as decrease GnRH gene expression (Leonhardt et al. 1995). There is also neurochemical evidence pointing to the role of GABA in tonic inhibition of presynaptic release of NE (Mansky et al. 1982; Lamberts et al. 1983).

In this study, we have used an ovariectomized, steroid-primed model to examine the role of GABA in IL-1β-induced suppression of NE levels in the MPA. The ovariectomized steroid-primed rat model consistently and reproducibly simulates the

proestrus LH surge and has been well documented (Kalra and Kalra 1979, 1983; Kalra et al. 1990a). In previous studies, we have used this model to show that IL-1β decreases NE levels in the MPA and suppresses the LH surge (MohanKumar and MohanKumar 2002). In the present study, we hypothesize that systemic IL-1β increases GABA levels in the MPA, concurrently decreases NE levels and thus blocks serum LH levels. We also intend to investigate if this decrease in NE and LH could be reversed by central administration of GABA-A and GABA-B receptor antagonists.

EXPERIMENTAL SETUP:

Three-to-four month-old female Sprague Dawley rats obtained from Harlan Inc. (Indianapolis, IN) were used in these experiments. The protocols used in this study were approved by the Institutional Animal Care and Use Committee at Michigan State University. After a two-week acclimatization period, all the animals were weighed and randomly divided into six groups. All animals were bilaterally ovariectomized and implanted with a push–pull cannula in the MPA and an icv cannula as described in detail in the Methods chapter.

Steroid treatment protocol was followed as previously described (MohanKumar and MohanKumar 2002). On the 8th day after surgery, the rats were treated with a subcutaneous injection of estrogen (20 μg/0.1 ml corn oil) at 1000 h. On the 10th day, the rats received a subcutaneous injection of progesterone (2 mg/0.1 ml of corn oil) at 1000 h and were subjected to push–pull perfusion. On the day of the push-pull perfusion, after collecting a pretreatment blood sample and perfusate at 1300 h, they were injected i.p. with either 250 μl of the vehicle for IL-1β (PBS-1.0% BSA; control; n=7) or 5 μg of recombinant rat IL-1β (Abazyme, Needham, MA; n=6) and intracerebroventricularly with 5 μL CSF. Other groups of rats were treated i.c.v with either a GABA-A antagonist, bicuculline (Sigma, St Louis, MO) (BIC+saline; 1pg in 5 μL; n=6), a GABA-B antagonist, CGP-35348 (Sigma; CGP+saline; 20 μg in 5 μL; n=6), in combination with either PBS-1.0% BSA i.p or 5 μg of IL-1β i.p (n=5-6 each for IL-1β +BIC, IL-1β +CGP) at 1300 h. The effective doses of BIC and CGP were titrated based on preliminary pilot studies such that they were low enough not to cause any convulsions.

RESULTS:

Effect of IL-1β on GABA:

The changes in levels of GABA in the MPA in rats treated with vehicle or IL-1β are shown in Fig 4-1. The effect of treatment [$F(1,47)= 46.71$], time [$F(11,38)=3.31$] as well as the interaction between treatment and time [$F(11,38)=3.19$] were found to be statistically significant ($p<0.05$). In the vehicle-treated controls, the levels of GABA at 1300 h were 62.43±15.87 and decreased to 9.12±16.15 at 1530 h and then regained baseline values. Treatment with IL-1β significantly increased GABA levels from 1300 h at 41.82±22.85 to 116.91±13.25 at 1500 h, 235.53±15.5 at 1530 h which gradually decreased to baseline values. The average levels of GABA in IL-1β-treated group were 189.3±29.14 which was significantly higher than the levels in controls (49.88±10.97; $p<0.05$).

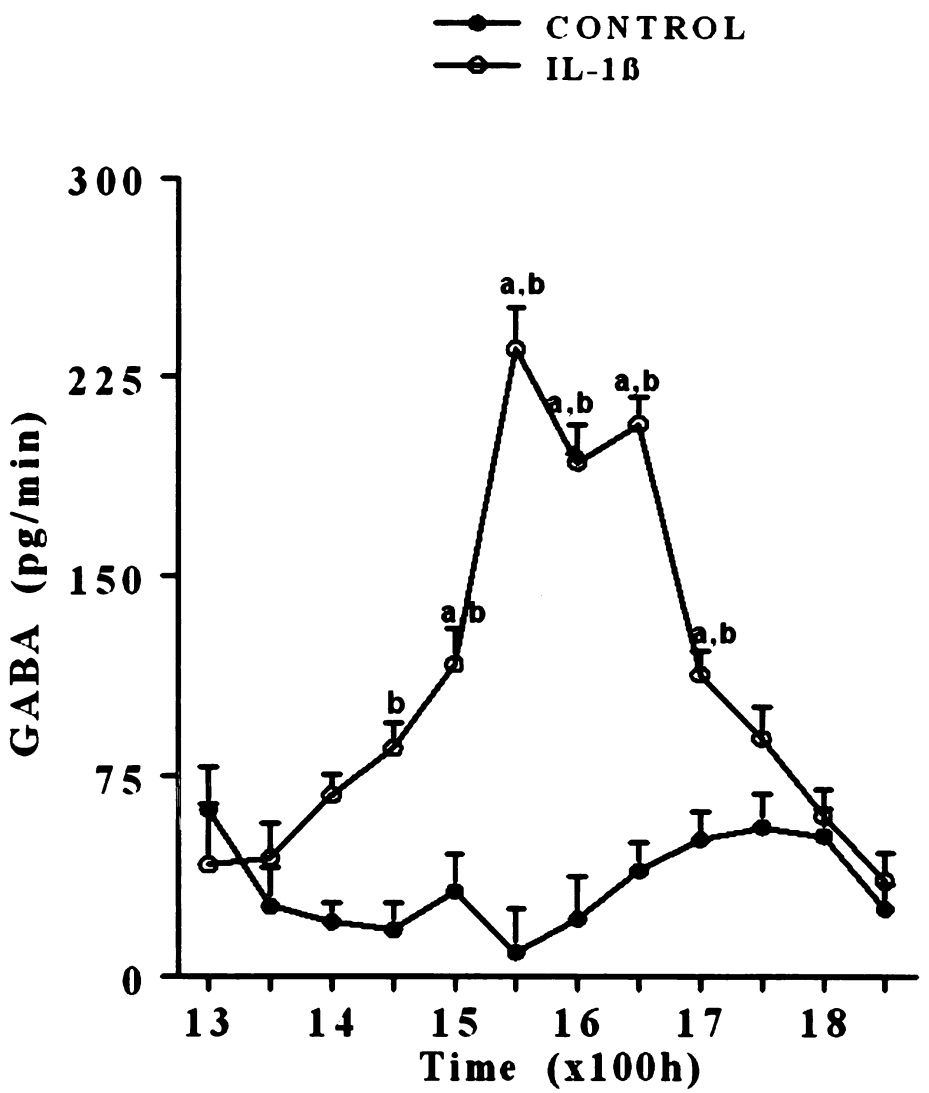

Figure 4-1A. *Effects of IL-1β on GABA release in the MPA.* GABA release (mean±SE;pg/min) profile in the MPA measured at 30-min intervals in ovariectomized steroid primed animals that were either treated with with PBS (Control, n=7), or 5 μg of IL-1β (n=6) at 1300 h. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h and 'b' indicates significant difference ($p<0.05$) from levels in control animals. Note that the GABA levels in IL-1β-treated animals increased within 2 hrs post injection and remain high until 4 hrs post injection.

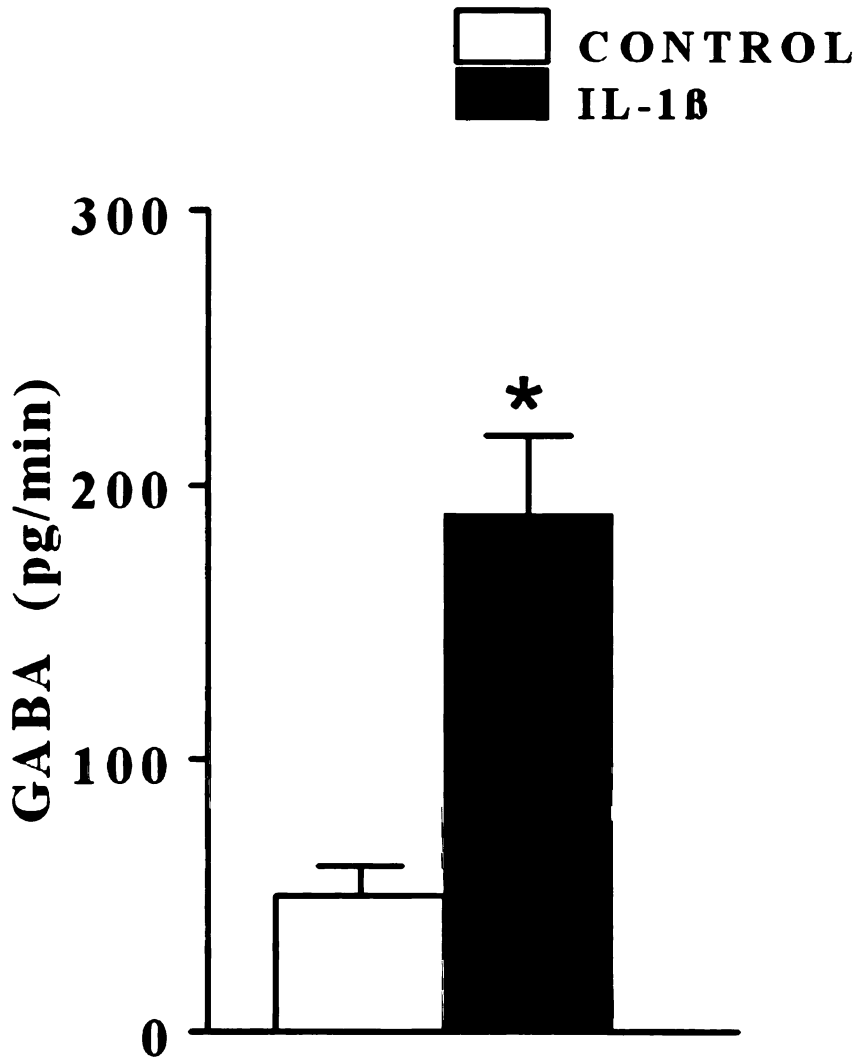

Figure 4-1B: Average GABA levels (mean±SE;pg/min) during the entire period of observation in the control and IL-1β treated animals (*$p<0.05$). Note that treatment with IL-1β significantly increased the levels of GABA measured during the entire treatment period.

Effect of IL-1β on NE: Role of GABA antagonists:

The profiles of NE release in the MPA in rats treated with vehicle, IL-1β, Bicuculline, CGP-35348 or IL-1β in combination with bicuculline or CGP-35348 are shown in Figs 4-2A, B, C and D. The effect of treatment [$F(5,184)=18.33$], time $F(11,108)=15.7$] as well as the interaction between treatment and time [$F(55,108)=3.07$] were found to be statistically significant ($p<0.05$). In control animals, NE levels (pg/min, Mean±SE) increased gradually from 1300 h (5.53±0.79), reached a peak at 1630 h (22.49±2.23; $p<0.05$) and declined to 4.8±1.6 at 1800 h. In contrast, treatment with IL-1β suppressed the rise in NE levels. NE levels in this group were 4.62±1.47 at 1300 h and remained at that level for the remaining period of observation. In the group treated with bicuculline alone, NE levels increased from 4.44±0.93 at 1300 h, by more than three-fold to 18.69±2.6 at 1630 h ($p<0.05$), were 15.01±2.6 at 1700 h. Treatment with bicuculline in combination with IL-1β partially reversed the suppressive effect of IL-1β on NE. The levels of NE in these animals increased significantly from 4.57±1.21 at 1300 h to 13.31±2.96 at 1630 h ($p<0.01$). These levels were not different from the group treated with bicuculline alone at 1630 h but were significantly higher compared to the IL-1β-treated group (5.41±3.31; $p<0.05$) at this time point. In the group treated with CGP-35348 alone, NE levels increased from 5.2±0.85 to 17.03±2.2 at 1630 h ($p<0.05$). The levels at 1530 and 1700 h were 13.43±1.21 and 12.06±2.35, respectively, which were different from the control values at those time points. The combination of CGP-35348 along with IL-1β failed to reverse the suppressive effect of IL-1β.

The average levels of NE over the entire period of observation are shown in Fig 4-F. NE levels in control animals (9.51±0.48), animals treated with bicuculline alone (9.16±0.83), CGP-35348 alone (9.43±1.14) and in animals treated with IL-1β+bicuculline (6.94±2.2) were significantly higher than those in animals treated with either IL-1β alone (5.3±0.59) or IL-1β+CGP-35348(4.18±0.95; $p<0.05$).

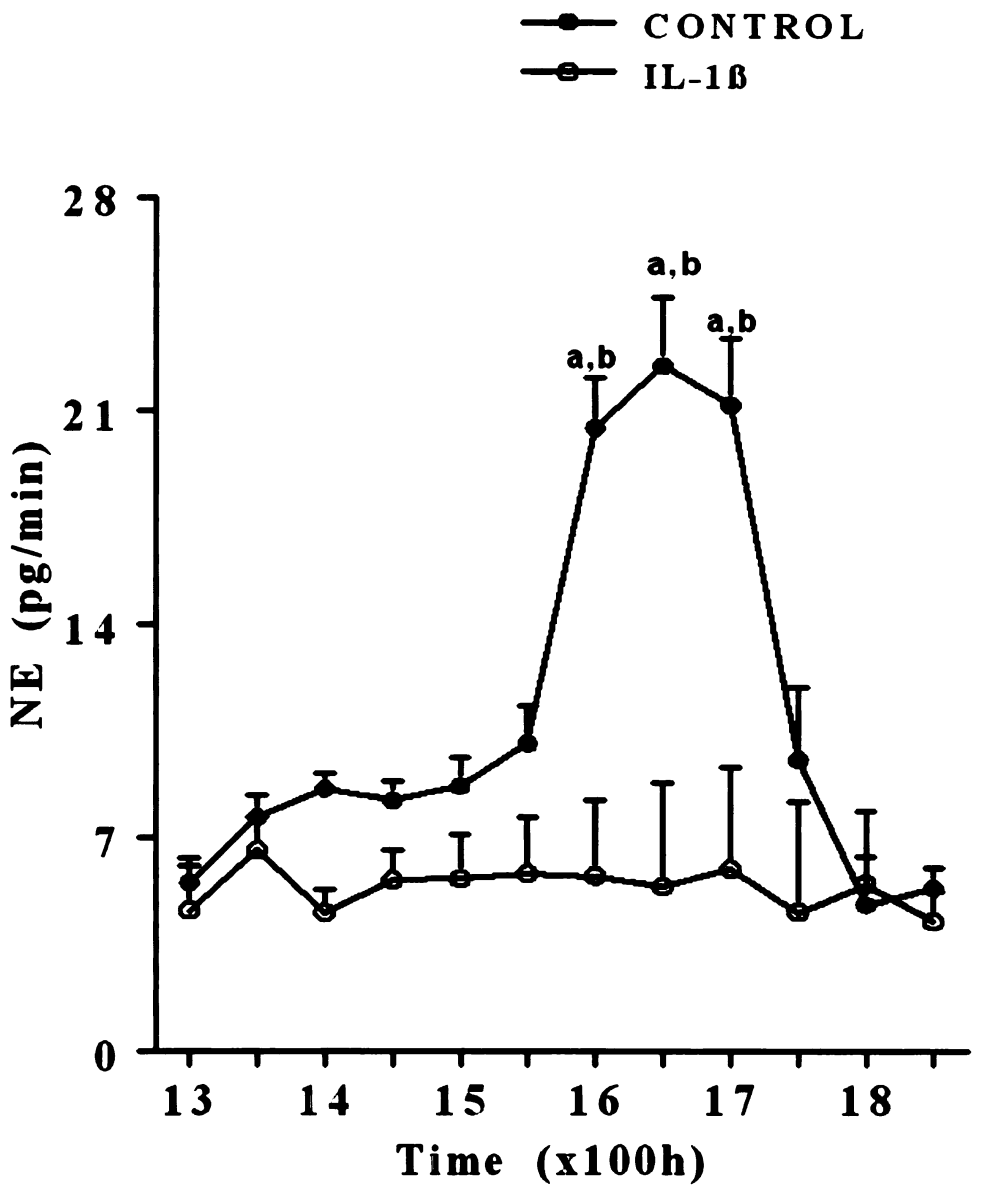

Figure 4-2A: *Effects of IL-1β on NE release in the MPA.* NE release (mean±SE; pg/min) profile in the MPA measured at 30-min intervals in ovariectomized steroid primed animals that were either treated with PBS (Control,n=7), or 5 μg of IL-1β (n=6) at 1300 h. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h and 'b' indicates difference ($p<0.05$) from levels in IL-1β-treated animals. Note that the NE levels in control animals increased from 1530 h and reached a peak at 1630 h. But the levels in IL-1β treated rats remained unchanged during the treatment period and were significantly lower than respective levels in controls.

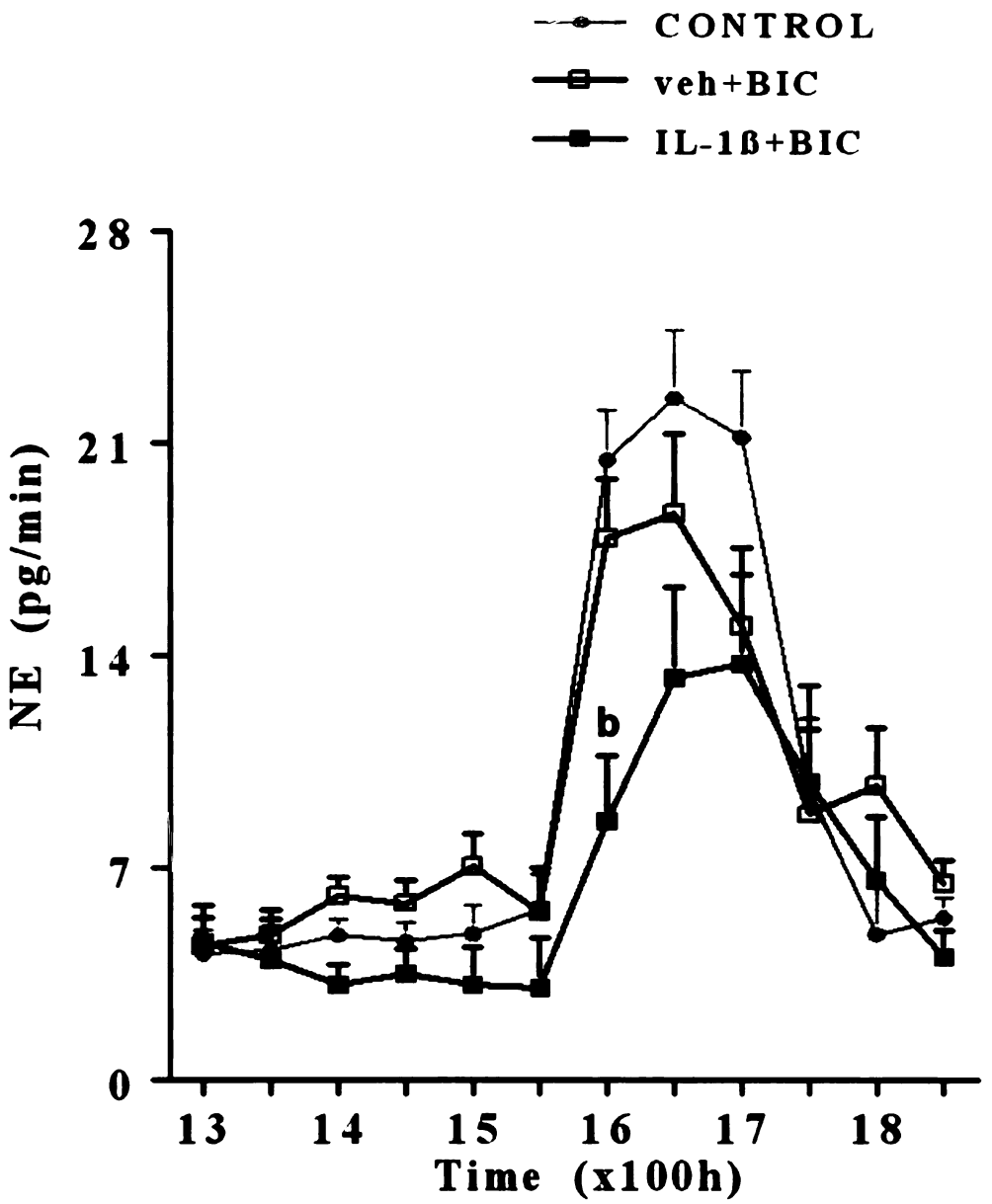

Figure 4-2B: *Effects of IL-1β on NE release in the MPA.* NE release (mean±SE; pg/min) profile in the MPA measured at 30-min intervals in ovariectomized steroid primed animals that were either treated with bicuculline (veh+BIC, n=6), or a combination of 5 μg of IL-1β and bicuculline (IL-1β +BIC,n=6) at 1300 h. The NE profile in control animals is provided for comparison purposes in grey. 'b' indicates difference ($p<0.05$) from levels in IL-1β-treated animals. Note that the NE levels in veh+BIC animals were not different from control levels. Treatment with bicuculline reversed the suppressive effect of IL-1β on NE.

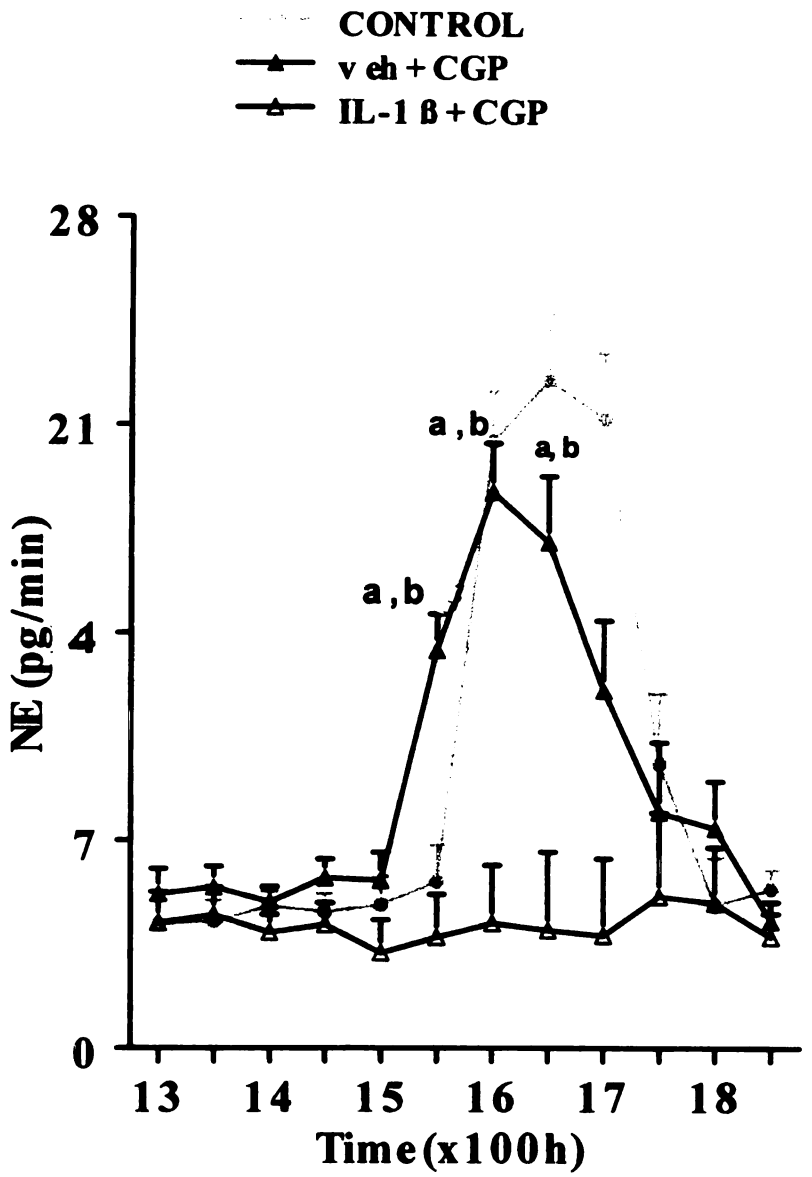

Figure 4-2C: *Effects of IL-1β on NE release in the MPA.* NE release (mean±SE; pg/min) profile in the MPA measured at 30-min intervals in ovariectomized steroid primed animals that were either treated with CGP-35348 (veh+CGP, n=6), or a combination of 5μg of IL-1β and bicuculline (IL-1β +CGP,n=6) at 1300h. The NE profile in control animals is provided for comparison purposes in grey. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h, 'b' indicates difference ($p<0.05$) from levels in IL-1β-treated animals and 'c' indicates difference from levels in control animals ($p<0.05$) . Note that the NE levels in veh+CGP animals were not different from control levels at 1630h. Treatment with CGP 35348 failed to reverse the suppressive effect of IL-1β on NE.

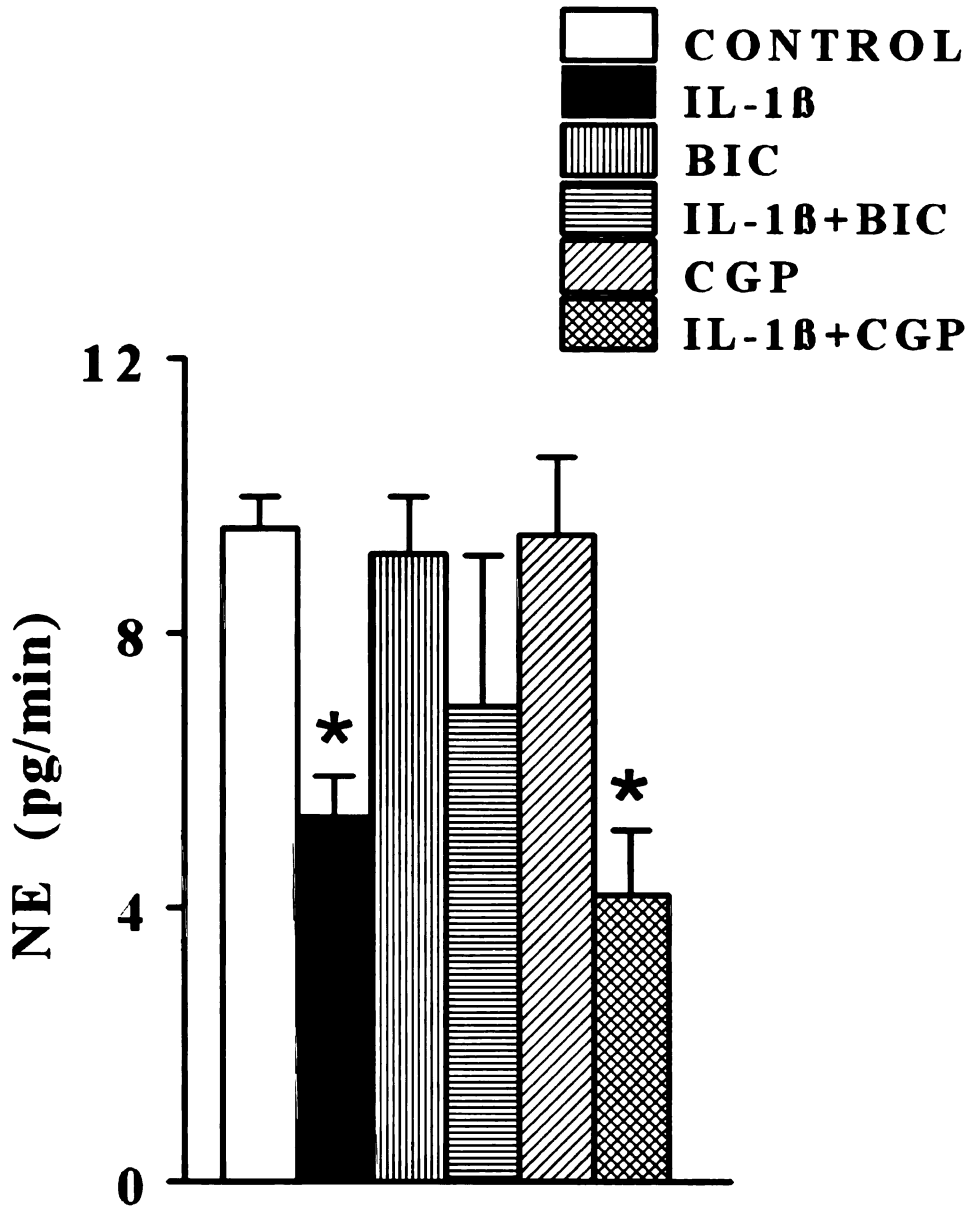

Figure 4-2D: Average NE levels (mean±SE;pg/min) during the entire period of observation in the control, IL-1β, veh+BIC, IL-1β +BIC, veh+CGP, IL-1β +CGP treated animals at 1300 h. Note that treatment with IL-1β significantly decreased the levels of NE measured during the entire treatment period and CGP 35348 failed to reverse it.

Effect of IL-1β on plasma LH levels - Role of GABA antagonists:

Concurrent changes in plasma LH levels in the animals subjected to push-pull perfusion are shown in Fig 4-3A,B,C and D. The effects of treatment [F (5, 163) =8.3], time [F (6, 11) =11.66] and the interaction between treatment and time [F (30,11)=3.35] were found to be significant ($p<0.05$). In control animals, LH levels (ng/ml, mean±SE) were 0.45 ± 0.19 at 1300 h and gradually increased to peak concentrations of 5.9 ± 1.02 at 1800 h ($p<0.05$), closely following the increase in NE levels in the MPA that occurred at 1630 and 1700 h. In contrast, in IL-1β treated animals, LH levels were 0.57±0.25 at 1300 h and remained at that level during the rest of the observation period, parallel to the low levels of NE observed in the MPA. In the group treated with bicuculline alone, LH levels increased from 0.99±0.22 at 1300h to 6.82±1.02 at 1700h ($p<0.05$) and decreased to 4.65±1.18 at 1800h. These levels at 1800h were not different from those in the control animals but the levels at 1700h were higher than those in controls ($P<0.05$) indicating that the peak in LH was advanced by bicuculline treatment . The suppressive effect of IL-1β on LH was reversed by co-treatment with bicuculline. In this group, LH levels at 1800 h (4.68±1.29) were significantly higher than the levels observed in the IL-1β-treated group (0.6±0.7; $p<0.05$). In the group treated with CGP-35348 alone, the levels at 1300h were 0.49±0.25, which significantly increased to 5.65±1.44 at 1800h ($p<0.05$). The levels at 1800h were not different from control levels but higher than CGP-35348+IL-1β treated group. In the IL-1β+CGP-35348 group, the levels of LH remained similar to IL-1β-treated group indicating that CGP-35348 did not reverse the effect of IL-1β. The average levels of serum LH during the entire observation period is shown in Fig 4-J. Average LH levels in control animals (2.41±0.23), animals treated with bicuculline alone (2.74±0.39),

CGP-35348 alone (2.025±0.19) and in animals treated with IL-1β+bicuculline (1.64±0.24) were significantly higher than those in animals treated with either IL-1β alone (0.67±0.05) or IL-1β+CGP-35348(1.1±0.0.27; $p<0.05$).

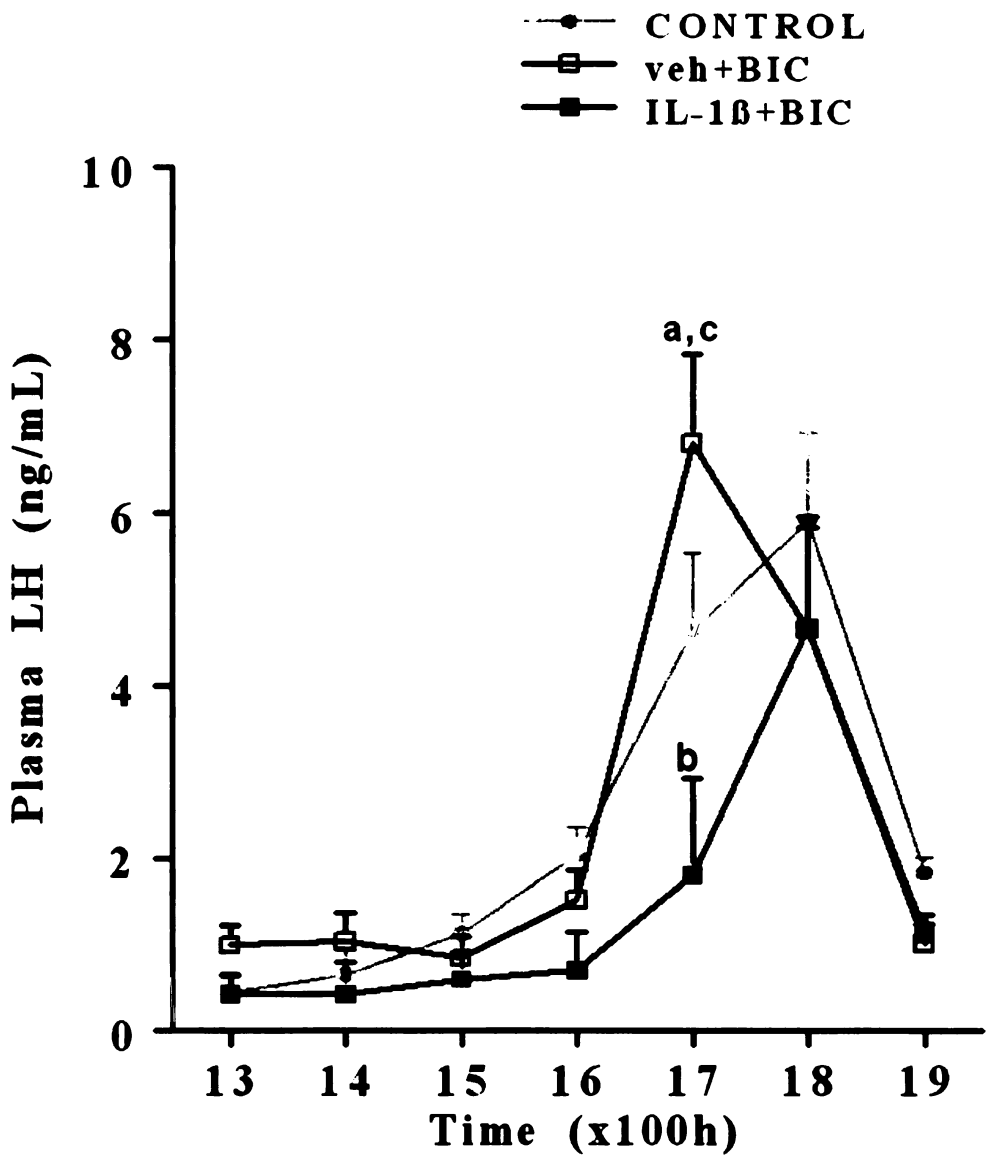

Figure 4-3B: *Effects of IL-1β on serum LH levels.* Serum LH (mean±SE; ng/mL) profile measured at hourly intervals in ovariectomized steroid primed animals that were either treated with bicuculline (veh+BIC, n=6), or a combination of 5μg of IL-1β and bicuculline (IL-1β +BIC,n=6) at 1300h. The LH profile in control animals is provided for comparison purposes in grey. 'a' indicates significant difference ($p<0.05$) from levels at 1300 h, 'b' indicates difference ($p<0.05$) from levels in IL-1β-treated animals and 'c' indicates difference from levels in control animals ($p<0.05$) . Note that the peak LH levels were advanced by an hour in veh+BIC animals but were not different from control levels at 1800h. Treatment with bicuculline reversed the suppressive effect of IL-1β on LH.

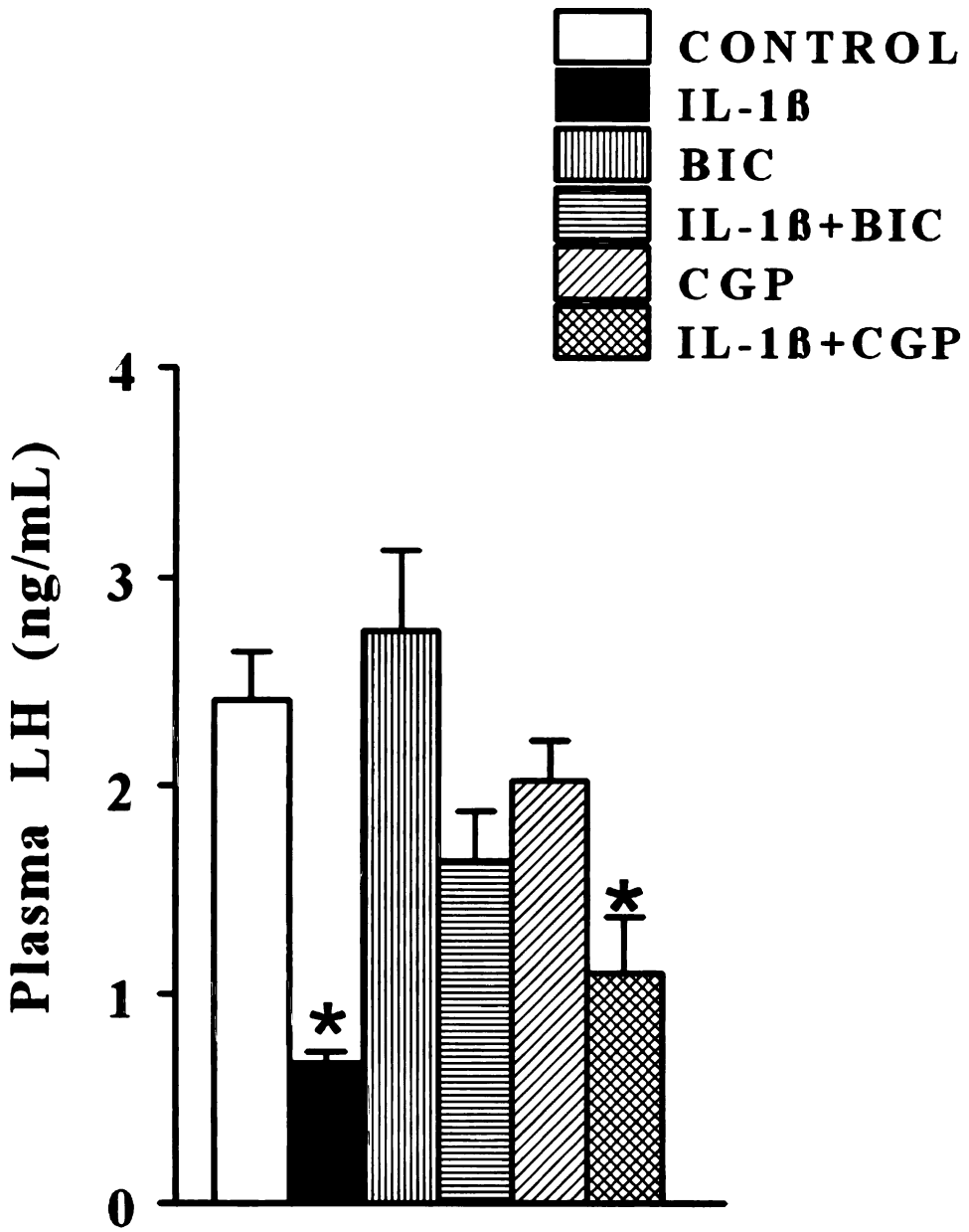

Figure 4-3D: Average serum LH levels (mean±SE;pg/min) during the entire period of observation in the control, IL-1β, veh+BIC, IL-1β +BIC, veh+CGP, IL-1β +CGP treated animals at 1300 h. Note that treatment with IL-1β significantly decreased the levels of LH measured during the entire treatment period and CGP 35348 failed to reverse it.

DISCUSSION:

The present study further explores the mechanisms by which systemic IL-1β produces a suppressive effect on the steroid-induced LH surge. The results of this study provide evidence for the involvement of GABA, the interaction between NE and GABA and additionally delineate the role of specific GABA receptors involved in transducing the effects of systemic IL-1β on LH secretion. This is in fact the first study to simultaneously measure NE and GABA levels in the MPA with corresponding measurements of serum LH during a systemic challenge with IL-1β.

Systemic IL-1β increased GABA levels in the MPA in ovariectomized-steroid primed rats. Parallel to this increase in GABA, there was a decrease in NE levels in the MPA as well as suppression of the steroid-induced LH surge. Furthermore, we attempted to reverse IL-1β's inhibitory effects on the HPG axis by using GABA antagonists. The GABA-A antagonist, bicuculline reversed the suppressive effect of IL-1β on LH by increasing the levels of NE while the GABA-B antagonist, CGP 35348 failed to produce such an effect. This suggests that systemic IL-1β is capable of modulating both stimulatory as well as inhibitory inputs on the GnRH system to influence the HPG axis.

The potentially adverse effect of cytokines on the reproductive axis is well known. IL-1β was believed to affect the HPG axis more likely at the hypothalamic level and inhibit ovulatory function (Rivier and Vale 1990). Several candidate molecules have been proposed to mediate the inhibitory effect of IL-1β on the HPG axis (reviewed in (Rivier and Rivest 1993; Kalra et al. 1998)) and the mechanistic evidence is constantly growing. This is because regulation of LH secretion is not simplistic but involves an intricate coordination of a horde of neurochemicals at the hypothalamic level (Levine 1997; Smith

and Jennes 2001). GABA is one of the important regulators of LH secretion. It has been shown to exert an inhibitory tone on LH surge (Donoso et al. 1994). Administration of GABA agonists was shown to suppress steroid-induced LH surge (Adler and Crowley 1986). In female rats during proestrus, when LH levels increase in the afternoon, the levels of GABA were found to be low (Demling et al. 1985; Jarry et al. 1988). This decrease in GABAergic input has been proposed to be permissive to the generation of the LH surge by increasing the responsiveness of GnRH neurons (Barraclough 1994; Dobson et al. 2003). The effects of pharmacological manipulations of GABA receptors on LH surge have yielded indecisive results, though. Bicuculline, GABA-A receptor antagonist is shown to cause an advance in peak of LH by one group (Kimura and Jinnai 1994) while another reports no such effect (Herbison and Dyer 1991).

The effects of GABA on LH secretion can be a result of i) its direct actions on the GnRH neurons or ii) by modulating the effects of other neurotransmitters like NE. There are several lines of evidence to point out the direct connections between GABA neurons and the GnRH neuronal network. Direct synaptic connectivity between GABAergic neurons with GnRH neurons at the MPA has been reported (Jennes et al. 1983; Leranth et al. 1985). Also, GnRH neurons express GABA receptors and electrophysiologically respond to different pharmacological manipulations, under both in vivo and in vitro conditions (Herbison and Dyer 1991; Favit et al. 1993; Jung et al. 1998).

Numerous studies point to the existence of a reciprocal relationship between GABA and NE (Mansky et al. 1982; Ondo et al. 1982; Demling et al. 1985). Also, direct synaptic connections between NE terminals and GABA interneurons has been described (Horvath et al. 1992). Release rates of GABA are low in the MPA while NE and LH

levels are high, and when LH levels are low in diestrus, levels of GABA were found to be high (Mitsushima et al. 2002). Moreover, administration of GABA-A agonist, muscimol has been shown to reduce NE turnover rate, paralleling an attenuation of the LH surge (Adler and Crowley 1986; Akema et al. 1990). The relationship between the levels of NE, GABA and LH found in this study do corroborate with existing results.

The levels of GABA in control animals during the steroid-induced LH surge in the present study (20-60 pg/min) are comparable to previous studies (Tin Tin Win et al. 2004) using a similar animal model (54 pg/min). In the present study, in control animals, corresponding to increase in NE levels in the MPA and the serum LH in the afternoon, the levels of GABA were low. In contrast, systemic IL-1β increased GABA release in the MPA allowing us to speculate that immune challenges are capable of influencing a wide range of aspects of the GABAergic system. Systemic administration of LPS has been shown to increase the synthesis of the GABA-synthetic enzyme, glutamic acid decarboxylase (GAD67) in the MPA as well as GABA levels (Feleder et al. 1996; Akema et al. 2005). Moreover, IL-1 has been shown to enhance inhibitory GABAergic post synaptic potentials and inhibit the activity of preoptic neurons by increasing presynaptic GABA release (Tabarean et al. 2006; Brambilla et al. 2007). It also has been shown to augment the expression of GABA-A receptors at the neuronal surface (Serantes et al. 2006).

On the role of GABA antagonists, we show here that bicuculline, a GABA-A receptor when administered along with IL-1β was able to block the effect of IL-1β on NE levels in the MPA and serum LH. No such effect was observed upon administration of CGP-35348, a GABA-B antagonist suggesting that IL-1β's modulation of the GABAergic

system to influence the HPG axis is probably mediated though GABA-A receptors. Several other studies provide substantial evidence for the preponderance of GABA-A receptor over GABA-B receptor in mediating LH secretion. Direct administration of GABA-A agonist, muscimol, was demonstrated to rapidly reduce LH release and decrease the expression of GnRH in the hypothalamus (Leonhardt et al. 1995) while the GABA-B agonist, baclofen, did not have such effect. Activation of GABA-A receptors is shown to be inhibitory to GnRH electrical activity (Kimura et al. 1993) while GABA-B receptor activation inhibits mRNA expression and peptide synthesis of GnRH (Bergen et al. 1991). Taken together, these studies support an inhibitory role for GABA in LH secretion. The involvement of NE in GABA-induced LH suppression has not been studied in great detail. The few studies that have examined the role of NE in this phenomenon were done in male rats. Nevertheless, these were found to be GABA-A receptor mediated (Feleder et al. 1999a; Sakamaki et al. 2003) strengthening the results of the present study that bicuculline alone and not CGP-35348 reversed IL-1β's effects on NE and LH.

The role of GABA-A receptor in modulating the inhibitory effects of stress on LH has been documented in the context of other stressors also. Metabolic stress following glucoprivation is known to increase central GABA levels and this effect suppresses reproductive function (Singh et al. 2004). During such metabolic stress, central GABA-A but not GABA-B receptors have been demonstrated to be responsible for the inhibition of LH surge (Singh and Briski 2005). In forskolin-induced suppression of LH surge, GABA-A receptors have been implicated (Taleisnik et al. 1993). This involvement of GABA receptors in mediating stress-induced inhibition of LH does not seem to be a

global phenomenon though. In a neurogenic stress paradigm, like acute restraint, GABA antagonists were ineffective in reversing the inhibition on LH (Roozendaal et al. 1997) suggesting that the role of GABA receptors is exclusive to homeostatic stress paradigms.

Apart from studies in rodents, experiments in other species also suggest the involvement of GABA-A receptors in bringing about the inhibitory effect of GABA on LH secretion. Activation of GABA-A receptors in sheep during the breeding season has been shown to inhibit LH secretion leading to anovulation (Scott and Clarke 1993).

The present study presents interesting findings that inhibition of the steroid-induced LH surge in female rats by systemic immune challenge is mediated by a central increase in GABAergic inhibitory input. This increase also corresponded to a decrease in noradrenergic stimulatory input. This study also provides insight into the receptor mechanisms involved in GABA-mediated inhibition of LH. Specifically, GABA-A receptor mediated mechanism was found to reverse the inhibitory effect of systemic IL-1β on levels of NE in the MPA and serum LH while GABA-B blockade did not have such effect. Taken together, these findings suggest that systemic IL-1β inhibits HPG activity by interaction between GABA and NE at the MPA and this is accomplished most probably by a GABA-A receptor mediated mechanism.

CHAPTER 5: SIMULTANEOUS EFFECTS OF SYSTEMIC INTERLEUKIN-1β ON THE HPA AND HPG AXES: ROLE OF NOREPINEPHRINE

INTRODUCTION:

Interaction between the immune and neuroendocrine systems during an immune challenge often results in activation of the hypothalamic-pituitary-adrenal (HPA) axis and suppression of the hypothalamo-pituitary-gonadal (HPG) axis (Kalra et al. 1998; Beishuizen and Thijs 2003; Webster and Sternberg 2004). Interleukins are proteins that are released by activated macrophages during a systemic immune challenge. They play a major role in conveying to the CNS the occurrence of immune stimulation (Besedovsky and del Rey 1996; Dinarello 1996). In particular, the cytokine, interleukin-1β (IL-1β) has been shown to affect both the HPA and the HPG axes (Berkenbosch et al. 1987; Sapolsky et al. 1987; Kalra et al. 1990a; Rivier and Vale 1990).

Central and peripheral administration of IL-1β has been shown to block reproduction. Specifically it has been shown to suppress the preovulatory LH surge and thereby block ovulation (Kalra et al. 1990a; Rivier and Vale 1990; MohanKumar and MohanKumar 2002). Numerous studies have demonstrated the involvement of central pathways in mediating this effect. IL-1β not only decreases c-fos immunoreactivity in the gonadotrophin releasing hormone (GnRH) neurons (Rivier and Rivest 1993), but is capable of blocking the transcription and translation of GnRH culminating in the decrease of GnRH from the hypothalamus (Rivier and Rivest 1993; Kang et al. 2000). The mechanism by which IL-1β mediates these effects is not well established. It is possible

that atleast some of the multitude of neurochemicals implicated in regulation of LH (Kalra 1993; Smith and Jennes 2001) might be involved in bringing about these effects.

IL-1β has been shown to activate the HPA axis by increasing c-fos immunoreactivity in corticotrophin releasing hormone (CRH) neurons, release of CRH from the paraventricular nucleus (PVN) effecting an increase in circulating glucocorticoids (Besedovsky et al. 1986; Berkenbosch et al. 1987; Sapolsky et al. 1987; Brady et al. 1994). Our previous studies indicate that systemic administration of IL-1β activates the HPA axis by increasing the noradrenergic stimulatory input to the CRH neurocircuitry in the PVN (MohanKumar and Quadri 1993; MohanKumar and MohanKumar 2005).

Using push-pull perfusion, we have demonstrated that suppression of the preovulatory LH surge involves decrease in norepinephrine (NE) levels in the medial preoptic area, one of the areas with highest density of GnRH neurons (MohanKumar and MohanKumar 2002; Sirivelu et al. 2006). It is interesting to note that the effects of IL-1β on both the HPA axis as well as the HPG axis have been mediated via NE. And, more importantly, it is clear that the effect of IL-1β on the noradrenergic system is not generalized but region-specific.

Studying the interaction between the HPA and the HPG axes during an immune challenge is essential for understanding the mechanisms by which immune stress compromises reproduction. Several studies have looked at the isolated effect of IL-1β on the HPA axis as well as the HPG axis. The simultaneous effects of IL-1β on both the HPA and the HPG axes in the same animal and the mechanisms involved in precipitating these changes have not been studied so far. The present study is the first of such

experiments to evaluate the concurrent changes in the HPA and the HPG axes during a systemic IL-1 challenge. The role of NE in such an interaction between these two systems, especially in female rats has not been studied so far. The objective of the present study is to examine the differential effects of IL-1β on noradrenergic activity in the HPA axis related areas as well as the HPG axis related areas of the brain.

EXPERIMENTAL SETUP:

The estrous cycle of the rats was monitored by daily vaginal cytology after a rest period of two weeks. The rats that showed regular estrous cycles were selected. On the day of proestrus, they were randomly subjected to one of the two treatments, Control (PBS-1.0% BSA), IL-1-β 5μg i.p at 1300 hrs. They were sacrificed at three time points: 1300, 1500 and 1700 hrs by rapid decapitation. The trunk blood was collected for analysis of IL-1β, LH and corticosterone levels. The brain was snap frozen in liquid nitrogen and stored in −70°C. Serial coronal sections (300 μm thick) of the brain were obtained using a cryostat (Slee Mainz, London, UK) maintained at −10°C. The brain areas – organum vasculosum lamina terminalis (OVLT), diagonal band of Broca (DBB), medial preoptic area (MPA), suprachiasmatic nucleus (SCN), arcuate nucleus (AN), paraventricular nucleus (PVN), central amygdala (CeA), bed nucleus of the stria terminalis (BNST) were microdissected by the Palkovits's micropunch technique. Tissue samples were obtained bilaterally, and all the subdivisions of the nuclei were included. The samples were used for neurotransmitter detection by HPLC-EC.

RESULTS:

Effect of IL-1β on circulating IL-1β levels:

The patterns of serum IL-1β (ng/ml; mean ±SE) in control and IL-1β treated animals are shown in Fig 5-1. The levels of IL-1β in the control animals at 1pm were 0.15 ± 0.1 and remained at similar levels at 3pm and 5pm. However, in the IL-1β-treated animals they increased to 2.08 ± 0.44 at 3pm, which were significantly different from levels at 1pm as well as control levels at 3pm (0.19 ± 0.03; $p<0.05$). At 5pm, the levels dropped to 0.69 ± 0.16 which were also higher than control values (0.26 ± 0.06).

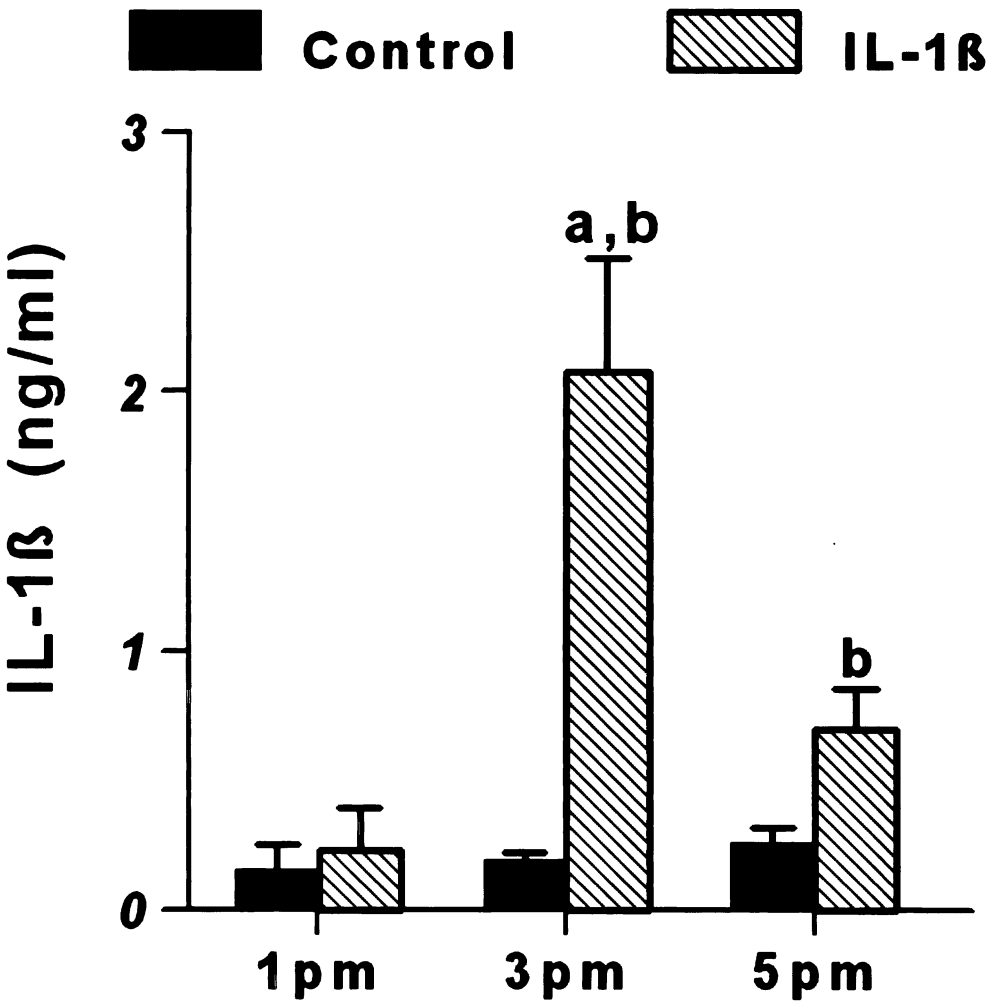

Figure 5-1. Serum IL-1β levels (mean±SE;ng/ml) in control (n=8+8+8) and IL-1β-treated female rats(n=8+8+8) on proestrus. 'a' indicates significant difference ($p<0.05$) from levels at 1pm and 'b' indicates difference ($p<0.05$) from levels in control animals. Note that treatment with IL-1β significantly increases serum IL-1β levels within 2 hrs while the levels in vehicle treated controls remain unchanged during the treatment period.

Effect of IL-1β on serum corticosterone levels:

The patterns of serum corticosterone (ng/ml; mean ±SE) in control and IL-1β treated animals are shown in Fig 5-2. At 1pm, corticosterone levels in control animals (492.87±96.98) were not different from levels in IL-1β-treated animals (524.9±102.86). At later time points of 3pm and 5pm, the levels in control animals gradually decreased. In contrast, treatment with IL-1β increased corticosterone levels to 812.18±112.68 at 3pm and 556.0±93.69 at 5pm which were significantly higher ($p<0.05$) than the levels in control animals (305.26±70.96, 271.92±43.32 respectively).

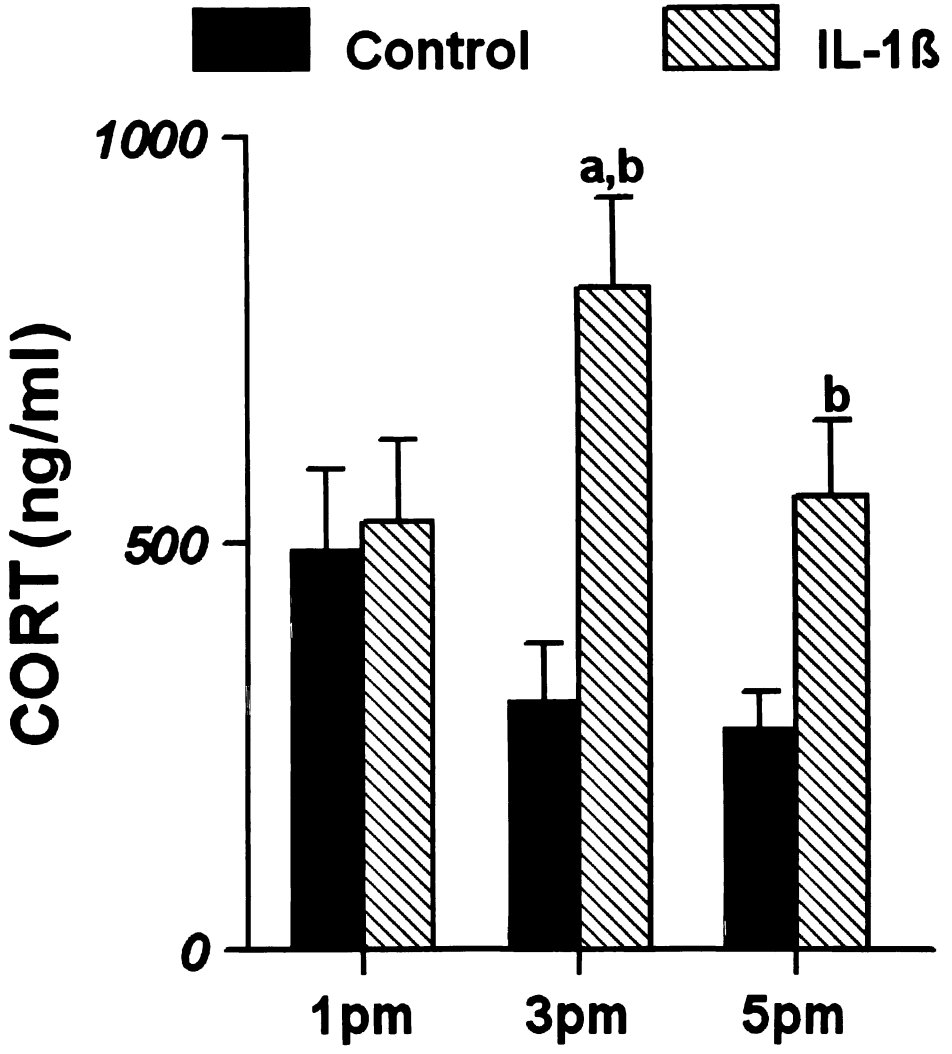

Figure 5-2. Serum corticosterone levels (mean±SE;ng/ml) in control (n=8+8+8) and IL-1β-treated(n=8+8+8) female rats on proestrus. 'a' indicates significant difference ($p<0.05$) from levels at 1pm and 'b' indicates difference ($p<0.05$) from levels in control animals. Note that treatment with IL-1β significantly increases serum corticosterone levels within 2 hrs while the levels in vehicle treated controls remain unchanged during the treatment period.

Effect of IL-1β on serum LH levels:

The levels of LH (ng/ml; mean ±SE) in control and IL-1β treated animals are shown in Fig 5-3. In control animals, LH levels at 1pm were 1.48±0.13 and gradually increased to 1.89±0.24 at 3pm and reached a peak at 5pm (4.91±1.12, $p<0.05$). However in IL-1β-treated animals, LH levels at 1pm were 1.29±0.11 and remained at similar levels at 3pm and 5pm. Treatment with IL-1β blocked the LH peak at 5pm compared to control animals ($p<0.05$).

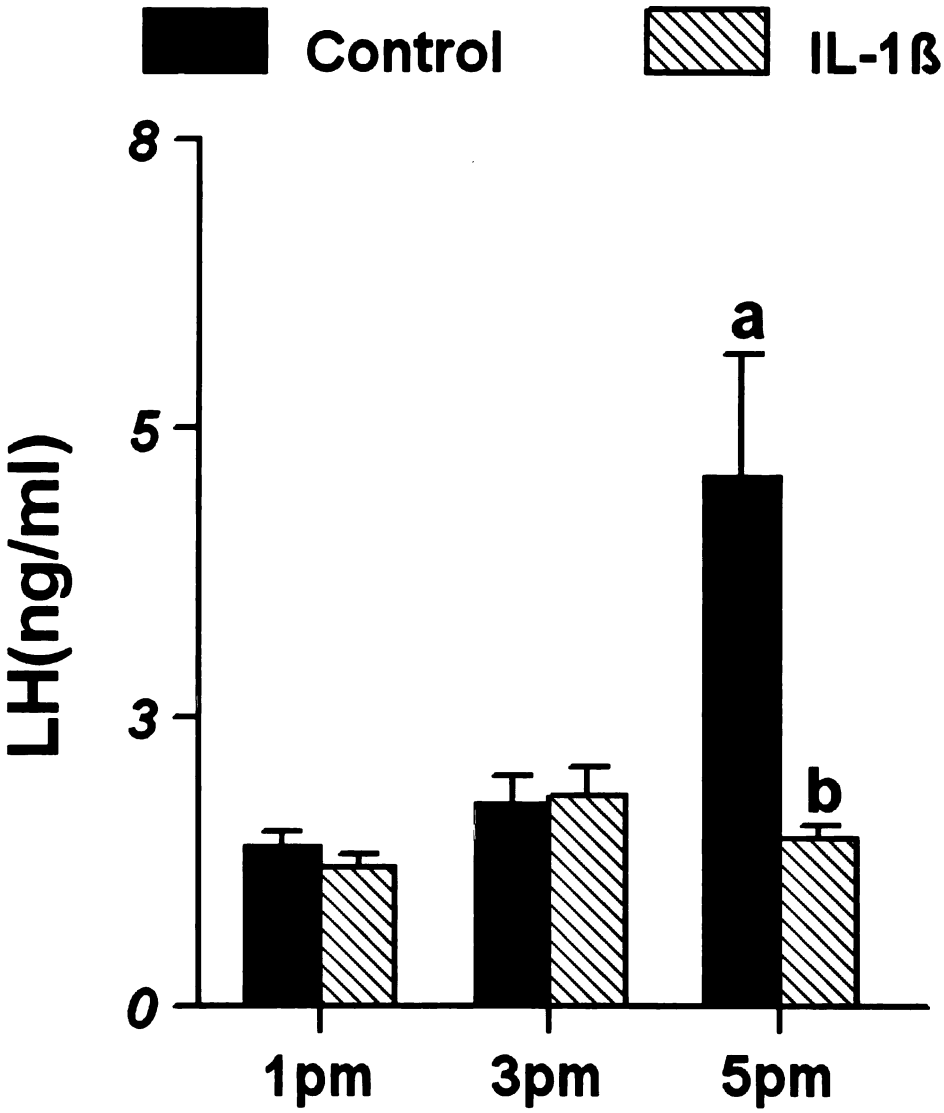

Figure 5-3. Serum LH levels (mean±SE;ng/ml) in control (n=8+8+8) and IL-1β-treated(n=8+8+8) female rats on proestrus. 'a' indicates significant difference ($p<0.05$) from levels at 1pm and 'b' indicates difference ($p<0.05$) from levels in control-treated animals. Note that serum LH level increased significantly in the control animals which was blocked by treatment with IL-1β.

Effect of IL-1β on NE levels in HPG axis-related areas:

The levels of NE (pg/μg protein; mean ±SE) in control and IL-1β treated animals in various areas related to the HPG axis are shown in Fig 5-4A-E.

MPA: The levels of NE in control animals at 1pm were 18.22±0.5, which significantly increased to 24.70±3.2 at 3pm ($p<0.05$; Fig3a). In IL-1β-treated animals, the levels of NE at 1pm were 13.07±1.55 which remained at similar levels at 3pm and 5pm. At 3pm, the levels of NE in IL-1β-treated animals (16.89±.87) were significantly lower compared to the levels in control animals ($p<0.05$). At 5pm also, the levels of NE in control animals (20.72±2.0) were significantly decreased ($p<0.05$) by the treatment with IL-1β (14.26±1.26) indicating a suppression of the reproductive axis.

DBB: In the control animals, the levels of NE at 1pm were 12.15±1.41, which significantly increased to 20.98±1.41 at 3pm ($p<0.05$; Fig 3b). This increase in NE levels at 3pm was blocked by treatment with IL-1β (11.76±2.5; $p<0.05$). The levels of NE in the IL-1β-treated animals did not change with time.

OVLT: The levels of NE in control animals at 1pm were 10.47±0.826 (Fig 3c) and remained at similar levels at 3pm (12.51±.805) and 5pm (10.96±1.96). In IL-1β-treated animals, the levels of NE at 1pm were 8.22±1.23 and remained at similar levels during 3pm (6.92±.89) and at 5pm (8.05±.66). However, at 3pm, the levels of NE in IL-1β-treated animals were significantly lower than those in control animals ($p<0.05$).

AN: In the control animals, the levels of NE did not change during the timepoints-1pm, 3pm and 5pm (Fig3d). In the IL-1β treated animals also, the levels of NE at 1pm were 15.97±1.5 and showed a trend to decrease over time. At 3pm, the levels of NE in IL-1β-

treated animals (13.01±1.07) were significantly lower ($p<0.05$) when compared to the levels in control animals (17.96 ±0.82).

SCN: The levels of NE in the control animals at 1pm were 16.26±2.81 (Fig3e) and remained at similar levels at 3pm and 5pm. In the IL-1β-treated animals, the levels of NE at 1pm were 15.26±1.84 which were not different from the levels in control animals (16.26±2.81). However at 3pm and 5pm, the levels of NE in IL-1β-treated animals (12.83±0.80 and 10.03±1.27 respectively) were significantly lower ($p<0.05$) compared to the levels in control animals (16.85±1.46 and 16.39±.94 respectively).

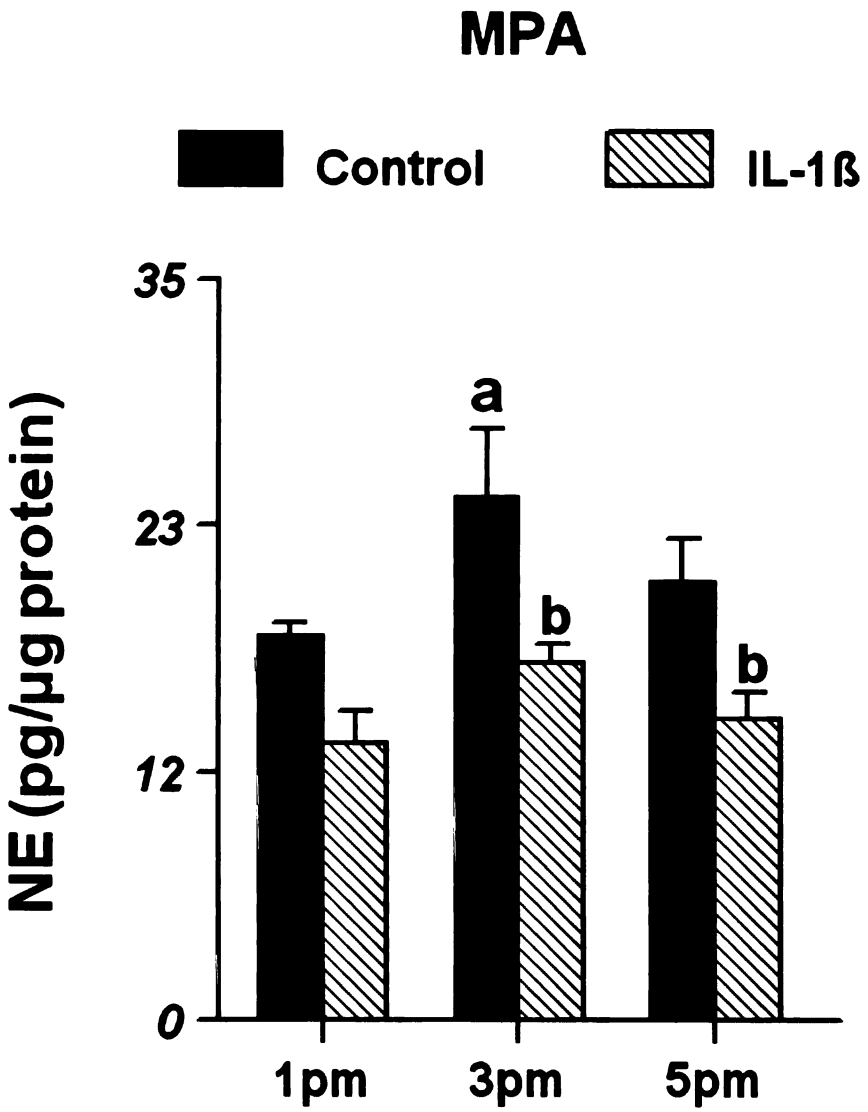

Figure 5-4A. Effect of systemic injections of PBS (control, n=8+8+8) and 5µg IL-1β (n=8+8+8) on the concentrations of NE (mean±SE;pg/µg protein) in the medial preoptic area. 'a' indicates significant difference ($p<0.05$) from levels at 1pm and 'b' indicates difference ($p<0.05$) from levels in control animals. Note that NE levels increased significantly in the control animals which were blocked by treatment with IL-1β.

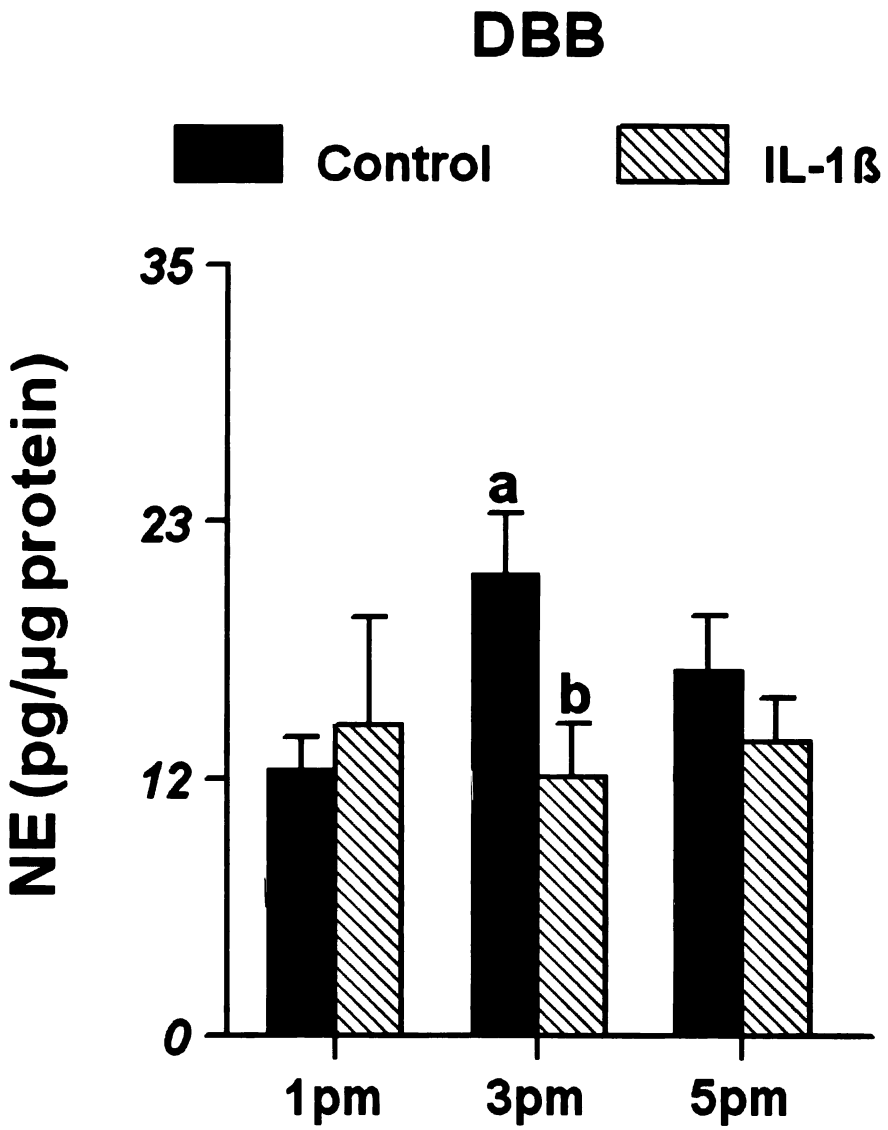

Figure 5-4B. Effect of systemic injections of PBS (control, n=8+8+8) and 5µg IL-1β (n=8+8+8) on the concentrations of NE (mean±SE;pg/µg protein) in the diagonal band of Broca. 'a' indicates significant difference ($p<0.05$) from levels at 1pm and 'b' indicates difference ($p<0.05$) from levels in control animals. Note that NE levels increased significantly in the control animals which were blocked by treatment with IL-1β.

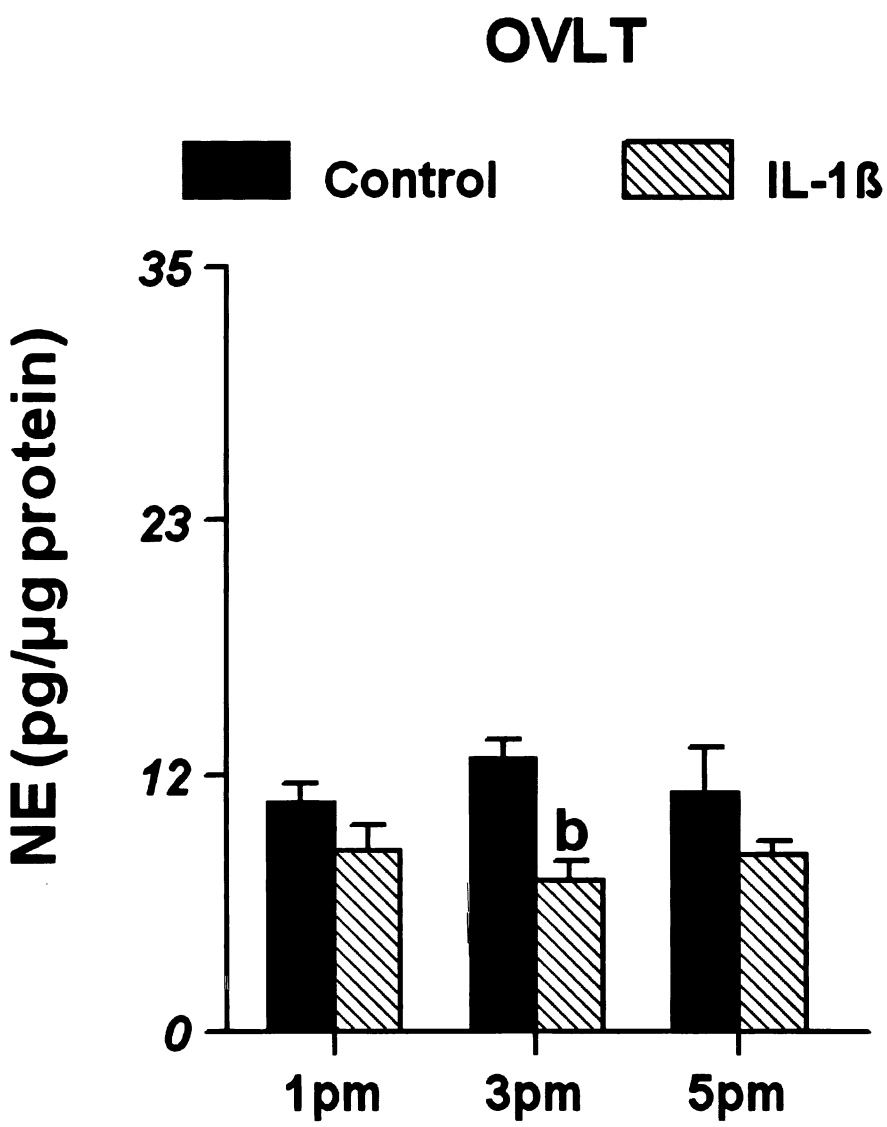

Figure 5-4C. Effect of systemic injections of PBS (control, n=8+8+8) and 5µg IL-1β (n=8+8+8) on the concentrations of NE (mean±SE;pg/µg protein) in the organum vasculosum lamina terminals. 'b' indicates difference ($p<0.05$) from levels in control animals. Note that NE levels decreased significantly in the IL-1β treated animals compared to the controls.

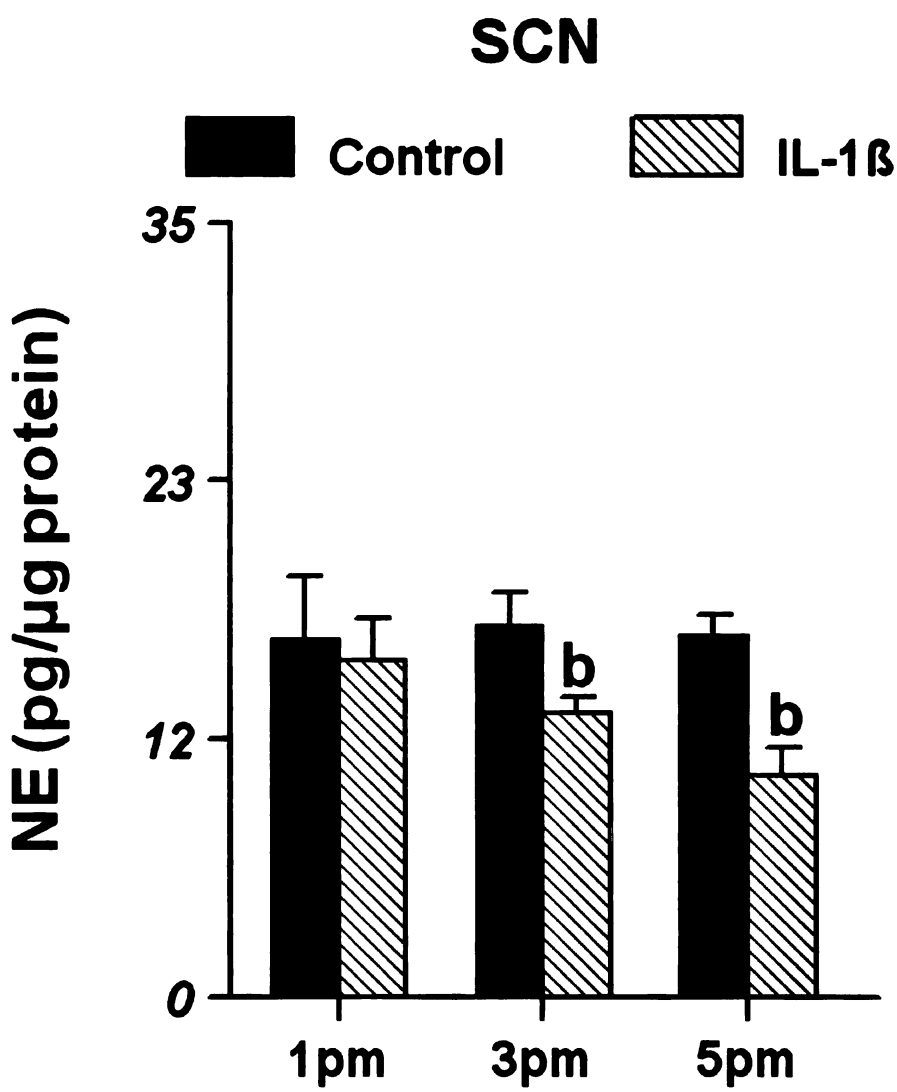

Figure 5-4D. Effect of systemic injections of PBS (control, n=8+8+8) and 5µg IL-1β (n=8+8+8) on the concentrations of NE (mean±SE;pg/µg protein) in the suprachiasmatic nucleus. 'b' indicates difference ($p<0.05$) from levels in control animals. Note that NE levels decreased significantly in the IL-1β treated animals compared to the controls.

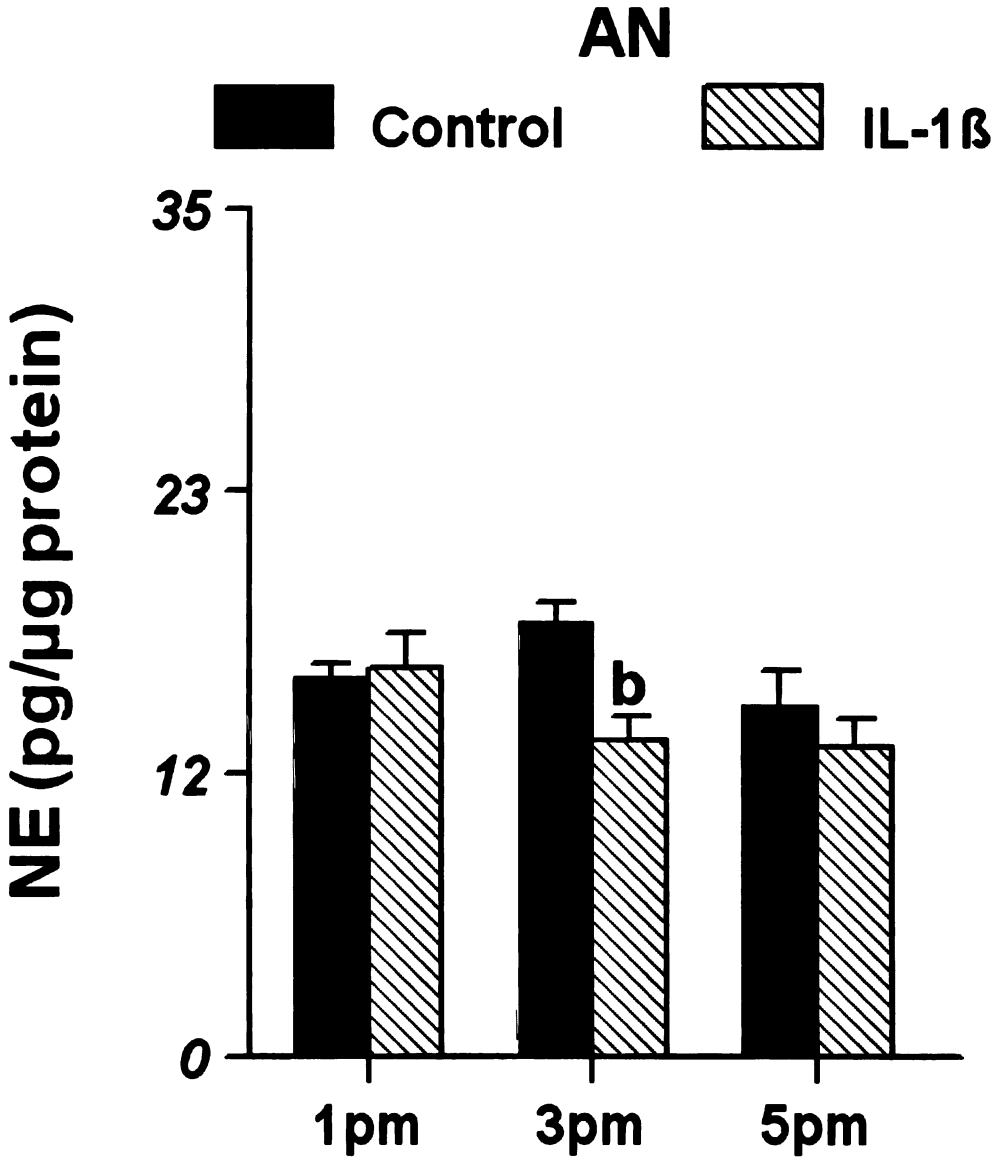

Figure 5-4E. Effect of systemic injections of PBS (control, n=8+8+8) and 5µg IL-1β (n=8+8+8) on the concentrations of NE (mean±SE;pg/µg protein) in the suprachiasmatic nucleus. 'b' indicates difference ($p<0.05$) from levels in control animals. Note that NE levels decreased significantly in the IL-1β treated animals compared to the controls.

Effect of IL-1β on NE levels in HPA axis-related areas:

The levels of NE (pg/μg protein; mean ±SE) in control and IL-1β treated animals in various areas related to the HPA axis are shown in Fig 5-5A-C.

PVN: The levels of NE in the control animals at 1pm were 11.67±2.78 (Fig 4a) and did not change at 3pm and 5pm. In the IL-1β-treated animals, the levels of NE at 1pm were 11.48±2.49 which significantly increased to 20.9±2.24 ($p<0.05$) at 3pm and remained at 13.14±2.98 at 5pm. At 3pm and 5pm, the levels of NE in IL-1β-treated animals were significantly higher ($p<0.05$) than the levels in control animals indicating an activation of the stress axis.

BNST: In control animals, the levels of NE at 1pm were 5.03±.54 (Fig 4b) which showed a trend to decrease at 3pm and 5pm (2.73±.49, 2.66±.41 respectively). In the animals treated with IL-1β, the levels of NE at 1pm were 5.36±0.61 which increased to 7.57±1.56 at 3pm and 6.71±.93 at 5pm. The levels at 3pm and 5pm were significantly higher in the IL-1β-treated animals when compared to the control animals ($p<0.05$).

CeA: In the control animals, the levels of NE at 1pm did not differ from the levels at 3pm and 5pm. Treatment with IL-1β significantly increased ($p<0.05$) the levels of NE (14.50±3.3) at 3pm when compared to the levels in control animals.

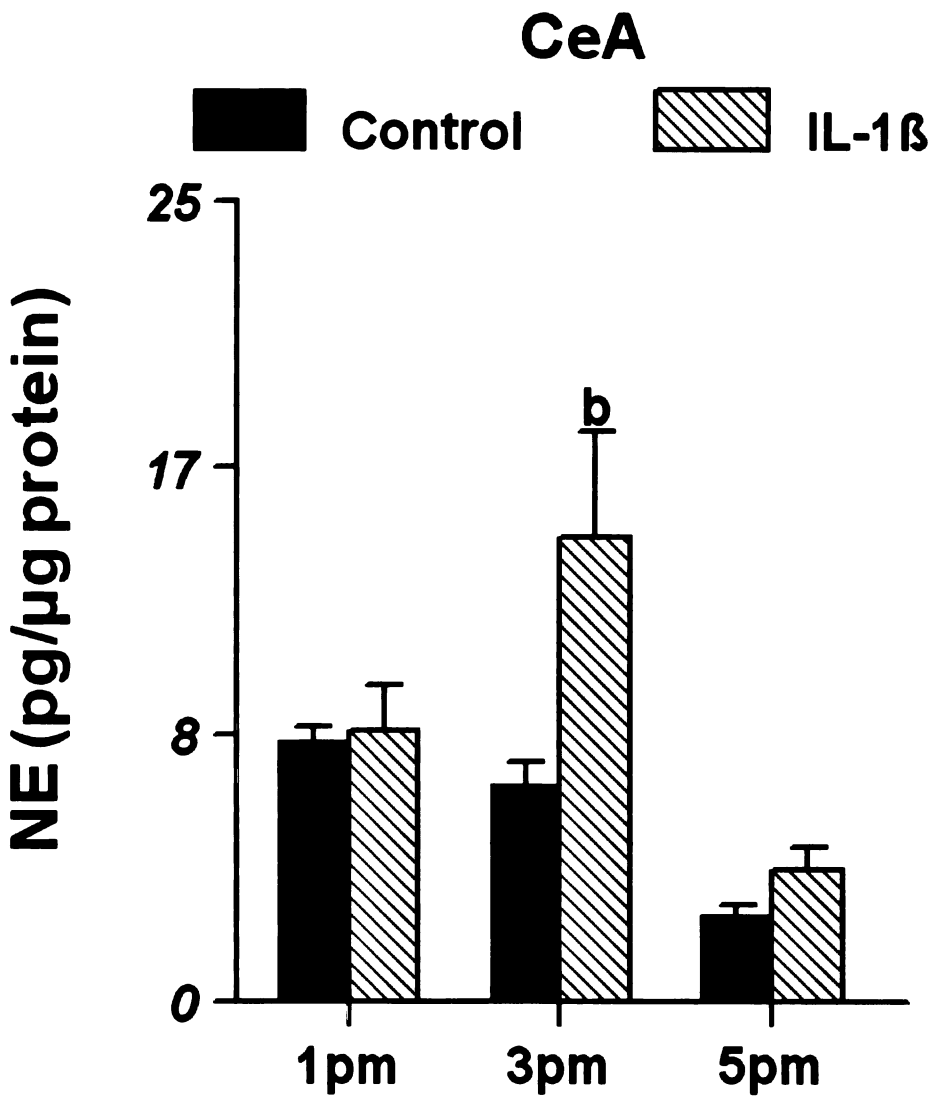

Figure 5-5C. Effect of systemic injections of PBS (control, n=8+8+8) and 5µg IL-1β (n=8+8+8) on the concentrations of NE (mean±SE;pg/µg protein) in the central amygdala. 'b' indicates difference ($p<0.05$) from levels in control animals. Note that NE levels increased significantly in the IL-1β treated animals compared to control animals

DISCUSSION:

We show here that NE mediates the effects of systemic IL-1β on both the HPG and the HPA axes. The present study provides the first evidence for the involvement of NE in the opposing effects produced by IL-1β on the two axes. More interestingly, as a general trend, there was a suppression of noradrenergic activity in the HPG axis related areas while NE levels were increased in the HPA related areas. This leads us to conclude that NE could be one of the key mediators by which a systemic immune challenge produces diametrically opposite effects: stimulatory effects on the HPA axis and inhibitory effects on the HPG axis.

There are several lines of evidence to support a stimulatory role for NE in IL-1β-induced activation of the stress axis. In our previous studies, we have shown that systemic IL-1β stimulates the release of NE from the PVN of the hypothalamus as early as 30 min and this increase continued for over 60 minutes, as measured by push-pull perfusion in male rats (MohanKumar and Quadri 1993). In another study using mice, it was found that the plasma levels of ACTH and corticosterone reached peak levels 2hrs after systemic IL-1β administration (Besedovsky et al. 1986). Corroborating with the aforementioned studies, in the present study too, systemic administration of IL-1β increased NE levels in the PVN as well as serum corticosterone levels at the measured intervals of 2 hrs and 4hrs post injection. However, the novel component of the present study is the measurement of these parameters on the afternoon of proestrus in female rats. Given the extensive literature on sexual dimorphic responses to stress (reviewed in (Gaillard and Spinedi 1998), this observation is of particular importance.

Apart from the activation of hypothalamic components of the CRH network, several other non-hypothalamic CRH rich areas like the central amygdala (CeA), bed nucleus of the stria terminalis (BNST) and the locus coeruleus could be involved in mediating the HPA responses to stress (Koob 1999). The amygdaloid CRF pathways form an important component of the neuroendocrine responses to stress. Central injections of CRH into the CeA can cause physiological changes similar to central injections of CRH. Acute stress paradigms have been shown to increase CRH levels in the CeA (Merali et al. 1998). Similarly, systemic IL-1β administration also results in activation of the CeA during the course of mounting a HPA response (Brady et al. 1994; Ericsson et al. 1997; Day et al. 1999; Xu et al. 1999). Studies involving lesions of the CeA suggested that it coordinates HPA responses between PVN the brainstem (Xu et al. 1999). NE has been shown to have a stimulatory input on the CRH neuronal system of the CeA (Raber et al. 1995). Immobilization stress which is associated with an increase in brain IL-1β levels, also induces NE release in the CeA as measured by in vivo microdialysis (Quirarte et al. 1998; Ma and Morilak 2005). Taken together, these results suggest that the neurocircuitry for NE-CRH interaction in the integration of HPA responses may be present in the CeA similar to the PVN. In the present study, we have shown that NE levels in the CeA increase at 2hrs and return to pre-treatment levels at 4hrs post injection. Since central CeA is the origin of afferent CRH-pathways to the brainstem, BNST and other hypothalamic areas also (Gray 1993; Gray and Bingaman 1996), this transient activation of CeA might play a role in integrating HPA responses between brainstem nuclei which in turn provide input to various hypothalamic areas.

The BNST plays an important role in the regulation of the HPA responses during stress and it is one of the principal extra-hypothalamic relay centers to the PVN from the CeA and the brainstem (Forray and Gysling 2004). Alpha-adrenergic receptors are thought to play a significant role in the BNST for coordinating these HPA responses (Cecchi et al. 2002; Banihashemi and Rinaman 2006). Acute immobilization stress increases NE levels in the BNST by 30 min and these levels remain elevated for about 2 hours in male Sprague-Dawley rats (Pacak et al. 1995). In the present study, systemic injection of IL-1β in female rats increased NE levels in the BNST at 2hrs and remained elevated at 4hrs post injection. This sustained increase might be due to the fact that HPA responses to systemic immune challenges in females are exaggerated especially during proestrus (Viau and Meaney 1991; Rivier 1994).

Our earlier studies as well as experiments in previous chapters have shown that systemic IL-1β is capable of inhibiting the HPG axis by decreasing NE levels in the MPA (MohanKumar and MohanKumar 2002). The critical role of MPA in transducing IL-1β's effects on the HPG axis is evident from the fact that direct infusion of IL-1β into the MPA was enough to suppress LH secretion (Rivier and Rivest 1993). However GnRH neurons that are scattered over a number of regions in the hypothalamus have been thought to be involved in the generation of LH surge as well (Smith and Jennes 2001). Specifically those include the OVLT, the DBB, the preopticosuprachiasmatic tuberoinfundibular system (PSTS) with perikarya in the MPA, SCN and the AN and terminals in the median eminence (Barraclough and Wise 1984; Hiatt et al. 1992; Herbison 2006). Simultaneous changes in all these neuronal populations in the face of an immune challenge, over a period of time have not been measured before.

In the present study, we measured NE levels in all these areas after an intraperitoneal injection of IL-1β. The levels of NE increased in the DBB and the MPA at 3pm, which seem to correlate with the increase in LH at 5pm. This increase in NE is in agreement with previous studies which have shown that changes in NE in the MPA correlate very well with the changes in the serum levels of LH on proestrus (Mohankumar et al. 1994; Szawka et al. 2007). Administration of IL-1β suppressed this increase in NE in the MPA as well as other GnRH-related areas like the DBB, OVLT, SCN and the AN. The reduction in NE levels could be attributed to three possible reasons: 1. IL-1β could act on brainstem noradrenergic neurons to decrease NE biosynthesis, 2. It could act on NE terminals in the hypothalamus to affect release or 3. It could act indirectly through the stress axis to suppress LH secretion.

Evidence for the first possibility comes from different sources. Using tract tracing combined with immunohistochemical/electron microscopic evidence various studies have shown that the input to the MPA and other GnRH areas is provided by the brainstem areas – ventrolateral medulla (VLM; A1) and nucleus of solitary tract (NTS; A2) (Day et al. 1980; Jennes et al. 1983; Woulfe et al. 1990; Wright and Jennes 1993). NE synthesis involves conversion of dietary amino acid, L-tyrosine to L-dopa by tyrosine hydroxylase, which is the rate-limiting step (Nagatsu et al. 1964). Synthesis of this enzyme takes place in the brainstem areas and it is transported to the terminals to produce NE. If IL-1β affects this step in the biosynthesis, then it could result in a decrease in NE at the terminal. This possibility has been explored in the previous chapter where we have reversed the suppressive effect of IL-1β on the HPG axis by using L-dopa, a NE precursor which by-passes this rate limiting step. The other effect on brainstem

noradrenergic neurons could be targeted towards the synthesis of tyrosine hydroxylase itself. Direct effects of IL-1β on the expression of TH mRNA will be examined in detail in Chapter 6.

IL-1β could also act at the level of NE terminals to decrease NE levels. This is possible by inhibiting NE release by involvement of other inhibitory neurotransmitters. One such inhibitory neurotransmitter is GABA. The levels of GABA in the MPA have been shown to be in a reciprocal relationship with NE levels during the generation of LH surge on proestrus (Adler and Crowley 1986; Akema et al. 1990). When NE levels increase during the afternoon of proestrus, GABA levels are low. Moreover, there is evidence for direct synaptic connections between GABA interneurons with the noradrenergic neurons in the MPA suggesting a direct modulation of NE outflow by GABA (Horvath et al. 1992). Taken together, it is very much conceivable that the decrease in NE levels could be mediated by an increase in GABA levels.

Apart from this, the decrease in NE and suppression of LH could involve the participation of the HPA axis. CRH has been shown to have an inhibitory effect on LH secretion (Rivest and Rivier 1993b). Moreover, there is evidence for direct synaptic connections between CRH afferents and GnRH neurons in the MPA (MacLusky et al. 1988). The role of CRH in mediating IL-1β-induced suppression of LH has been inconclusive, however. Passive immunoneutralization of CRH or use of α-helical CRH failed to reverse the suppressive effect of IL-1β on LH (Bonavera et al. 1993b; Rivier and Rivest 1993). However, other groups have shown that administration of α-helical CRH was capable of reversing the inhibitory effect of IL-1β on LH (Maeda et al. 1994; Tsukamura et al. 1994; Tsukahara et al. 1999). Besides CRH, glucocorticoids can also be

involved in the suppression of HPG activity. In studies using LPS in a sheep model, there is evidence that glucocorticoids might be involved in mediating the activated HPA-induced inhibitory influence on LH (Tilbrook et al. 2000; Karsch et al. 2002). However, this contention too has been questioned by later studies in sheep as well as rat models (Debus et al. 2002; Watanobe and Habu 2003). Though one cannot discount the role of stress mediated suppression of HPG activity in the context of an immune challenge, further mechanistic studies are required to clarify this possibility.

In conclusion, systemic IL-1β decreased NE levels in GnRH-rich areas while it increased NE levels in CRH-related areas. These changes were well correlated with the decreased serum levels of luteinizing hormone and increased corticosterone supporting the view that noradrenergic input is a useful index of the state of HPG and HPA axes during short term activation. The novel finding that NE is differentially regulated by IL-1β in female rats on proestrous opens up exciting future research possibilities.

CHAPTER 6. NEUROENDOCRINE EFFECTS OF SYSTEMIC INTERLEUKIN-1β: ROLE OF BRAINSTEM NORADRENERGIC NUCLEI

INTRODUCTION:

The far ranging neuroendocrine effects of IL-1β have been discussed in the previous chapters. In the earlier experiments described in chapters 3-5, systemic administration of IL-1β was shown to affect the noradrenergic system and in turn suppress LH. We have shown that this suppression could be reversed at the hypothalamic, pituitary and ovarian levels by treatment with a noradrenergic precursor, L-dopa. This provides evidence that probably IL-1β produces its effects at the level of NE biosynthesis. Also in chapter 5, we have shown that the levels of NE in the GnRH-rich areas of hypothalamus were decreased by IL-1β. In the same study, we showed that NE levels in CRH-rich areas were increased upon treatment with IL-1β. These diametrically opposite effects could be a result of IL-1β's effects on the brainstem noradrenergic neurons that provide innervations to these hypothalamic areas. Retrograde tracing techniques have identified the role of A1 and A2 nuclei in providing noradrenergic input to the MPA as well as the PVN (Day et al. 1980; Sawchenko and Swanson 1982). Using double immunostaining and electron-microscopy, it has been demonstrated that GnRH neurons in the MPA are innervated predominantly by A1 and A2 noradrenergic nuclei. The functional changes in the expression of TH and *c-fos* in the brainstem noradrenergic nuclei during estradiol induced LH surge has also been demonstrated (Conde et al. 1995; Temel et al. 2002). Systemic IL-1β administration has also been shown to induce *c-fos* expression in A2, A6 nuclei of the brainstem (Brady et

al. 1994). Several studies have pointed to regulation of tyrosine hydroxylase at the level of gene expression by transcriptional control (Kilbourne et al. 1992; Nankova et al. 1994; Sabban et al. 1995; Serova et al. 1999). Also, the localization of IL-1-RI has been described in the brainstem (Yabuuchi et al. 1994). IL-1β has show to modulate its effects on the CNS by multiple pathways (Srinivasan et al. 2004). So we hypothesized that IL-1β could affect the expression patterns of TH mRNA in noradrenergic nuclei in the brainstem and this could involve changes in the molecules associated with IL-1β's signaling network.

EXPERIMENTAL SETUP:

The estrous cycle of the rats was monitored by daily vaginal cytology after a rest period of two weeks. The rats that showed regular estrous cycles were selected. On the day of proestrus, they were randomly subjected to one of the two treatments, Control (PBS-1.0% BSA), IL-1-β 5μg i.p at 1300 hrs. They were sacrificed at three time points: 1300, 1500 and 1700 hrs by rapid decapitation. The brainstem was removed and snap-frozen in liquid nitrogen and stored in −70°C. The brainstem noradrenergic nuclei were microdissected by Palkovits technique and RNA was extracted from those areas. Quantitative RT-PCR for TH, β-actin was performed for all the areas at each of the time points. The details of which are provided in the Methods Chapter. For the RT^2-PCR profiler array, the 1500 time point was chosen.

RESULTS:

Effect of IL-1β on TH mRNA in the brainstem noradrenergic nuclei:

The levels of TH mRNA (mean±SE) expressed as normalized ratio with respect to β-actin mRNA in control and IL-1 treated animals in the brainstem areas are shown in Fig 6A-C.

A1: In the control animals, the normalized levels of TH mRNA were 1.6±0.1 at 1pm and significantly decreased ($p<0.05$) to 1.1±0.1 at 3pm and 0.7±0.2 at 5pm. At each time point, these levels were not different from those in the IL-1β-treated animals. However, in the IL-1β-treated animals also, the levels of TH mRNA at 3pm and 5pm were significantly lower than those at 1pm ($p<0.05$).

A2: The levels of normalized TH mRNA in the control animals at 1pm were 1.6±0.25 and remained at similar levels at 3pm and 5pm. The levels in the IL-1β-treated group were 1.4±0.2 at 1pm and were at similar levels at 3pm. However, at 5pm, the levels decreased to 0.6±0.1, which was significantly lower ($p<0.05$) than the levels observed in control animals (1.1±0.2).

A6: In the control animals, the TH levels at 1pm were 1.64±0.38 and were at similar levels at 3pm and 5pm. In the IL-1β-treated group, the TH levels were 1.59±0.27 at 1pm and were not different from the levels at 3pm. The levels of TH at 5pm in the IL-1β-treated group were 1.87±0.26, which were significantly higher ($p<0.05$) than the levels in the control animals (1.12±0.28).

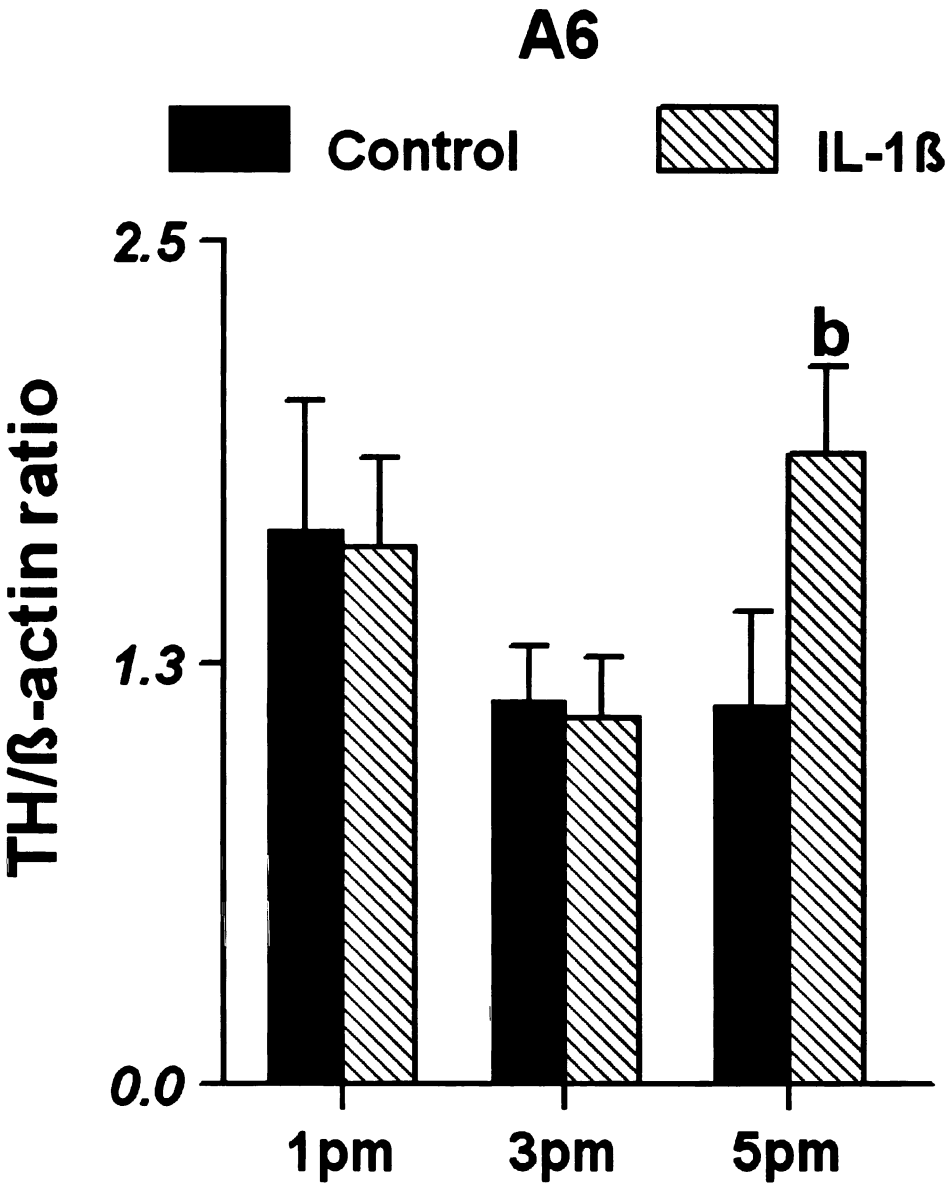

Figure 6-1C. TH mRNA expression in the noradrenergic A6 region in the brainstem (n=6-8 per group). The values were normalized to β-actin mRNA. Note the there were no differences in the expression of TH at 1pm and 3pm while levels increased in IL-1β treated rats compared to controls at 5pm. 'b' indicates difference ($p<0.05$) from levels in control animals.

Effect of IL-1β on expression of genes associated with IL-1β signaling in the brainstem noradrenergic nuclei:

A list of genes that were significantly up regulated or down regulated is given in Table 6A which was generated using the following criteria: significant difference between the two groups $p < 0.05$ (t test), and absolute value of fold change greater than two. Between the noradrenergic nuclei, there were expression changes in numerous genes and there was a degree of overlap too. We thus evaluated the spatial expression of 84 genes in the noradrenergic nuclei and out of which 32 (38%) showed significant up/down regulation in at least one of these areas. In A1 region, 21 (25%) genes showed significant fold changes of which 3 were down regulated and 18 were up regulated. In the A2 region, 25 (29%) genes displayed a significant fold change, of which 8 were down regulated and 17 were up regulated. In the A6 region, 20 (24%) genes showed significant fold changes and of which 6 were down regulated and 14 were up regulated. Among all three regions, there was 38% overlap in the fold change among all the three regions and about 50% overlap among each of the combinations of A1, A2 and A6.

One of the striking aspects in the expression profiling was the up regulation of the chemokines-CCL2 and CXCL10 in the noradrenergic nuclei. Compared to the expression in the controls, the fold changes in CCL2 were 40, 117 and 23 in A1, A2 and A6 respectively. The corresponding fold changes for CXCL10 were 32, 81 and 24 respectively. Among the cytokines, significant up regulation of IL-1β (2 fold) took place only in the A2 region. It is to be noted that the cognate receptor, IL-1RI is also up regulated (2 fold) in the same region but not in other regions. Another signaling molecule interleukin-1 receptor associated kinase-1 (IRAK-2) was up regulated (2 fold, 3 fold) in

the A1 and A6 regions. Between the two major pathways that IL-1β activates in the CNS, the members of the NF-κB pathway were up regulated while the members of the MAPK pathway were not affected or downregulated. As a general trend, other proinflammatory cytokines like IL-6, TNF-α and were upregulated. The other member of the TNF family, lymphotoxin-A (TNF-β) was up regulated (5 fold) in A1 region while it was down regulated in A6 region (3 fold). The expression of granulocyte-colony stimulating factor, G-CSF also was up regulated in all the brainstem areas by 7 fold, 21 fold and 7 fold respectively.

Among the members of the interferon family, IFN-β was up regulated (3 fold) in the A1 region while it was down regulated (3 fold) in the A2 region. IFN-γ was down regulated (4 fold) in the A2 region alone. A transcription factor, which serves as an activator of IFN-α and β, interferon regulatory factor-1 (IRF-1) was found to be up regulated (3, 6, 11 fold respectively) in all the noradrenergic areas. A classic anti-inflammatory cytokine, IL-10 was down regulated (4 fold, 3 fold respectively) in the A2 and A6 regions. IL-2 was also down regulated in all the noradrenergic areas.

COX-2 is a known downstream effector of IL-1 and it was up regulated in all the brainstem areas by 18, 12 and 15 folds respectively. c-fos, a marker of neuronal activation was also up regulated in all areas; though was statistically significant only in A1 and A2 (3 fold and 5 fold). The transcription factor-C/EBP-β was also up regulated in all the areas by 3-, 3-and 4-folds respectively.

Table 6-1: Effect of IL-1β on expression of genes associated with IL-1β signaling in the brainstem noradrenergic nuclei

Symbol	Description	A1	A2	A6
	RECEPTORS			
Cd14	CD14 antigen		2	
Clecsf9	Macrophage-inducible C-type lectin	4		
Cd180	CD180 antigen (predicted)		-3	-2
Tlr2	Toll-like receptor 2	-2		
Tlr5	Toll-like receptor 5		-3	
	DOWNSTREAM SIGNALING EFFECTORS			
Map3k1	Mitogen activated protein kinase kinase kinase 1		-2	
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105			2
Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	5	5	6
Nfkbib	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	4	4	
Ptgs2	Prostaglandin-endoperoxide synthase 2	18	12	15
Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	2	5	8
Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2		2	4
Ube2n	Ubiquitin-conjugating enzyme E2N	-4		

Table 6-1 continued

Symbol	Description	A1	A2	A6
	TRANSCRIPTION FACTORS			
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	3	3	4
Fos	FBJ murine osteosarcoma viral oncogene homolog	3	5	
	CHEMOKINE FAMILY			
Ccl2	Chemokine (C-C motif) ligand 2	40	117	23
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	2		-2
Csf3	Colony stimulating factor 3 (granulocyte)	7	21	7
Cxcl10	Chemokine (C-X-C motif) ligand 10	32	81	24
	CYTOKINE FAMILY			
Ifnb1	Interferon beta 1, fibroblast	3	-3	
Ifng	Interferon gamma		-4	
Il10	Interleukin 10		-4	-3
Il1a	Interleukin 1 alpha		-4	-3
Il1b	Interleukin 1 beta		2	
Il1r1	Interleukin 1 receptor, type I		2	
Il2	Interleukin 2	-2	-6	-3
Il6	Interleukin 6	3	6	2
Il6ra	Interleukin 6 receptor, alpha	3	3	3
Irak2	Interleukin-1 receptor-associated kinase 2	2		3
Irf1	Interferon regulatory factor 1	3	6	11
Lta	Lymphotoxin A	5		-3
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	4	3	2

DISCUSSION:

Using gene expression profiling we have provided an assessment of the expression of TH mRNA and the downstream signaling pathways of IL-1β in the noradrenergic brainstem nuclei along with in female rats on proestrus. Our analysis indicates that distinct pattern in the expression of TH mRNA exists between noradrenergic nuclei. We have also looked at differential expression between major cytokines, chemokines, downstream signaling effectors, transcription factors and receptors affected by IL-1β signaling pathway among these brainstem areas.

Results from our studies in previous chapters showed that the levels of NE decreased in the areas related to the HPG axis while it increased in areas related to HPA axis. Brainstem noradrenergic areas provide innervation to these hypothalamic areas involved in neuroendocrine regulation (Day et al. 1980; Sawchenko and Swanson 1982; Wright and Jennes 1993). During the various stages of estrous cycle, the levels of activation of brainstem neurons in A1 and A2 cell groups have been shown to be different. In the present study, no significant differences between the control and the IL-1β-treated rats were found in the A1 region at each of the time points. However, compared to the level of expression at 1pm, the levels at 3pm and 5pm were significantly lower on proestrus. In A2 region, there were no temporal or treatment differences at 1pm and 3pm, but a decrease in expression by IL-1β treatment at 5pm was observed. On a comparative basis, it was found that maximal activation of TH neurons in the A1 and A2 regions took place took place between 9-11am on proestrus (Conde et al. 1995) among all the stages of estrous cycle. Even in gonadectomized-steroid primed models, TH levels increased from 8am, 10 am before reaching a peak at 12 am then gradually decreasing

after that in the A1 region; while they kept increasing until 2pm in A2 region before declining (Curran-Rauhut and Petersen 2003). The expression changes in the control animals in the present study concur with these findings. However, one of the reasons why IL-1β might not have had any effect on TH mRNA levels could be the timing of the injection. By the time IL-1β was injected at 1pm, the kinetics of the TH transcription machinery responsible for inducing LH surge would have already started to take effect taking into account, the results of the studies described above. So the injected IL-1β could not have had the chance to alter the dynamics of TH expression at that point. Alternatively, it may be argued that IL-1β could be injected at an earlier time point to observe changes in TH expression if any. However, that involves a different experimental predicament. Pioneering work by Everett in the 50s introduced the concept of critical period in the regulation of LH (Everett et al. 1949; Everett and Sawyer 1949). With respect to studying the interaction between IL-1β and LH regulation, it was found that administration at 8.30 am or 2.30 pm had minimal effects on GnRH secretion compared to administration at 12 noon (Rivest and Rivier 1993c), emphasizing the role of timing of external influences in LH regulation. So simultaneously capturing the effects of systemic IL-1β on the dynamics of both TH and LH in proestrus rats might rather be an onerous experimental task. In A6 region, the expression of TH mRNA did not change in the controls, while it increased significantly 4 hrs after IL-1β administration. Even in other stress paradigms like immobilization or cold stress, it was shown that induction of TH mRNA and increase in TH activity in the A6 region was relatively less rapid compared to mediated by noradrenergic neurons might not be by transcriptionally regulated.

Expression profiling of the molecules involved in the IL-1β signaling pathway revealed certain general patterns. The expression of pro-inflammatory cytokines like IL-6, TNF was increased while the expression of anti-inflammatory cytokines like IL-10 was down regulated. One of the significant findings is that the expression of chemokines CCL2 and CXCL-10 were increased by numerous folds along with other chemoattractants like GM-CSF. The expression of transcription factor C/EBP beta was also upregulated. Among the two major signaling pathways that IL-1β employs, there was a preferential induction of the NF-κB pathway over the MAPK pathway.

The expression of immediate early gene, c-fos was increased in all the noradrenergic areas, though significantly in A1 and A2 which is in agreement with previous findings (Brady et al. 1994). Also, another downstream modulator COX-2 was upregulated in all the noradrenergic areas 2 hrs after IL-1β treatment. IL-1β is known to induce expression of the enzyme COX-2 in various parts of the brain, which is involved in the synthesis of prostaglandins(Cao et al. 1997; Cao et al. 2001). Prostaglandins have been the inflammatory intermediates produced by IL-1β in many instances (Cao et al. 1997; Engblom et al. 2002; Nie et al. 2003; Turrin and Rivest 2004). Effects of IL-1β like the pyrogenic response and the activation of HPA axis have been shown to be mediated by prostaglandins.(Scammell et al. 1998; Cao et al. 2001; Engblom et al. 2002; Zhang et al. 2003). Some of the effects of IL-1β at the brain are blocked by using prostaglandin inhibitors (Scammell et al. 1998). PGE_2 has been shown to be involved IL-1α mediated suppression of LH secretion in an in vitro model (Rettori et al. 1991).However it is not known if the actions of IL-1β on NE system are also mediated by prostaglandins. The findings of the present study provide allow us to speculate that possibility.

Chemokines are small secreted proteins whose function in the periphery was initially confined to chemoattraction and activation of the immune system until recent discoveries into their involvement in central nervous inflammatory pathologies (Bajetto et al. 2001; Callewaere et al. 2007). In the present study, within 2 hrs after IL-1β treatment, there was a dramatic upregulation of chemokine transcripts-CCL-2 and CXCL-10 in all the noradrenergic nuclei. This is the first study to demonstrate such novel changes in the chemokine profile at the level of brainstem noradrenergic areas in intact female rats during a systemic immune challenge.

Though few, there are studies to demonstrate the involvement of central chemokines in stress induced neuroendocrine alterations. In a transcriptome analysis of the paraventricular nucleus after peripheral LPS administration, it was observed that levels of CCL-2 and CXCL-10 were upregulated by 3-5 fold by 1hr and 20-40 fold, 3 hrs following the immune challenge (Reyes et al. 2003). In another study, systemic administration of IL-1β induced robust expression of CCL-2 in the circumventricular organs and the choroid plexus (Thibeault et al. 2001). In the same lines as their roles in the periphery, it was considered that they would serve to recruit lymphocytes and monocytes at the barrier-related areas. In other studies, cytokine induced increase in CCL-2 and CXCL-10 in the components of the blood brain barrier was demonstrated which was associated with increased leukocytic infiltration (Boztug et al. 2002; Harkness et al. 2003; Shaftel et al. 2007). However, considering the relatively slow time of leukocyte infiltration, it was unlikely that acute HPA responses observed by systemic immune challenge could be explained by this action of chemokines alone. In response to immune stimuli, chemokines are shown to be produced not only by vascular elements but

by components of the CNS like astrocytes also (Weiss et al. 1998; Oh et al. 1999), microglia (Ambrosini and Aloisi 2004) and some chemokines, by even neurons (Harrison et al. 1998). Recently, the possibility has been raised that they might act as a novel class of neurotransmitters (Rostene et al. 2007). In their recent review arguing the case for chemokines as neurotransmitters, Rostene et al., provide evidence for all the criteria put forth previously (Rotsztejn et al. 1981) for a substance to be considered as a neurotransmitter. Chemokines are localized in nerve terminal vesicles, are colocalized with other neurotransmitter systems, are released after membrane depolarization, have electrophysiological effects and do have pre- and postsynaptic receptors. All the members of the chemokine family may not satisfy all the above mentioned criteria, but definitely as a class, chemokines, do fulfill these criteria. CCL2 has been shown to be involved in regulation of other neuroendocrine functions like osmotic balance and feeding (Plata-Salaman and Borkoski 1994; Banisadr et al. 2005). Considering the results of the present study and the other studies which involved stress-induced upregulation of these chemokines, it is possible that they might serve as neurotransmitters to modulate neuroendocrine functions.

It has been shown that IL-1β is capable of specific activation of several downstream pathways which include MAPK pathway and NF-κB pathways (Srinivasan et al. 2004; Subramaniam et al. 2004; Miggin and O'Neill 2006). NF-κB production has been shown to be a key marker of IL-1β signaling in the CNS and its blockade has shown to reduce sickness behavior induced by IL-1β (Nadjar et al. 2003; Nadjar et al. 2005). In the present study, there was a selective upregulation of the genes associated with the NF-κB signaling pathway while genes associated with MAPK signaling were either

unaffected or downregulated. In a neurogenic stress paradigm like immobilization, it was shown that signaling molecules like p38, JNK1/2/3 and ERK-1/2 associated with the MAPK pathway were selectively upregulated in the A6 region (Hebert et al. 2005). This provides novel insight into the differences in downstream transcriptional mechanisms involved in transducing stress responses at the level of brainstem on the basis of whether the stressor is metabolic or neurogenic.

In the present study, we found several changes in the expression of members of cytokine family after treatment with IL-1β. This could be a direct effect or mediated by the activation of transcription factors or downstream signaling molecules. The mRNA levels of proinflammatory cytokines like IL-1β, IL-6, TNF-α, TNF-β were increased while those of anti-inflammatory molecules like IL-10 were decreased. Proinflammatory cytokines have been associated with activation of the HPA axis and suppression of the HPG axis (Rivest and Rivier 1993b; Karsch et al. 2002). Surprisingly, IL-2 which is known to activate the HPA axis (Hanisch and Quirion 1995) is downregulated in the present study; the reasons for which are not clear. IL-10 has been shown to be involved in abrogating the neuroinflammatory effects of LPS and it confers neuroprotection in models of LPS-induced neurotoxicity (Lynch et al. 2004; Qian et al. 2006). The observed decrease in anti-inflammatory cytokine expression could be one of the strategies by which IL-1β produces its neuroendocrine effects.

Systemic administration of IL-1β has been shown to induce it own expression as well as induce other proinflammatory cytokines like IL-6 and TNF-α (Churchill et al. 2006). In the present study, the genes for colony stimulating factors GCSF and GM-CSF were also upregulated. Apart from their properties of chemoattraction, they have also

been shown to be involved in production of proinflammatory cytokines like IL-1 and IL-6 by brain microglia either by direct administration or in neuroinflammatory conditions (Murphy et al. 1998; Fischer and Reichmann 2001). In the present study the expression of transcription factor- C/EBP-β increased several fold in the noradrenergic nuclei upon stimulation by IL-1β. Another isoform, C/EBP-δ has been shown to be upregulated in the PVN following LPS treatment (Reyes et al. 2003). In astrocytes and microglia, LPS is capable of specifically upregulating levels of C/EBP-β (Cardinaux et al. 2000; Ejarque-Ortiz et al. 2007). CEBP-β binding motifs have been described in the promoters of various inflammatory cytokines including IL-1β, IL-6, TNF, GM-CSF and other genes associated with inflammation like COX-2 and iNOS (reviewed in (Poli 1998)). So it is possible that IL-1β induced cytokine expression might involve modulation by transcription factors like C/EBP or CSFs or they might work in a feed-forward system to enhance the production of proinflammatory cytokines in the brainstem during a systemic immune challenge.

Our quest to dissect out the individual roles of these nuclei in explaining the several neuroendocrine alterations produced by IL-1β has not been very successful due to the overlap in the signaling molecules and expected redundancy in molecular pathways. Nevertheless, this study has provided novel insights into understanding molecular pathways activated during a systemic immune challenge at the level of brainstem and how this might serve to integrate neuroendocrine functions.

CHAPTER 7. SUMMARY AND CONCLUSIONS

. Stress and reproduction seem to be at loggerheads, almost always. As an adaptation to stress, higher mammals have evolved mechanisms to budget their limited energy resources intelligently. At this decision branch point between survival and procreation, the logical strategy appears to persist with pro-survival strategies and shut down reproduction which is the oft taken route (Moberg 1991; Cameron 1997). During activation of stress, the pathways leading to successful reproduction are often turned off. Beneficial though it may appear, sometimes this supposedly transient shutting off of reproduction can have undesirable and often permanent changes in the reproductive axis. It can also result in the organism's impaired ability to regain its full potential to reproduce even after the threat of stress has been staved off.

During systemic infections, a complex interaction takes place between immune, endocrine and the nervous systems. IL-1β, a paradigm of immune stress is capable of mimicking these changes when administered systemically (Licinio and Frost 2000; Dinarello 2001; Quan and Banks 2007). It is one of the important mediators of the central effects of LPS, a hallmark product of bacterial infections (Watanobe and Hayakawa 2003). The effects of IL-1β on reproductive axis include reduction in LH, anovulation, persistent corpora lutea and pseudo-pregnant like state. Elevated levels of IL-1β have been reported in serum and peritoneal fluids of women with fertility problems (Akoum et al. 2007; Kalu et al. 2007). Apart from this, several stressor paradigms like immobilization have been shown to increase hypothalamic levels of IL-1β (Minami et al., 1991; Shintani et al., 1995). So not only in disease conditions, but in other stressful

conditions also, elevations in IL-1 can compromise reproductive function. This aspect is not limited to human beings but can be applied to breeding of wild animals in captivity that is constantly challenged by various stressors. Elevated stress levels have been reported in some captive species (Baker et al., 1998; Terio et al., 2004) and reproductive failure in some species has been attributed to lower LH and disrupted ovarian cyclicity (Faulkes et al., 1990). Hence it is important to understand the effect of IL-1 on the reproductive axis. The results of the present study have not only provided insights into the systems level, but into the intricate molecular crosstalk between the various components of the neuroimmune machinery.

IL-1β can cross the BBB through saturable transport mechanisms or can also enter through the circum ventricular organs or through a neurally mediated route (Banks et al. 1989; Plotkin et al. 1996; Goehler et al. 1999). After intravenous injection of IL-1, it has been estimated that 0.08% of that dose would reach the brain by using kinetics of iodinated-IL-1(Banks and Kastin 1991), based on which we estimate that 4 ng of the injected 5μg IL-1β might have reached the brain. We also measured the serum levels of IL-1β after i.p injection and found that they significantly increased at the measured 2hrs and 4hrs post injection. The levels of IL-1β in the treatment group (0.7-2.4 ng/ml) were comparable to the levels (~1.2-1.6 ng/ml) found in previous studies which measured serum IL-1β levels during acute endotoxemia and adjuvant-induced arthritis (Saito et al. 2003; Barsante et al. 2005). So the model we used in series of experiments in this study truly mimics an inflammatory process.

It was first demonstrated that IL-1β was capable of decreasing LH secretion in male rats (Rivier and Vale 1989). Subsequently studies in female rats showed that IL-1β

could block LH surge and inhibit ovulation also (Rivier and Vale 1990). Continuous infusions of IL-1β for 4-6 days can also disrupt estrus cyclicity, decrease LHRH mRNA levels and suppress LH resulting in pseudopregnant-like state in rats with numerous corporea lutea (Rivest et al. 1993; Rivier and Erickson 1993). The mechanism by which IL-1β modulates these effects on the HPG axis has been an open question. Numerous neurotransmitters like opioids, neuropeptides, CRH, prostaglandins have emerged as candidate molecules for transducing this effect. However, the role of NE, one of the important stimulatory inputs on GnRH neurons has not been clearly investigated.

A large body of elegant experiments (reviewed in (Barraclough and Wise 1984; Barraclough 1994; Herbison 1997; Pau and Spies 1997) do point out an important role for catecholamines in the regulation of GnRH and LH. The regulation of GnRH neurons in the MPA involves a complex interplay between stimulatory neurotransmitters like NE and inhibitory ones like GABA. NE dynamics include the changes in the release and reuptake patterns in the axon terminals as well as the changes in the biosynthesis at the level of cell bodies. The cell bodies of the NE neurons that project to the MPA are located in the brainstem. We have shown that peripheral administration of IL-1β drastically disrupts rhythmic release of NE in the MPA and thereby blocks LH surge in steroid-primed ovariectomized rat model (MohanKumar and MohanKumar 2002). IL-1β could affect locally at the MPA or rather more globally at the level of brainstem. However the questions of how IL-1β interacts with this complex network of neurotransmitters and would GABA come into this equation remains unexplored. The logical extension of this idea would be to explore how this decrease in NE is brought

about by IL-1β and at what level of the noradrenergic system does IL-1β act to produce these effects.

For the first set of studies, we hypothesized that IL-1β could affect the HPG axis by affecting TH activity and we attempted to reverse this effect using a NE precursor, L-dopa. Extending the previous studies in which IL-1β suppressed LH surge in ovariectomized-steroid primed model, we used intact cycling rats in this experiment. In the control animals, the LH levels gradually increased from 1pm and reached peak concentrations at 5pm and declined thereafter. This was tightly correlated with the rhythmic increase in NE levels in the MPA from 1pm which reached highest levels at 4.30 pm and decreased thereafter. In sharp contrast to this profile, in the group treated with IL-1β, LH levels remained unchanged from 1300h and NE levels did not rise indicating that systemic IL-1β blocks NE to suppress LH surge. Since GnRH neurons have been shown to contain adrenergic receptors, as a mechanistic extension of the above finding, we treated animals with adrenergic agonists in combination with IL-1β to examine if they could reverse the suppressive effect of IL-1β. However, the NE agonists failed to block the inhibitory effect of IL-1β on LH surge. Counter intuitive it may sound; this could be due to several possible reasons. In the present study, a single bolus injection of the agonist was administered which would not have simulated the endogenous pattern of NE release. In previous studies, administration of NE itself as a bolus was found to block LH secretion without simultaneous electrochemical stimulation (Cramer and Barraclough 1978). In phenobarbital-blocked LH models and in male rats also, NE agonists failed to reverse the blockade (Al-Hamood et al. 1985; Koh et al. 1985). This underlines the importance of rhythmic release and not single or continuous dosage for LH

surge to take place. For instance, in monkeys with hypothalamic lesions continuous administration of synthetic GnRH failed to restore LH release (Belchetz et al. 1978). It could be argued that NE agonists could be administered in a pulsatile manner simulating the endogenous pattern of NE release. However, titration of the dose of the agonist and the periodicity with simultaneous measurements of the hormonal profile in the same rat would not only be extremely impractical but would also be so difficult to be consistently repeatable. To circumvent this experimental predicament, we used a NE precursor, L-dopa which would relieve the block on NE synthesis if any and not probably restore the endogenous NE pattern.

L-dopa, on the other hand was able to partially reverse the effect of IL-1β on NE levels in the MPA and the LH surge. L-dopa is the product of the enzymatic action of tyrosine hydroxylase (TH) on L-tyrosine. This also happens to be the rate limiting step in NE biosynthesis (Nagatsu 1995) and therefore TH is critically involved in LH regulation. TH activity increases significantly in the MPA during the afternoon of proestrus along with the LH surge (Mohankumar et al. 1997b). This suggests the possibility that IL-1β can decrease TH activity to suppress the LH surge. To test this, we used L-dopa to by-pass TH and provided the substrate required for NE biosynthesis. L-dopa crosses the blood-brain barrier easily and has been used to reverse acyclicity and increase LH levels in old animals that have low hypothalamic NE levels (Huang et al. 1976; Forman et al. 1980). The results from the present study indicate that administration of L-dopa along with IL-1β can indeed partially reverse the IL-1β-induced suppression of the LH surge. This was accompanied by a partial but significant restoration of NE levels in the MPA. The partial rather than complete reversal suggests that IL-1β may affect enzymes

downstream of TH such as dopa decarboxylase or dopamine β-hydroxylase that are involved in NE synthesis. This needs further investigation.

Further support for L-dopa's ability to reverse the effect of IL-1β on the HPG axis comes from the histological examination of the ovary. Ovaries of animals treated with IL-1β alone had less numbers of fresh CL compared to the rest of the groups suggesting suppressed ovulation. In contrast, control rats and the rats treated with L-dopa alone or IL-1β+L-dopa had larger numbers of freshly formed CL suggesting that ovulation had occurred in these groups. Moreover, the numbers of old CL were higher in the IL-1β treated animals. This supports an earlier study that observed retention of CL with chronic IL-1β treatment (Rivier and Erickson 1993). A previous study reported failure of ovulation after central administration of IL-1β (Rivier and Vale 1990). Results from the present study indicate a failure of ovulation in IL-1β-treated rats. In contrast, control rats had larger numbers of freshly formed CL suggesting that ovulation had occurred in this group. Moreover, the numbers of old CL were higher in the IL-1β treated animals suggesting lack of luteolysis. Results from the present study also indicate that L-dopa was effective in reversing IL-1β's actions on the ovary. Since L-dopa also reversed the effects of IL-1β on hypothalamic NE and plasma LH levels, it is possible that the effects observed on the ovary are mediated through LH rather than through a direct action on the ovary. Since the minimum levels of LH required for successful ovulation are only a fraction of the peak levels (Gosden *et al.* 1976), it is possible that the modest increase in the LH levels produced by L-dopa was sufficient to cause ovulation in IL-1β treated animals.

In this first series of experiments we have provided evidence for the potential for noradrenergic precursor, L-dopa to reverse the inhibitory effect of IL-1β. This could be an action at the level of NE terminals at the MPA. At the level of the MPA, apart from NE, another prominent input to the GnRH comes from GABA neurons. Moreover, GABA has been shown to have a reciprocal relationship with NE in the MPA. During proestrus surge in female rats, paralleling the increase in the levels of NE, the levels of GABA are found to decrease in the MPA (Demling et al. 1985; Akema et al. 1990). Taking these into consideration, we intended to investigate the role of GABA in mediating the suppressive effect of IL-1β on LH and how GABA interacts with NE at the level of MPA and how this interaction affects IL-1β's effects on LH in the second set of studies.

In this study we used an ovariectomized steroid primed model to investigate the role of GABA in IL-1β mediated suppression of HPG axis. This model has been well characterized and has advantages of consistent reproducibility and low variability. In control animals, the levels of GABA in the MPA did not vary significantly from 1pm while in the IL-1β treated group, the levels increased significantly. Conversely, the levels of NE in the same control animals in the MPA increased rhythmically paralleling the LH surge while in IL-1β-treated rats there was a decrease in NE and LH levels. This provides evidence that there exists a strong inverse relationship between levels of GABA and NE in the MPA during LH surge and positive relationship between NE and serum levels. Numerous studies point to the existence of a reciprocal relationship between GABA and NE (Mansky et al. 1982; Ondo et al. 1982; Demling et al. 1985). Also, direct synaptic connections between NE terminals and GABA interneurons have been described

(Horvath et al. 1992). Release rates of GABA are low in the MPA while NE and LH levels are high, and when LH levels are low in diestrus, levels of GABA were found to be high (Mitsushima et al. 2002). Though the evidence in the present study is only correlative, this provides the basis for further experimental characterization of the role of the interaction between GABA and NE. IL-1β could independently act on both GABAergic and catecholaminergic neurons to produce its effects on LH. Or there could be an interaction between these two systems to modulate IL-1β's effects on LH. To mechanistically test such possibility we extended the above mentioned idea by using GABA antagonists. We hypothesized that if such an interaction does exist, GABA antagonists might be capable of increasing NE levels in the MPA and counter the inhibitory effect of IL-1β on LH. The results of our studies discussed in Chapter 4 show that bicuculline, a GABA-A receptor when administered along with IL-1β was able to block the effect of IL-1β on NE levels in the MPA and serum LH. No such effect was observed upon administration of CGP-35348, a GABA-B antagonist suggesting that IL-1β's modulation of the GABAergic system to influence the HPG axis is probably mediated though GABA-A receptors. Several other studies provide substantial evidence for the preponderance of GABA-A receptor over GABA-B receptor in mediating LH secretion. Direct administration of GABA-A agonist, Muscimol, was demonstrated to rapidly reduce LH release and decrease the expression of GnRH in the hypothalamus (Leonhardt et al. 1995) while the GABA-B agonist, Baclofen, did not have such effect. Activation of GABA-A receptors is shown to be inhibitory to GnRH electrical activity (Kimura et al. 1993) while GABA-B receptor activation inhibits mRNA expression and peptide synthesis of GnRH (Bergen et al. 1991). Taken together, these findings suggest

that systemic IL-1β inhibits HPG activity by interaction between GABA and NE at the MPA and this is accomplished most probably by a GABA-A receptor mediated mechanism.

IL-1β has been shown to activate the HPA axis by increasing c-fos immunoreactivity in corticotrophin releasing hormone (CRH) neurons, release of CRH from the paraventricular nucleus (PVN) effecting an increase in circulating glucocorticoids (Besedovsky et al. 1986; Berkenbosch et al. 1987; Sapolsky et al. 1987; Brady et al. 1994). Our previous studies indicate that systemic administration of IL-1β activates the HPA axis by increasing the noradrenergic stimulatory input to the CRH neurocircuitry in the PVN (MohanKumar and Quadri 1993; MohanKumar and MohanKumar 2005). As described in the series of studies in Chapters 3, 4 we show that the suppressive effects of IL-1β on the HPG axis are also modulated by NE. It is interesting to note that the effects of IL-1β on both the HPA axis as well as the HPG axis have been mediated via NE. The simultaneous effects of IL-1β on both the HPA and the HPG axes in the same animal and the mechanisms involved in precipitating these changes have not been studied so far. We chose to measure the concentrations of NE in specific brain areas from rats sacrificed at three time points on proestrus. This would provide a spatial and temporal analysis of the changes in NE concentrations in various brain areas associated with the HPA and the HPG axes.

The findings in Chapter 5 show here that NE mediates the effects of systemic IL-1β on both the HPG and the HPA axes. More interestingly, as a general trend, there was a suppression of noradrenergic activity in the HPG axis related areas while NE levels were increased in the HPA related areas. This study provides the first evidence for the

involvement of NE in the opposing effects produced by IL-1β on the two axes. This leads us to conclude that NE could be one of the key mediators by which a systemic immune challenge produces diametrically opposite effects: stimulatory effects on the HPA axis and inhibitory effects on the HPG axis.

Our earlier studies as well as experiments in previous chapters have shown that systemic IL-1β is capable of inhibiting the HPG axis by decreasing NE levels in the MPA (MohanKumar and MohanKumar 2002).However GnRH neurons that are scattered over a number of regions in the hypothalamus have been thought to be involved in the generation of LH surge as well (Smith and Jennes 2001). Specifically those include the OVLT, the DBB, the preopticosuprachiasmatic tuberoinfundibular system (PSTS) with perikarya in the MPA, SCN, AN and terminals in the median eminence (Barraclough and Wise 1984; Hiatt et al. 1992; Herbison 2006). Simultaneous changes in all these neuronal populations in the face of an immune challenge, over a period of time have not been measured before.

In the present study, we measured NE levels in all these areas after an intraperitoneal injection of IL-1β. The levels of NE increased in the DBB and the MPA at 3pm, which seem to correlate with the increase in LH at 5pm. This increase in NE is in agreement with previous studies which have shown that changes in NE in the MPA correlate very well with the changes in the serum levels of LH on proestrus (Mohankumar et al. 1994; Szawka et al. 2007). Administration of IL-1β suppressed this increase in NE in the MPA as well as other GnRH-related areas like the DBB, OVLT, SCN and the AN. The reduction in NE levels could be attributed to three possible reasons: 1.IL-1β could act on brainstem noradrenergic neurons to decrease NE

biosynthesis. 2. It could act on NE terminals in the hypothalamus to affect release. 3. Since IL-1β activates the stress axis; the products of this activation can in turn act to suppress the HPG axis.

In the present study, the serum levels of corticosterone increased within 2 hrs and remained higher than the controls until 4 hrs post injection of IL-1β. Corresponding to this increase in corticosterone, there was an increase in the levels of NE in the PVN also by almost two folds. This suggests that in female rats also, systemic immune challenge is capable of activating the stress axis activity with the involvement of NE. Also, CRH has been shown to have an inhibitory effect on LH secretion (Rivest and Rivier 1993b). Moreover, there is evidence for direct synaptic connections between CRH afferents and GnRH neurons in the MPA (MacLusky et al. 1988). The role of CRH in mediating IL-1β-induced suppression of LH has been inconclusive, however. Passive immunoneutralization of CRH or use of α-helical CRH failed to reverse the suppressive effect of IL-1β on LH (Bonavera et al. 1993b; Rivier and Rivest 1993). However, other groups have shown that administration of α-helical CRH was capable of reversing the inhibitory effect of IL-1β on LH (Maeda et al. 1994; Tsukamura et al. 1994; Tsukahara et al. 1999). Besides CRH, glucocorticoids can also be involved in the suppression of HPG activity. In studies using LPS in a sheep model, there is evidence that glucocorticoids might be involved in mediating the activated HPA-induced inhibitory influence on LH (Tilbrook et al. 2000; Karsch et al. 2002). However, this contention too has been questioned by later studies in sheep as well as rat models (Debus et al. 2002; Watanobe and Habu 2003). Though one cannot discount the role of stress mediated suppression of

HPG activity in the context of an immune challenge, further mechanistic studies are required to clarify this possibility.

IL-1β could also act at the level of NE terminals to decrease NE levels. This is possible by inhibiting NE release by involvement of other inhibitory neurotransmitters. One such inhibitory neurotransmitter is GABA. The interaction between GABA and NE has been examined in detail in Chapter 4.

Noradrenergic input to the MPA and other GnRH areas is provided by the brainstem areas – ventrolateral medulla (VLM; A1) and nucleus of solitary tract (NTS; A2) (Day et al. 1980; Jennes et al. 1983; Woulfe et al. 1990; Wright and Jennes 1993). NE synthesis involves conversion of dietary amino acid, L-tyrosine to L-dopa by tyrosine hydroxylase, which is the rate-limiting step (Nagatsu et al. 1964). Synthesis of this enzyme takes place in the brainstem areas and it is transported to the terminals to produce NE. If IL-1β affects this step in the biosynthesis, then it could result in a decrease in NE at the terminal. This possibility has been explored in the previous chapter where we have reversed the suppressive effect of IL-1β on the HPG axis by using L-dopa, a NE precursor which by-passes this rate limiting step. The other effect on brainstem noradrenergic neurons could be targeted towards the synthesis of tyrosine hydroxylase itself. Direct effects of IL-1β on the expression of TH mRNA were examined in detail in Chapter 6.

Apart from the activation of hypothalamic components of the CRH network, several other non-hypothalamic CRH rich areas like the central amygdala (CeA), bed nucleus of the stria terminalis (BNST) and the locus coeruleus could be involved in mediating the HPA responses to stress (Koob 1999). Similarly, systemic IL-1β

administration also results in activation of the CeA during the course of mounting a HPA response (Brady et al. 1994; Ericsson et al. 1997; Day et al. 1999; Xu et al. 1999). Studies involving lesions of the CeA suggested that it coordinates HPA responses between PVN the brainstem (Xu et al. 1999). NE has been shown to have a stimulatory input on the CRH neuronal system of the CeA (Raber et al. 1995). Also, BNST plays an important role in the regulation of the HPA responses during stress and it is one of the principal extra-hypothalamic relay centers to the PVN from the CeA and the brainstem (Forray and Gysling 2004). So we chose to investigate the changes in NE concentrations in these areas during systemic IL-1β administration. In the present study, we have shown that NE levels in the CeA increase at 2hrs and return to pre-treatment levels at 4hrs post injection. In contrast, NE levels in the BNST increased at 2hrs and remained elevated at 4hrs post injection. Since central CeA is the origin of afferent CRH-pathways to the brainstem, BNST and other hypothalamic areas also (Gray 1993; Gray and Bingaman 1996), this transient activation of CeA might play a role in integrating HPA responses between brainstem nuclei which in turn provide input to various hypothalamic areas.

Previous studies have noted an exaggerated HPA response during systemic immune challenge compared to their male counter parts, which was negated upon gonadectomy (Frederic et al. 1993, Rivier 1994, Spinedi et al. 1992). The prolonged HPA response observed in the present study could be a result of such sexually dimorphic gonadal steroid-dependent response. During the various stages of the estrous cycle, the HPA responses to stress were more pronounced on proestrus compared to estrus or diestrus. Also, in ovariectomized rats, administration of estrogen and progesterone accentuated the HPA responses compared to oil-treated controls indicating the role of

gonadal steroids in mediating these responses (Viau & Meaney 1991). Since the present studies were conducted in female rats on proestrus, it is possible that the hormonal profile during this stage might have influenced the outcome of the HPA response.

Though NE is stimulatory to both HPA and HPG axis activities, it is interesting to note that systemic IL-1β selectively increased NE levels in HPA related areas while it decreased NE levels in HPG related areas. The noradrenergic innervation to these areas in the hypothalamus is provided from the brain stem regions A1 and A2 and to some extent A6 (Day et al. 1980; Sawchenko and Swanson 1982). Brainstem neurons have shown to have differential responses to systemic IL-1β. By selective neurotoxic lesioning, it was shown that A1 and A2 participate in IL-1β mediated increases in PVN while A2 additionally is involved in the recruitment of CeA neurons (Buller *et al.* 2001). It is possible that the differential effects of IL-1β on NE profile in these areas are a result of differential expression of TH at these brainstem noradrenergic areas. So we investigated the effects of systemic IL-1β on the brain stem expression of TH mRNA.

In the present study, no significant differences between the control and the IL-1β-treated rats were found in the A1 region at each of the time points. However, compared to the level of expression at 1pm, the levels at 3pm and 5pm were significantly lower on proestrus. In A2 region, there were no temporal or treatment differences at 1pm and 3pm, but a decrease in expression by IL-1β treatment at 5pm was observed. On a comparative basis, it was found that maximal activation of TH neurons in the A1 and A2 regions took place took place between 9-11am on proestrus (Conde et al. 1995) among all the stages of estrous cycle. Even in gonadectomized-steroid primed models, TH levels increased from 8am, 10 am before reaching a peak at 12 am then gradually decreasing after that in the

A1 region; while they kept increasing until 2pm in A2 region before declining (Curran-Rauhut and Petersen 2003). The expression changes in the control animals in the present study concur with these findings. However, one of the reasons why IL-1β might not have had any effect on TH mRNA levels could be the timing of the injection. By the time IL-1β was injected at 1pm, the kinetics of the TH transcription machinery responsible for inducing LH surge would have already started to take effect taking into account, the results of the studies described above. So the injected IL-1β could not have had the chance to alter the dynamics of TH expression at that point. Alternatively, it may be argued that IL-1β could be injected at an earlier time point to observe changes in TH expression if any. However, that involves a different experimental predicament. Pioneering work by Everett in the 50s introduced the concept of critical period in the regulation of LH (Everett et al. 1949; Everett and Sawyer 1949). With respect to studying the interaction between IL-1β and LH regulation, it was found that administration at 8.30 am or 2.30 pm had minimal effects on GnRH secretion compared to administration at 12 noon (Rivest and Rivier 1993c), emphasizing the role of timing of external influences in LH regulation. So simultaneously capturing the effects of systemic IL-1β on the dynamics of both TH and LH in proestrus rats might rather be an onerous experimental task. In A6 region, the expression of TH mRNA did not change in the controls, while it increased significantly 4 hrs after IL-1β administration. Even in other stress paradigms like immobilization or cold stress, it was shown that induction of TH mRNA and increase in TH activity in the A6 region was relatively less rapid suggesting that the effects of IL-1β on TH at the noradrenergic neurons might not be by transcriptionally regulated.

The expression of immediate early gene, c-fos was increased in all the noradrenergic areas, though significantly in A1 and A2 which is in agreement with previous findings (Brady et al. 1994). Also, another downstream modulator COX-2 was upregulated in all the noradrenergic areas 2 hrs after IL-1β treatment. IL-1β is known to induce expression of the enzyme COX-2 in various parts of the brain, which is involved in the synthesis of prostaglandins(Cao et al. 1997; Cao et al. 2001). Prostaglandins have been the inflammatory intermediates produced by IL-1β in many instances (Cao et al. 1997; Engblom et al. 2002; Nie et al. 2003; Turrin and Rivest 2004). However it is not known if the actions of IL-1β on NE system are also mediated by prostaglandins. The findings of the present study provide allow us to speculate that possibility.

In the present study, within 2 hrs after IL-1β treatment, there was a dramatic upregulation of chemokine transcripts-CCL-2 and CXCL-10 in all the noradrenergic nuclei. This is the first study to demonstrate such novel changes in the chemokine profile at the level of brainstem noradrenergic areas in intact female rats during a systemic immune challenge. Considering the results of the present study and the other studies which involved stress-induced upregulation of these chemokines (Thibeault et al. 2001; Reyes et al. 2003), it is possible that they might serve as neurotransmitters to modulate neuroendocrine functions.

Systemic administration of IL-1β has been shown to induce it own expression as well as induce other proinflammatory cytokines like IL-6 and TNF-α (Churchill et al. 2006). In the present study, the genes for colony stimulating factors GCSF and GM-CSF were also upregulated. Apart from their properties of chemoattraction, they have also been shown to be involved in production of proinflammatory cytokines like IL-1 and IL-

6 by brain microglia either by direct administration or in neuroinflammatory conditions (Murphy et al. 1998; Fischer and Reichmann 2001). In the present study the expression of transcription factor- C/EBP-β increased several fold in the noradrenergic nuclei upon stimulation by IL-1β. Another isoform, C/EBP-δ has been shown to be upregulated in the PVN following LPS treatment (Reyes et al. 2003). In astrocytes and microglia, LPS is capable of specifically upregulating levels of C/EBP-β (Cardinaux et al. 2000; Ejarque-Ortiz et al. 2007). CEBP-β binding motifs have been described in the promoters of various inflammatory cytokines including IL-1β, IL-6, TNF, GM-CSF and other genes associated with inflammation like COX-2 and iNOS (reviewed in (Poli 1998)). So it is possible that IL-1β induced cytokine expression might involve modulation by transcription factors like C/EBP or CSFs or they might work in a feed-forward system to enhance the production of proinflammatory cytokines in the brainstem during a systemic immune challenge.

How do the present findings extend to the context of generalized stress-induced suppression of LH secretion is an interesting point to contemplate. The present study used systemic administration of IL-1β as a paradigm for immune stress and studied its effects on the HPG axis. There are other studies which point to the role of NE and GABA in mediating the effects of other kinds of stressors. Suppression of LH surge following food deprivation has been shown to involve catecholaminergic input to the PVN and central CRH mechanisms are shown to induce LH suppression (Maeda et al. 1994). Further, this effect is shown to involve alpha adrenergic receptor in the PVN (Tsukamura et al. 1994). The source of NE input to the PVN during fasting induced stress has been shown to be emerging from brainstem A2 regions and this effect is estrogen dependent (Nagatani *et*

al. 1994). Another metabolic stress paradigm commonly employed is glucoprivation or lipoprivation and both these strategies have been shown to suppress LH (Schneider 2004). Acute glucoprivation increases NE in the PVN and blocking this increase by targeted immunolesioning was able to prevent suppression of LH (Nagatani et al. 1996; I'Anson et al. 2003). Similar findings were obtained in the other paradigm, lipoprivation too (Sajapitak *et al.* 2008). Apart from NE, glucoprivation is known to increase central GABA levels and this effect suppresses reproductive function (Singh et al. 2004) and this inhibition is shown to involve central GABA-A, but not GABA-B receptors (Singh & Briski 2005).

The findings of the present study exemplify the effects of a metabolic stressor on the HPG regulation. The response against the stressor seems to differ based on the nature of the stressor – whether it is metabolic or neurogenic. Direct injection of CRH into the noradrenergic A6 region induced GAD 67 neuronal activation in the MPA with subsequent suppression of LH. In the same study, administration of alpha-helical CRF(9-41) blocked restraint stress-induced suppression of LH pulses, without affecting the inhibitory response to hypoglycemia (Mitchell *et al.* 2005). In a neurogenic stress paradigm, like acute restraint, GABA antagonists were ineffective in reversing the inhibition on LH (Roozendaal et al. 1997) suggesting that the role of GABA receptors is exclusive to homeostatic stress paradigms. In the present study, there was a selective upregulation of the genes associated with the NF-κB signaling pathway while genes associated with MAPK signaling were either unaffected or downregulated. In a neurogenic stress paradigm like immobilization, it was shown that signaling molecules like p38, JNK1/2/3 and ERK-1/2 associated with the MAPK pathway were selectively

upregulated in the A6 region (Hebert et al. 2005). This provides novel insight into the differences in downstream transcriptional mechanisms involved in transducing stress responses at the level of brainstem on the basis of whether the stressor is metabolic or neurogenic.

In conclusion, we show that the effects of systemic IL-1β on the reproductive axis takes place at numerous levels. It can act at the level of brainstem or at the hypothalamus or directly on the ovary. At the brainstem, it can cause an increase in chemokines and downstream effectors of IL-1β and this inturn can influence the levels of NE in the hypothalamus. At the hypothalamus, IL-1β increases NE in the CRH areas while it decreases NE in the GnRH areas. This decrease in NE in the GnRH areas can cause suppression of LH and block ovulation. Numerous factors could contribute to this decrease in NE which include increase in GABA, increase in corticosterone or increased CRH. It is important to understand the complex interactions at the neuro-immune interface to device possible strategies to counter the undesirable suppressive effects of stress on reproduction.

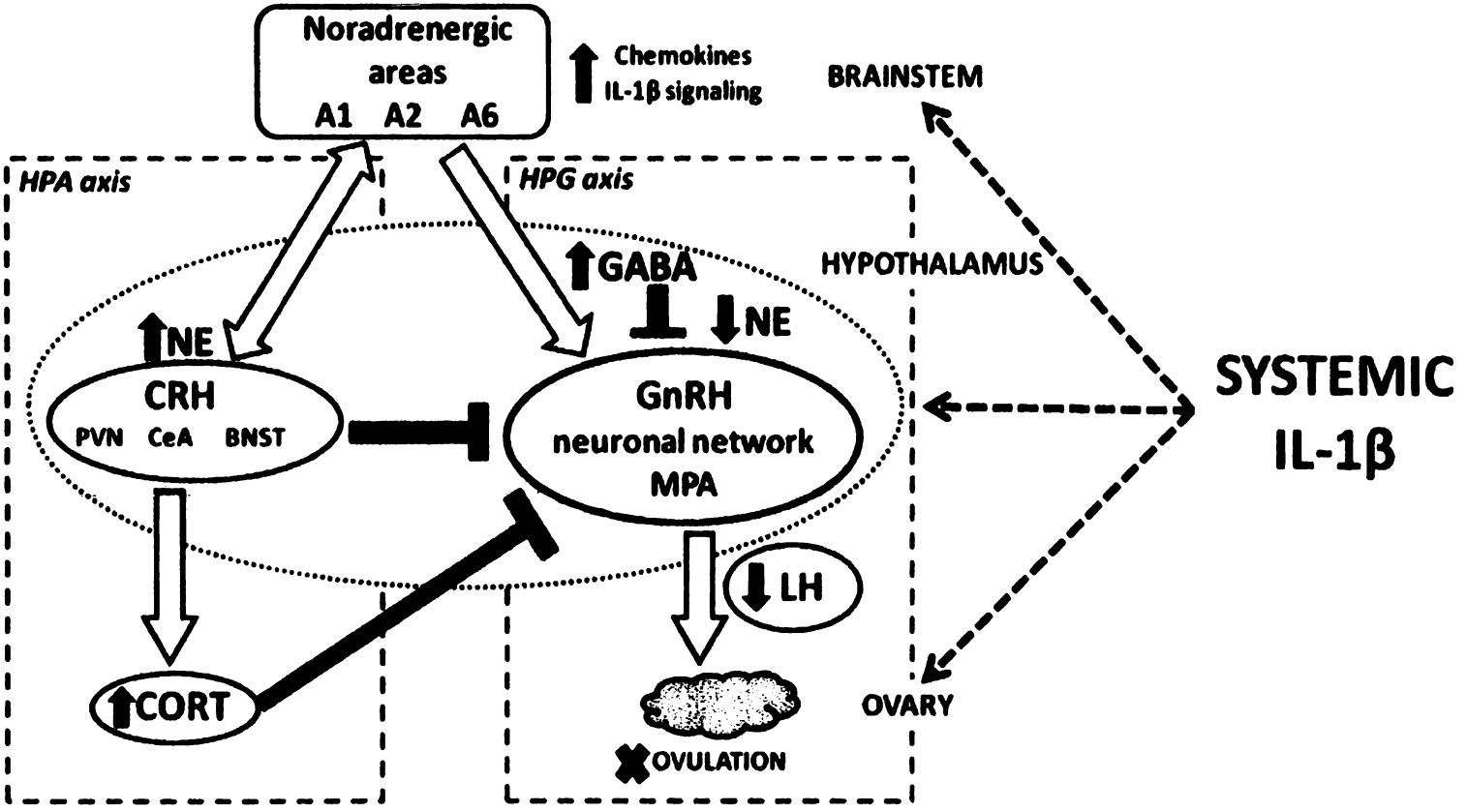

Fig 7-1. Overview of the mechanisms by which systemic IL-1β suppresses HPG axis. An immune stressor, IL-1β can act at the level of brainstem or hypothalamus or directly at the ovary to affect HPG function. At the brainstem, it can cause an increase in chemokines and downstream effectors of IL-1β and this inturn can influence the levels of NE in the hypothalamus. At the hypothalamus, IL-1β increases NE in the CRH areas while it decreases NE in the GnRH areas. This decrease in NE in the GnRH areas can cause suppression of LH and block ovulation. Numerous factors could contribute to this decrease in NE which include increase in GABA, increase in corticosterone or increased CRH.

REFERENCES

Adler B. A. and Crowley W. R. (1986) Evidence for gamma-aminobutyric acid modulation of ovarian hormonal effects on luteinizing hormone secretion and hypothalamic catecholamine activity in the female rat. *Endocrinology* **118,** 91-97.

Ajika K. (1979) Simultaneous localization of LHRH and catecholamines in rat hypothalamus. *Journal of anatomy* **128,** 331-347.

Akema T., Chiba A. and Kimura F. (1990) On the relationship between noradrenergic stimulatory and GABAergic inhibitory systems in the control of luteinizing hormone secretion in female rats. *Neuroendocrinology* **52,** 566-572.

Akema T., He D. and Sugiyama H. (2005) Lipopolysaccharide increases gamma-aminobutyric acid synthesis in medial preoptic neurones in association with inhibition of steroid-induced luteinising hormone surge in female rats. *Journal of neuroendocrinology* **17,** 672-678.

Akira S. and Takeda K. (2004) Toll-like receptor signalling. *Nature reviews* **4,** 499-511.

Akoum A., Al-Akoum M., Lemay A., Maheux R. and Leboeuf M. (2007) Imbalance in the peritoneal levels of interleukin 1 and its decoy inhibitory receptor type II in endometriosis women with infertility and pelvic pain. *Fertility and sterility.*

Al-Hamood M. H., Gilmore D. P. and Wilson C. A. (1985) Evidence for a stimulatory beta-adrenergic component in the release of the ovulatory LH surge in pro-oestrous rats. *The Journal of endocrinology* **106,** 143-151.

Ambrosini E. and Aloisi F. (2004) Chemokines and glial cells: a complex network in the central nervous system. *Neurochemical research* **29,** 1017-1038.

Anselmo-Franci J. A., Franci C. R., Krulich L., Antunes-Rodrigues J. and McCann S. M. (1997) Locus coeruleus lesions decrease norepinephrine input into the medial preoptic area and medial basal hypothalamus and block the LH, FSH and prolactin preovulatory surge. *Brain research* **767,** 289-296.

Avitsur R., Cohen E. and Yirmiya R. (1997) Effects of interleukin-1 on sexual attractivity in a model of sickness behavior. *Physiology & behavior* **63,** 25-30.

Bajetto A., Bonavia R., Barbero S., Florio T. and Schettini G. (2001) Chemokines and their receptors in the central nervous system. *Frontiers in neuroendocrinology* **22,** 147-184.

Banihashemi L. and Rinaman L. (2006) Noradrenergic inputs to the bed nucleus of the stria terminalis and paraventricular nucleus of the hypothalamus underlie hypothalamic-pituitary-adrenal axis but not hypophagic or conditioned avoidance responses to systemic yohimbine. *J Neurosci* **26,** 11442-11453.

Banisadr G., Gosselin R. D., Mechighel P., Kitabgi P., Rostene W. and Parsadaniantz S. M. (2005) Highly regionalized neuronal expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) in rat brain: evidence for its colocalization with neurotransmitters and neuropeptides. *The Journal of comparative neurology* **489,** 275-292.

Banks W. A. and Kastin A. J. (1991) Blood to brain transport of interleukin links the immune and central nervous systems. *Life sciences* **48,** PL117-121.

Banks W. A., Kastin A. J. and Durham D. A. (1989) Bidirectional transport of interleukin-1 alpha across the blood-brain barrier. *Brain research bulletin* **23,** 433-437.

Banks W. A., Ortiz L., Plotkin S. R. and Kastin A. J. (1991) Human interleukin (IL) 1 alpha, murine IL-1 alpha and murine IL-1 beta are transported from blood to brain in the mouse by a shared saturable mechanism. *The Journal of pharmacology and experimental therapeutics* **259,** 988-996.

Barbanel G., Ixart G., Szafarczyk A., Malaval F. and Assenmacher I. (1990) Intrahypothalamic infusion of interleukin-1 beta increases the release of corticotropin-releasing hormone (CRH 41) and adrenocorticotropic hormone (ACTH) in free-moving rats bearing a push-pull cannula in the median eminence. *Brain research* **516,** 31-36.

Barraclough C. A. (1983) The role of catecholamines in the regulation of gonadotropin secretion. *Acta morphologica Hungarica* **31,** 101-115.

Barraclough C. A. (1992) Neural control of the synthesis and release of luteinizing hormone-releasing hormone. *Ciba Foundation symposium* **168,** 233-246; discussion 246-251.

Barraclough C. A. (1994) Neurotransmitter regulation of luteinizing hormone-releasing hormone neuronal function. *Acta Biol Hung* **45,** 189-206.

Barraclough C. A. and Sawyer C. H. (1957) Blockade of the release of pituitary ovulating hormone in the rat by chlorpromazine and reserpine: possible mechanisms of action. *Endocrinology* **61,** 341-351.

Barraclough C. A. and Wise P. M. (1982) The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. *Endocrine reviews* **3,** 91-119.

Barraclough C. A. and Wise P. M. (1984) The role of brain catecholamines in the regulation of pituitary luteinizing hormone and follicle stimulating hormone secretion. *Endocrine reviews* **3,** 91-119.

Barsante M. M., Roffe E., Yokoro C. M., Tafuri W. L., Souza D. G., Pinho V., Castro M. S. and Teixeira M. M. (2005) Anti-inflammatory and analgesic effects of atorvastatin in a rat model of adjuvant-induced arthritis. *European journal of pharmacology* **516,** 282-289.

Bartolomucci A., Palanza P., Parmigiani S., Pederzani T., Merlot E., Neveu P. J. and Dantzer R. (2003) Chronic psychosocial stress down-regulates central cytokines mRNA. *Brain research bulletin* **62,** 173-178.

Beishuizen A. and Thijs L. G. (2003) Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *Journal of endotoxin research* **9,** 3-24.

Belchetz P. E., Plant T. M., Nakai Y., Keogh E. J. and Knobil E. (1978) Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. *Science (New York, N.Y* **202,** 631-633.

Bergen H. T., Hejtmancik J. F. and Pfaff D. W. (1991) Effects of gamma-aminobutyric acid receptor agonists and antagonist on LHRH-synthesizing neurons as detected by immunocytochemistry and in situ hybridization. *Experimental brain research. Experimentelle Hirnforschung* **87,** 46-56.

Berkenbosch F., Vanoers J., Delrey A., Tilders F. and Besedovsky H. (1987) Corticotropin-Releasing Factor Producing Neurons In The Rat Activated By Interleukin-1. *Science (New York, N.Y* **238,** 524-526.

Besedovsky H., del Rey A., Sorkin E. and Dinarello C. A. (1986) Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science (New York, N.Y* **233,** 652-654.

Besedovsky H. O. and del Rey A. (1996) Immune-neuro-endocrine interactions: facts and hypotheses. *Endocrine reviews* **17,** 64-102.

Blamire A. M., Anthony D. C., Rajagopalan B., Sibson N. R., Perry V. H. and Styles P. (2000) Interleukin-1beta -induced changes in blood-brain barrier permeability, apparent diffusion coefficient, and cerebral blood volume in the rat brain: a magnetic resonance study. *J Neurosci* **20,** 8153-8159.

Bluthe R. M., Walter V., Parnet P., Laye S., Lestage J., Verrier D., Poole S., Stenning B. E., Kelley K. W. and Dantzer R. (1994) Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. *Comptes rendus de l'Academie des sciences* **317,** 499-503.

Bonavera J. J., Kalra S. P. and Kalra P. S. (1993a) Evidence That Luteinizing-Hormone Suppression In Response To Inhibitory Neuropeptides, Beta-Endorphin, Interleukin-1-Beta, And Neuropeptide-K, May Involve Excitatory Amino-Acids. *Endocrinology* **133,** 178-182.

Bonavera J. J., Kalra S. P. and Kalra P. S. (1993b) Mode of action of interleukin-1 in suppression of pituitary LH release in castrated male rats. *Brain research* **612,** 1-8.

Boraschi D. and Tagliabue A. (2006) The interleukin-1 receptor family. *Vitamins and hormones* **74,** 229-254.

Boztug K., Carson M. J., Pham-Mitchell N., Asensio V. C., DeMartino J. and Campbell I. L. (2002) Leukocyte infiltration, but not neurodegeneration, in the CNS of transgenic mice with astrocyte production of the CXC chemokine ligand 10. *J Immunol* **169,** 1505-1515.

Brady L. S., Lynn A. B., Herkenham M. and Gottesfeld Z. (1994) Systemic interleukin-1 induces early and late patterns of c-fos mRNA expression in brain. *J Neurosci* **14,** 4951-4964.

Brambilla D., Franciosi S., Opp M. R. and Imeri L. (2007) Interleukin-1 inhibits firing of serotonergic neurons in the dorsal raphe nucleus and enhances GABAergic inhibitory post-synaptic potentials. *The European journal of neuroscience* **26,** 1862-1869.

Brann D. W. and Mahesh V. B. (1991) Detailed examination of the mechanism and site of action of progesterone and corticosteroids in the regulation of gonadotropin secretion: hypothalamic gonadotropin-releasing hormone and catecholamine involvement. *Biology of reproduction* **44,** 1005-1015.

Breder C. D., Dinarello C. A. and Saper C. B. (1988) Interleukin-1 immunoreactive innervation of the human hypothalamus. *Science (New York, N.Y* **240,** 321-324.

Brown-Grant K., Exley D. and Naftolin F. (1970) Peripheral plasma oestradiol and luteinizing hormone concentrations during the oestrous cycle of the rat. *The Journal of endocrinology* **48,** 295-296.

Buchanan D. L., Kurita T., Taylor J. A., Lubahn D. B., Cunha G. R. and Cooke P. S. (1998) Role of stromal and epithelial estrogen receptors in vaginal epithelial proliferation, stratification, and cornification. *Endocrinology* **139,** 4345-4352.

Buller K., Xu Y., Dayas C. and Day T. (2001) Dorsal and ventral medullary catecholamine cell groups contribute differentially to systemic interleukin-1beta-induced hypothalamic pituitary adrenal axis responses. *Neuroendocrinology* **73,** 129-138.

Bulun S. and Adashi E. (2008) The physiology and Pathology of the female reproductive axis, in *Williams Textbook of Endocrinology* Vol. 11th Edition (Kronenberg HM M., S, Polonsky, KS, and Larsen, PR, ed.). WB Saunders Philadelphia.

Butcher R. L., Collins W. E. and Fugo N. W. (1974) Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. *Endocrinology* **94,** 1704-1708.

Callewaere C., Banisadr G., Rostene W. and Parsadaniantz S. M. (2007) Chemokines and chemokine receptors in the brain: implication in neuroendocrine regulation. *Journal of molecular endocrinology* **38,** 355-363.

Cameron J. L. (1997) Stress and behaviorally induced reproductive dysfunction in primates. *Semin Reprod Endocrinol* **15,** 37-45.

Campbell R. E. and Herbison A. E. (2007) Definition of brainstem afferents to gonadotropin-releasing hormone neurons in the mouse using conditional viral tract tracing. *Endocrinology* **148,** 5884-5890.

Cannon J. G. (1998) Adaptive interactions between cytokines and the hypothalamic-pituitary-gonadal axis. *Annals of the New York Academy of Sciences* **856,** 234-242.

Cao C., Matsumura K. and Watanabe Y. (1997) Induction of cyclooxygenase-2 in the brain by cytokines. *Ann N Y Acad Sci* **813,** 307-309.

Cao C., Matsumura K., Shirakawa N., Maeda M., Jikihara I., Kobayashi S. and Watanabe Y. (2001) Pyrogenic cytokines injected into the rat cerebral ventricle induce cyclooxygenase-2 in brain endothelial cells and also upregulate their receptors. *Eur J Neurosci* **13,** 1781-1790.

Cardinaux J. R., Allaman I. and Magistretti P. J. (2000) Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. *Glia* **29,** 91-97.

Cecchi M., Khoshbouei H., Javors M. and Morilak D. A. (2002) Modulatory effects of norepinephrine in the lateral bed nucleus of the stria terminalis on behavioral and neuroendocrine responses to acute stress. *Neuroscience* **112,** 13-21.

Champagne D., Beaulieu J. and Drolet G. (1998) CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. *Journal of neuroendocrinology* **10,** 119-131.

Chuluyan H. E., Saphier D., Rohn W. M. and Dunn A. J. (1992) Noradrenergic innervation of the hypothalamus participates in adrenocortical responses to interleukin-1. *Neuroendocrinology* **56,** 106-111.

Churchill L., Taishi P., Wang M., Brandt J., Cearley C., Rehman A. and Krueger J. M. (2006) Brain distribution of cytokine mRNA induced by systemic administration of interleukin-1beta or tumor necrosis factor alpha. *Brain research* **1120,** 64-73.

Civantos Calzada B. and Aleixandre de Artinano A. (2001) Alpha-adrenoceptor subtypes. *Pharmacol Res* **44,** 195-208.

Clark K. A., Shin A. C., Sirivelu M. P., Mohankumar S. M. and Mohankumar P. S. (2008) Systemic administration of leptin decreases plasma corticosterone levels: Role of hypothalamic norepinephrine. *Brain research* **1195,** 89-95.

Clarke I. J., Scott C. J., Pereira A. and Pompolo S. (2006) The role of noradrenaline in the generation of the preovulatory LH surge in the ewe. *Domestic animal endocrinology* **30,** 260-275.

Clifton D. K. and Sawyer C. H. (1979) LH release and ovulation in the rat following depletion of hypothalamic norepinephrine: chronic vs. acute effects. *Neuroendocrinology* **28,** 442-449.

Coen C. W. and Coombs M. C. (1983) Effects of manipulating catecholamines on the incidence of the preovulatory surge of of luteinizing hormone and ovulation in the rat: evidence for a necessary involvement of hypothalamic adrenaline in the normal or 'midnight' surge. *Neuroscience* **10,** 187-206.

Colotta F., Re F., Muzio M., Bertini R., Polentarutti N., Sironi M., Giri J. G., Dower S. K., Sims J. E. and Mantovani A. (1993) Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science (New York, N.Y* **261,** 472-475.

Conde G. L., Bicknell R. J. and Herbison A. E. (1995) Changing patterns of Fos expression in brainstem catecholaminergic neurons during the rat oestrous cycle. *Brain research* **672,** 68-76.

Coppola J. A., Leonardi R. G. and Lippmann W. (1966) Ovulatory failure in rats after treatment with brain norepinephrine depletors. *Endocrinology* **78,** 225-228.

Cramer O. M. and Barraclough C. A. (1978) The actions of serotonin, norepinephrine, and epinephrine on hypothalamic processes leading to adenohypophyseal luteinizing hormone release. *Endocrinology* **103,** 694-703.

Curran-Rauhut M. A. and Petersen S. L. (2003) Oestradiol-dependent and -independent modulation of tyrosine hydroxylase mRNA levels in subpopulations of A1 and A2 neurones with oestrogen receptor (ER)alpha and ER beta gene expression. *Journal of neuroendocrinology* **15,** 296-303.

Da Silva J. A., Peers S. H., Perretti M. and Willoughby D. A. (1993) Sex steroids affect glucocorticoid response to chronic inflammation and to interleukin-1. *The Journal of endocrinology* **136,** 389-397.

Dauffenbach L. M., Khan S. M. and Yeh J. (2003) Corpus luteum regression in the rat--in vivo and in vitro studies of apoptotic mechanisms. *Journal of medicine* **34,** 87-100.

Day H. E., Curran E. J., Watson S. J., Jr. and Akil H. (1999) Distinct neurochemical populations in the rat central nucleus of the amygdala and bed nucleus of the stria terminalis: evidence for their selective activation by interleukin-1beta. *The Journal of comparative neurology* **413,** 113-128.

Day T. A., Blessing W. and Willoughby J. O. (1980) Noradrenergic and dopaminergic projections to the medial preoptic area of the rat. A combined horseradish peroxidase/catecholamine fluorescence study. *Brain research* **193,** 543-548.

Deak T., Bordner K. A., McElderry N. K., Barnum C. J., Blandino P., Jr., Deak M. M. and Tammariello S. P. (2005) Stress-induced increases in hypothalamic IL-1: a systematic analysis of multiple stressor paradigms. *Brain research bulletin* **64,** 541-556.

Debus N., Breen K. M., Barrell G. K., Billings H. J., Brown M., Young E. A. and Karsch F. J. (2002) Does cortisol mediate endotoxin-induced inhibition of pulsatile luteinizing hormone and gonadotropin-releasing hormone secretion? *Endocrinology* **143,** 3748-3758.

Demling J., Fuchs E., Baumert M. and Wuttke W. (1985) Preoptic catecholamine, GABA, and glutamate release in ovariectomized and ovariectomized estrogen-primed rats utilizing a push-pull cannula technique. *Neuroendocrinology* **41,** 212-218.

Dinarello C. A. (1994) The interleukin-1 family: 10 years of discovery. *Faseb J* **8,** 1314-1325.

Dinarello C. A. (1996) Biologic basis for interleukin-1 in disease. *Blood* **87,** 2095-2147.

Dinarello C. A. (2001) IL-1 beta, in *Cytokine Reference*, Vol. I: Ligands (Oppenheim J. and Feldman M., eds). Academic Press, San Fransisco.

Dobson H., Ghuman S., Prabhakar S. and Smith R. (2003) A conceptual model of the influence of stress on female reproduction. *Reproduction (Cambridge, England)* **125,** 151-163.

Donoso A. O., Bishop W., Fawcett C. P., Krulich L. and McCann S. M. (1971) Effects of drugs that modify brain monoamine concentrations on plasma gonadotropin and prolactin levels in the rat. *Endocrinology* **89,** 774-784.

Donoso A. O., Seltzer A. M., Navarro C. E., Cabrera R. J., Lopez F. J. and Negro-Vilar A. (1994) Regulation of luteinizing hormone-releasing hormone and luteinizing hormone

secretion by hypothalamic amino acids. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al* **27,** 921-932.

Drouva S. V., Laplante E. and Kordon C. (1982) alpha 1-adrenergic receptor involvement in the LH surge in ovariectomized estrogen-primed rats. *European journal of pharmacology* **81,** 341-344.

Ebisui O., Fukata J., Tominaga T., Murakami N., Kobayashi H., Segawa H., Muro S., Naito Y., Nakai Y., Masui Y. and et al. (1992) Roles of interleukin-1 alpha and -1 beta in endotoxin-induced suppression of plasma gonadotropin levels in rats. *Endocrinology* **130,** 3307-3313.

Eisenberg S. P., Brewer M. T., Verderber E., Heimdal P., Brandhuber B. J. and Thompson R. C. (1991) Interleukin 1 receptor antagonist is a member of the interleukin 1 gene family: evolution of a cytokine control mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 5232-5236.

Ejarque-Ortiz A., Medina M. G., Tusell J. M., Perez-Gonzalez A. P., Serratosa J. and Saura J. (2007) Upregulation of CCAAT/enhancer binding protein beta in activated astrocytes and microglia. *Glia* **55,** 178-188.

Engblom D., Ek M., Saha S., Ericsson-Dahlstrand A., Jakobsson P. J. and Blomqvist A. (2002) Prostaglandins as inflammatory messengers across the blood-brain barrier. *J Mol Med* **80,** 5-15.

Ericsson A., Arias C. and Sawchenko P. E. (1997) Evidence for an intramedullary prostaglandin-dependent mechanism in the activation of stress-related neuroendocrine circuitry by intravenous interleukin-1. *J Neurosci* **17,** 7166-7179.

Ericsson A., Liu C., Hart R. P. and Sawchenko P. E. (1995) Type 1 interleukin-1 receptor in the rat brain: distribution, regulation, and relationship to sites of IL-1-induced cellular activation. *The Journal of comparative neurology* **361,** 681-698.

Everett J. W. (1989) *Neurobiology of Reproduction in the Female Rat*, Vol. 32, p 133. Springer-Verlag, Berlin Heidelberg.

Everett J. W. and Sawyer C. H. (1949) A neural timing factor in the mechanism by which progesterone advances ovulation in the cyclic rat. *Endocrinology* **45,** 581-595, illust.

Everett J. W., Markee J. E. and Hollinshead W. H. (1949) A neurogenic timing factor in control of ovulatory discharge of luteinizing hormone in the cycling rat. Endocrinology. *Endocrinology* **44,** 234-243.

Favit A., Wetsel W. C. and Negro-Vilar A. (1993) Differential expression of gamma-aminobutyric acid receptors in immortalized luteinizing hormone-releasing hormone neurons. *Endocrinology* **133,** 1983-1989.

Feleder C., Wuttke W. and Moguilevsky J. A. (1999a) Effects of the GABA-A receptor agonist and antagonist on the in vitro release of hypothalamic catecholamines: apparent parallelism between these effects and the LHRH secretion in adult male rats. *Exp Clin Endocrinol Diabetes* **107,** 80-84.

Feleder C., Refojo D., Jarry H., Wuttke W. and Moguilevsky J. A. (1996) Bacterial endotoxin inhibits LHRH secretion following the increased release of hypothalamic GABA levels. Different effects on amino acid neurotransmitter release. *Neuroimmunomodulation* **3,** 342-351.

Feleder C., Ginzburg M., Wuttke W., Moguilevsky J. A. and Arias P. (1999b) GABAergic activation inhibits the hypothalamic-pituitary-ovaric axis and sexual development in the immature female rat. Associated changes in hypothalamic glutamatergic and taurinergic systems. *Brain Res Dev Brain Res* **116,** 151-157.

Fernandez-Galaz C., Dyer R. G. and Herbison A. E. (1994) Analysis of brainstem A1 and A2 noradrenergic inputs to the preoptic area using microdialysis in the rat. *Brain research* **636,** 227-232.

Fischer H. G. and Reichmann G. (2001) Brain dendritic cells and macrophages/microglia in central nervous system inflammation. *J Immunol* **166,** 2717-2726.

Forman L. J., Sonntag W. E., Miki N. and Meites J. (1980) Maintenance by L-DOPA treatment of estrous cycles and LH response to estrogen in aging female rats. *Exp Aging Res* **6,** 547-554.

Forray M. I. and Gysling K. (2004) Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis. *Brain Res Brain Res Rev* **47,** 145-160.

France E. S. (1970) Reversal by pargyline of reserpine block of induced ovulation--direct ovarian effects. *Neuroendocrinology* **6,** 77-89.

Franci J. A. and Antunes-Rodrigues J. (1985) Effect of locus ceruleus lesion on luteinizing hormone secretion under different experimental conditions. *Neuroendocrinology* **41,** 44-51.

Francis J., MohanKumar S. M. and MohanKumar P. S. (2000) Correlations of norepinephrine release in the paraventricular nucleus with plasma corticosterone and leptin after systemic lipopolysaccharide: blockade by soluble IL-1 receptor. *Brain research* **867,** 180-187.

Frederic F., Oliver C., Wollman E., Delhaye-Bouchaud N. and Mariani J. (1993) IL-1 and LPS induce a sexually dimorphic response of the hypothalamo-pituitary-adrenal axis in several mouse strains. *European cytokine network* **4,** 321-329.

Freeman M. E. (2004) Neuroendocrine control of the ovarian cycle in rat, in *Knobil and Neill's Physiology of Reproduction*, Vol. 2 (Neill J., ed.). Academic Press, San Diego.

Fuchs E., Mansky T., Stock K. W., Vijayan E. and Wuttke W. (1984) Involvement of catecholamines and glutamate in GABAergic mechanism regulatory to luteinizing hormone and prolactin secretion. *Neuroendocrinology* **38,** 484-489.

Gaillard R. C. and Spinedi E. (1998) Sex- and stress-steroids interactions and the immune system: evidence for a neuroendocrine-immunological sexual dimorphism. *Domestic animal endocrinology* **15,** 345-352.

Galand P., Leroy F. and Chretien J. (1971) Effect of oestradiol on cell proliferation and histological changes in the uterus and vagina of mice. *The Journal of endocrinology* **49,** 243-252.

Gallo R. V. (1980) Neuroendocrine regulation of pulsatile luteinizing hormone release in the rat. *Neuroendocrinology* **30,** 122-131.

Gallo R. V. (1984) Further studies on norepinephrine-induced suppression of pulsatile luteinizing hormone release in ovariectomized rats. *Neuroendocrinology* **39,** 120-125.

Gallo R. V. and Drouva S. V. (1979) Effect of intraventricular infusion of catecholamines on luteinizing hormone release in ovariectomized and ovariectomized, steroid-primed rats. *Neuroendocrinology* **29,** 149-162.

Gay N. J. and Keith F. J. (1991) Drosophila Toll and IL-1 receptor. *Nature* **351,** 355-356.

Gay V. L., Midgley A. R., Jr. and Niswender G. D. (1970) Patterns of gonadotrophin secretion associated with ovulation. *Federation proceedings* **29,** 1880-1887.

Gaykema R. P., Goehler L. E., Hansen M. K., Maier S. F. and Watkins L. R. (2000) Subdiaphragmatic vagotomy blocks interleukin-1beta-induced fever but does not reduce IL-1beta levels in the circulation. *Auton Neurosci* **85,** 72-77.

Gayle D., Ilyin S. E. and Plata-Salaman C. R. (1997) Interleukin-1 receptor type I mRNA levels in brain regions from male and female rats. *Brain research bulletin* **42,** 463-467.

Gearing M. and Terasawa E. (1991) The alpha-1-adrenergic neuronal system is involved in the pulsatile release of luteinizing hormone-releasing hormone in the ovariectomized female rhesus monkey. *Neuroendocrinology* **53,** 373-381.

Goehler L. E., Relton J. K., Dripps D., Kiechle R., Tartaglia N., Maier S. F. and Watkins L. R. (1997) Vagal paraganglia bind biotinylated interleukin-1 receptor antagonist: a possible mechanism for immune-to-brain communication. *Brain research bulletin* **43,** 357-364.

Goehler L. E., Gaykema R. P., Nguyen K. T., Lee J. E., Tilders F. J., Maier S. F. and Watkins L. R. (1999) Interleukin-1beta in immune cells of the abdominal vagus nerve: a link between the immune and nervous systems? *J Neurosci* **19,** 2799-2806.

Goldman J. M., Murr A. S. and Cooper R. L. (2007) The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth defects research* **80,** 84-97.

Goldman J. M., Stoker T. E., Cooper R. L., McElroy W. K. and Hein J. F. (1994) Blockade of ovulation in the rat by the fungicide sodium N-methyldithiocarbamate: relationship between effects on the luteinizing hormone surge and alterations in hypothalamic catecholamines. *Neurotoxicol Teratol* **16,** 257-268.

Gosden R. G., Everett J. W. and Tyrey L. (1976) Luteinizing hormone requirements for ovulation in the pentobarbital-treated proestrous rat. *Endocrinology* **99,** 1046-1053.

Gray T. S. (1993) Amygdaloid CRF pathways. Role in autonomic, neuroendocrine, and behavioral responses to stress. *Annals of the New York Academy of Sciences* **697,** 53-60.

Gray T. S. and Bingaman E. W. (1996) The amygdala: corticotropin-releasing factor, steroids, and stress. *Critical reviews in neurobiology* **10,** 155-168.

Han S. K., Todman M. G. and Herbison A. E. (2004) Endogenous GABA release inhibits the firing of adult gonadotropin-releasing hormone neurons. *Endocrinology* **145,** 495-499.

Hancke J. L. and Wuttke W. (1979) Effects of chemical lesion of the ventral noradrenergic bundle or the medial preoptic area on preovulatory LH release in rats. *Experimental brain research. Experimentelle Hirnforschung* **35,** 127-134.

Hanisch U. K. and Quirion R. (1995) Interleukin-2 as a neuroregulatory cytokine. *Brain Res Brain Res Rev* **21,** 246-284.

Hansen M. K., Daniels S., Goehler L. E., Gaykema R. P., Maier S. F. and Watkins L. R. (2000) Subdiaphragmatic vagotomy does not block intraperitoneal lipopolysaccharide-induced fever. *Auton Neurosci* **85,** 83-87.

Harkness K. A., Sussman J. D., Davies-Jones G. A., Greenwood J. and Woodroofe M. N. (2003) Cytokine regulation of MCP-1 expression in brain and retinal microvascular endothelial cells. *Journal of neuroimmunology* **142,** 1-9.

Harrison J. K., Jiang Y., Chen S., Xia Y., Maciejewski D., McNamara R. K., Streit W. J., Salafranca M. N., Adhikari S., Thompson D. A., Botti P., Bacon K. B. and Feng L. (1998) Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 10896-10901.

Hartman R. D., He J. R. and Barraclough C. A. (1990) Gamma-aminobutyric acid-A and -B receptor antagonists increase luteinizing hormone-releasing hormone neuronal responsiveness to intracerebroventricular norepinephrine in ovariectomized estrogen-treated rats. *Endocrinology* **127,** 1336-1345.

Hashimoto I. and Wiest W. G. (1969) Correlation of the secretion of ovarian steroids with function of a single generation of corpora lutea in the immature rat. *Endocrinology* **84,** 873-885.

Hashimoto I., Henricks D. M., Anderson L. L. and Melampy R. M. (1968) Progesterone and pregn-4-en-20 alpha-ol-3-one in ovarian venous blood during various reproductive states in the rat. *Endocrinology* **82,** 333-341.

Havran R. T. and Oster G. (1977) Trypsin-like activity in the vaginal epithelial cells of the rat. *J Histochem Cytochem* **25,** 1178-1181.

Hebert M. A., Serova L. I. and Sabban E. L. (2005) Single and repeated immobilization stress differentially trigger induction and phosphorylation of several transcription factors and mitogen-activated protein kinases in the rat locus coeruleus. *Journal of neurochemistry* **95,** 484-498.

Helena C. V., Franci C. R. and Anselmo-Franci J. A. (2002) Luteinizing hormone and luteinizing hormone-releasing hormone secretion is under locus coeruleus control in female rats. *Brain Res* **955,** 245-252.

Herbison A. E. (1997) Noradrenergic regulation of cyclic GnRH secretion. *Reviews of reproduction* **2,** 1-6.

Herbison A. E. (2006) Physiology of Gonadotrophin-Releasing hormone neuronal network, in *Knobil and Neill's Physiology of Reproduction*

Third edition Edition (Neill J. D., ed.). Academic Press, San Diego.

Herbison A. E. and Dyer R. G. (1991) Effect on luteinizing hormone secretion of GABA receptor modulation in the medial preoptic area at the time of proestrous luteinizing hormone surge. *Neuroendocrinology* **53,** 317-320.

Herman J. P., Figueiredo H., Mueller N. K., Ulrich-Lai Y., Ostrander M. M., Choi D. C. and Cullinan W. E. (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Frontiers in neuroendocrinology* **24,** 151-180.

Hiatt E. S., Brunetta P. G., Seiler G. R., Barney S. A., Selles W. D., Wooledge K. H. and King J. C. (1992) Subgroups of luteinizing hormone-releasing hormone perikarya defined by computer analyses in the basal forebrain of intact female rats. *Endocrinology* **130,** 1030-1043.

Honma K. and Wuttke W. (1980) Norepinephrine and dopamine turnover rates in the medial preoptic area and the mediobasal hypothalamus of the rat brain after various endocrinological manipulations. *Endocrinology* **106,** 1848-1853.

Horvath T. L., Naftolin F. and Leranth C. (1992) GABAergic and catecholaminergic innervation of mediobasal hypothalamic beta-endorphin cells projecting to the medial preoptic area. *Neuroscience* **51,** 391-399.

Hosny S. and Jennes L. (1998) Identification of alpha1B adrenergic receptor protein in gonadotropin releasing hormone neurones of the female rat. *Journal of neuroendocrinology* **10,** 687-692.

Huang H. H., Marshall S. and Meites J. (1976) Induction of estrous cycles in old non-cyclic rats by progesterone, ACTH, ether stress or L-dopa. *Neuroendocrinology* **20,** 21-34.

I'Anson H., Sundling L. A., Roland S. M. and Ritter S. (2003) Immunotoxic destruction of distinct catecholaminergic neuron populations disrupts the reproductive response to glucoprivation in female rats. *Endocrinology* **144,** 4325-4331.

Ibata Y., Watanabe K., Kinoshita H., Kubo S., Sano Y., Sin S., Hashimura E. and Imagawa K. (1979) The location of LH-RH neurons in the rat hypothalamus and their pathways to the median eminence. Experimental immunohistochemistry and radioimmunoassay. *Cell and tissue research* **198,** 381-395.

Jarry H., Perschl A. and Wuttke W. (1988) Further evidence that preoptic anterior hypothalamic GABAergic neurons are part of the GnRH pulse and surge generator. *Acta endocrinologica* **118,** 573-579.

Jarry H., Hirsch B., Leonhardt S. and Wuttke W. (1992) Amino acid neurotransmitter release in the preoptic area of rats during the positive feedback actions of estradiol on LH release. *Neuroendocrinology* **56,** 133-140.

Jennes L., Stumpf W. E. and Tappaz M. L. (1983) Anatomical relationships of dopaminergic and GABAergic systems with the GnRH-systems in the septo-hypothalamic area. Immunohistochemical studies. *Experimental brain research. Experimentelle Hirnforschung* **50,** 91-99.

Jennes L., Jennes M. E., Purvis C. and Nees M. (1992) c-fos expression in noradrenergic A2 neurons of the rat during the estrous cycle and after steroid hormone treatments. *Brain Res* **586,** 171-175.

Jung H., Shannon E. M., Fritschy J. M. and Ojeda S. R. (1998) Several GABAA receptor subunits are expressed in LHRH neurons of juvenile female rats. *Brain research* **780,** 218-229.

Kaba H., Saito H., Otsuka K., Seto K. and Kawakami M. (1983) Effects of estrogen on the excitability of neurons projecting from the noradrenergic A1 region to the preoptic and anterior hypothalamic area. *Brain Res* **274,** 156-159.

Kabiersch A., del Rey A., Honegger C. G. and Besedovsky H. O. (1988) Interleukin-1 induces changes in norepinephrine metabolism in the rat brain. *Brain, behavior, and immunity* **2,** 267-274.

Kalra P. S., Sahu A. and Kalra S. P. (1990a) Interleukin-1 inhibits the ovarian steroid-induced luteinizing hormone surge and release of hypothalamic luteinizing hormone-releasing hormone in rats. *Endocrinology* **126,** 2145-2152.

Kalra P. S., Fuentes M., Sahu A. and Kalra S. P. (1990b) Endogenous opioid peptides mediate the interleukin-1-induced inhibition of the release of luteinizing hormone (LH)-releasing hormone and LH. *Endocrinology* **127,** 2381-2386.

Kalra P. S., Kalra S. P., Krulich L., Fawcett C. P. and McCann S. M. (1972) Involvement of norepinephrine in transmission of the stimulatory influence of progesterone on gonadotropin release. *Endocrinology* **90,** 1168-1176.

Kalra P. S., Edwards T. G., Xu B., Jain M. and Kalra S. P. (1998) The anti-gonadotropic effects of cytokines: the role of neuropeptides. *Domestic animal endocrinology* **15,** 321-332.

Kalra S. P. (1975) Observations on facilitation of the preovulatory rise of LH by estrogen. *Endocrinology* **96,** 23-28.

Kalra S. P. (1977) Suppression of serum LH-RH and LH in rats by an inhibitor of norepinephrine synthesis. *Journal of reproduction and fertility* **49,** 371-373.

Kalra S. P. (1993) Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. *Endocrine reviews* **14,** 507-538.

Kalra S. P. and McCann S. M. (1974) Effects of drugs modifying catecholamine synthesis on plasma LH and ovulation in the rat. *Neuroendocrinology* **15,** 79-91.

Kalra S. P. and Kalra P. S. (1979) Dynamic changes in hypothalamic LH-RH levels associated with the ovarian steroid-induced gonadotrophin surge. *Acta endocrinologica* **92,** 1-7.

Kalra S. P. and Kalra P. S. (1983) Neural regulation of luteinizing hormone secretion in the rat. *Endocrine reviews* **4,** 311-351.

Kalra S. P. and Crowley W. R. (1992) Neuropeptide Y: a novel neuroendocrine peptide in the control of pituitary hormone secretion, and its relation to luteinizing hormone. *Frontiers in neuroendocrinology* **13,** 1-46.

Kalu E., Sumar N., Giannopoulos T., Patel P., Croucher C., Sherriff E. and Bansal A. (2007) Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis. *The journal of obstetrics and gynaecology research* **33,** 490-495.

Kam K., Park Y., Cheon M., Son G. H., Kim K. and Ryu K. (2000) Effects of immobilization stress on estrogen-induced surges of luteinizing hormone and prolactin in ovariectomized rats. *Endocrine* **12,** 279-287.

Kang S. S., Kim S. R., Leonhardt S., Jarry H., Wuttke W. and Kim K. (2000) Effect of interleukin-1beta on gonadotropin-releasing hormone (GnRH) and GnRH receptor gene expression in castrated male rats. *Journal of neuroendocrinology* **12,** 421-429.

Kang S. S., Son G. H., Seong J. Y., Choi D., Kwon H. B., Lee C. C. and Kim K. (1998) Noradrenergic neurotoxin suppresses gonadotropin-releasing hormone (GnRH) and GnRH receptor gene expression in ovariectomized and steroid-treated rats. *Journal of neuroendocrinology* **10,** 911-918.

Karsch F. J., Battaglia D. F., Breen K. M., Debus N. and Harris T. G. (2002) Mechanisms for ovarian cycle disruption by immune/inflammatory stress. *Stress (Amsterdam, Netherlands)* **5,** 101-112.

Katsuura G., Gottschall P. E., Dahl R. R. and Arimura A. (1988) Adrenocorticotropin release induced by intracerebroventricular injection of recombinant human interleukin-1 in rats: possible involvement of prostaglandin. *Endocrinology* **122,** 1773-1779.

Khadra A. and Li Y. X. (2006) A model for the pulsatile secretion of gonadotropin-releasing hormone from synchronized hypothalamic neurons. *Biophysical journal* **91,** 74-83.

Kilbourne E. J., Nankova B. B., Lewis E. J., McMahon A., Osaka H., Sabban D. B. and Sabban E. L. (1992) Regulated expression of the tyrosine hydroxylase gene by membrane depolarization. Identification of the responsive element and possible second messengers. *The Journal of biological chemistry* **267,** 7563-7569.

Kim Y. I., Dudley C. A. and Moss R. L. (1987) A1 noradrenergic input to medial preoptic-medial septal area: an electrophysiological study. *Neuroendocrinology* **45,** 77-85.

Kimura F. and Jinnai K. (1994) Bicuculline infusions advance the timing of luteinizing hormone surge in proestrous rats: comparisons with naloxone effects. *Hormones and behavior* **28,** 424-430.

Kimura F. and Funabashi T. (1998) Two Subgroups of Gonadotropin Releasing Hormone Neurons Control Gonadotropin Secretion in Rats. *News Physiol Sci* **13,** 225-231.

Kimura F., Sano A., Hiruma H. and Funabashi T. (1993) Effects of gamma-aminobutyric acid-A receptor antagonist, bicuculline, on the electrical activity of luteinizing hormone-releasing hormone pulse generator in the ovariectomized rat. *Neuroendocrinology* **57,** 605-614.

Kitchen J. H., Ruf K. B. and Younglai E. V. (1974) Effects of intraventricular injections of 6-hydroxydopamine on plasma LH and FSH in male rats. *Journal of reproduction and fertility* **40,** 249-257.

Koh T., Nakai Y., Kinoshita F., Tsukada T., Tsujii S. and Imura H. (1985) Modulation of luteinizing hormone(LH) release by clonidine and opioid peptides in castrated male rats. *Endocrinologia japonica* **32,** 653-659.

Komaki G., Arimura A. and Koves K. (1992) Effect of intravenous injection of IL-1 beta on PGE2 levels in several brain areas as determined by microdialysis. *The American journal of physiology* **262,** E246-251.

Koob G. F. (1999) Corticotropin-releasing factor, norepinephrine, and stress. *Biological psychiatry* **46,** 1167-1180.

Kopin I. J. and Gordon E. K. (1962) Metabolism of norepinephrine-H3 released by tyramine and reserpine. *The Journal of pharmacology and experimental therapeutics* **138,** 351-359.

Kreda S. M., Sumner M., Fillo S., Ribeiro C. M., Luo G. X., Xie W., Daniel K. W., Shears S., Collins S. and Wetsel W. C. (2001) alpha(1)-adrenergic receptors mediate LH-releasing hormone secretion through phospholipases C and A(2) in immortalized hypothalamic neurons. *Endocrinology* **142,** 4839-4851.

Lamberts R., Vijayan E., Graf M., Mansky T. and Wuttke W. (1983) Involvement of preoptic-anterior hypothalamic GABA neurons in the regulation of pituitary LH and prolactin release. *Experimental brain research. Experimentelle Hirnforschung* **52,** 356-362.

Larsen B., Markovetz A. J. and Galask R. P. (1977) Relationship of Vaginal Cytology to Alterations of the Vaginal Microflora of Rats During the Estrous Cycle. *Applied and environmental microbiology* **33,** 556-562.

Le W.-W., Berghorn K. A., Smith M. S. and Hoffman G. E. (1997a) Alpha1-adrenergic receptor blockade blocks LH secretion but not LHRH cFos activation. *Brain research* **747,** 236-245.

Le W. W., Berghorn K. A., Smith M. S. and Hoffman G. E. (1997b) Alpha1-adrenergic receptor blockade blocks LH secretion but not LHRH cFos activation. *Brain research* **747,** 236-245.

Lee A., Talley E., Rosin D. L. and Lynch K. R. (1995) Characterization of alpha 2A-adrenergic receptors in GT1 neurosecretory cells. *Neuroendocrinology* **62,** 215-225.

Lee S. and Rivier C. (1996) Gender differences in the effect of prenatal alcohol exposure on the hypothalamic-pituitary-adrenal axis response to immune signals. *Psychoneuroendocrinology* **21,** 145-155.

Lee W. S., Smith M. S. and Hoffman G. E. (1990) Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone. *Proceedings of the National Academy of Sciences of the United States of America* **87,** 5163-5167.

Leonhardt S., Seong J. Y., Kim K., Thorun Y., Wuttke W. and Jarry H. (1995) Activation of central GABAA-but not of GABAB-receptors rapidly reduces pituitary LH release and GnRH gene expression in the preoptic/anterior hypothalamic area of ovariectomized rats. *Neuroendocrinology* **61,** 655-662.

Leranth C., MacLusky N. J., Shanabrough M. and Naftolin F. (1988) Catecholaminergic innervation of luteinizing hormone-releasing hormone and glutamic acid decarboxylase immunopositive neurons in the rat medial preoptic area. An electron-microscopic double immunostaining and degeneration study. *Neuroendocrinology* **48,** 591-602.

Leranth C., MacLusky N. J., Sakamoto H., Shanabrough M. and Naftolin F. (1985) Glutamic acid decarboxylase-containing axons synapse on LHRH neurons in the rat medial preoptic area. *Neuroendocrinology* **40,** 536-539.

Levine J. E. (1997) New concepts of the neuroendocrine regulation of gonadotropin surges in rats. *Biology of reproduction* **56,** 293-302.

Liaw J. J., He J. R., Hartman R. D. and Barraclough C. A. (1992) Changes in tyrosine hydroxylase mRNA levels in medullary A1 and A2 neurons and locus coeruleus following castration and estrogen replacement in rats. *Brain Res Mol Brain Res* **13,** 231-238.

Licinio J. and Frost P. (2000) The neuroimmune-endocrine axis: pathophysiological implications for the central nervous system cytokines and hypothalamus-pituitary-adrenal hormone dynamics. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al* **33,** 1141-1148.

Lynch A. M., Walsh C., Delaney A., Nolan Y., Campbell V. A. and Lynch M. A. (2004) Lipopolysaccharide-induced increase in signalling in hippocampus is abrogated by IL-10--a role for IL-1 beta? *Journal of neurochemistry* **88,** 635-646.

Ma S. and Morilak D. A. (2005) Norepinephrine release in medial amygdala facilitates activation of the hypothalamic-pituitary-adrenal axis in response to acute immobilisation stress. *Journal of neuroendocrinology* **17,** 22-28.

MacLusky N. J., Naftolin F. and Leranth C. (1988) Immunocytochemical evidence for direct synaptic connections between corticotrophin-releasing factor (CRF) and gonadotrophin-releasing hormone (GnRH)-containing neurons in the preoptic area of the rat. *Brain research* **439,** 391-395.

Maeda K., Cagampang F. R., Coen C. W. and Tsukamura H. (1994) Involvement of the catecholaminergic input to the paraventricular nucleus and of corticotropin-releasing hormone in the fasting-induced suppression of luteinizing hormone release in female rats. *Endocrinology* **134,** 1718-1722.

Mansky T., Mestres-Ventura P. and Wuttke W. (1982) Involvement of GABA in the feedback action of estradiol on gonadotropin and prolactin release: hypothalamic GABA and catecholamine turnover rates. *Brain research* **231,** 353-364.

Martinez de la Escalera G., Choi A. L. and Weiner R. I. (1992) Beta 1-adrenergic regulation of the GT1 gonadotropin-releasing hormone (GnRH) neuronal cell lines: stimulation of GnRH release via receptors positively coupled to adenylate cyclase. *Endocrinology* **131,** 1397-1402.

Martinovic J. and McCann S. (1977) Effect of lesions in the ventral noradrenergic tract produced by microinjection of 6-hydroxydopamine on gonadotropin release in the rat. *Endocrinology* **100,** 1206-1213.

Martins-Afferri M. P., Ferreira-Silva I. A., Franci C. R. and Anselmo-Franci J. A. (2003) LHRH release depends on Locus Coeruleus noradrenergic inputs to the medial preoptic area and median eminence. *Brain research bulletin* **61,** 521-527.

Matta S., Singh J., Newton R. and Sharp B. M. (1990) The adrenocorticotropin response to interleukin-1 beta instilled into the rat median eminence depends on the local release of catecholamines. *Endocrinology* **127,** 2175-2182.

Merali Z., McIntosh J., Kent P., Michaud D. and Anisman H. (1998) Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. *J Neurosci* **18,** 4758-4766.

Merriman C. R., Pulliam L. A. and Kampschmidt R. F. (1977) Comparison of leukocytic pyrogen and leukocytic endogenous mediator. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y* **154,** 224-227.

Meyerson B. J. and Sawyer C. H. (1968) Monoamines and ovulation in the rat. *Endocrinology* **83,** 170-176.

Miggin S. M. and O'Neill L. A. (2006) New insights into the regulation of TLR signaling. *Journal of leukocyte biology* **80,** 220-226.

Mitchell J. C., Li X. F., Breen L., Thalabard J. C. and O'Byrne K. T. (2005) The role of the locus coeruleus in corticotropin-releasing hormone and stress-induced suppression of pulsatile luteinizing hormone secretion in the female rat. *Endocrinology* **146,** 323-331.

Mitsushima D., Shwe T. T., Funabashi T., Shinohara K. and Kimura F. (2002) GABA release in the medial preoptic area of cyclic female rats. *Neuroscience* **113,** 109-114.

Moberg G. P. (1991) How behavioral stress disrupts the endocrine control of reproduction in domestic animals. *J Dairy Sci* **74,** 304-311.

MohanKumar P. S. and Quadri S. K. (1993) Systemic administration of interleukin-1 stimulates norepinephrine release in the paraventricular nucleus. *Life sciences* **52,** 1961-1967.

Mohankumar P. S., Thyagarajan S. and Quadri S. K. (1994) Correlations of catecholamine release in the medial preoptic area with proestrous surges of luteinizing hormone and prolactin: effects of aging. *Endocrinology* **135,** 119-126.

Mohankumar P. S., Thyagarajan S. and Quadri S. K. (1995) Cyclic and age-related changes in norepinephrine concentrations in the medial preoptic area and arcuate nucleus. *Brain research bulletin* **38,** 561-564.

MohanKumar P. S., MohanKumar S. M. and Quadri S. K. (1997a) Deprenyl stimulates the release of luteinizing hormone from the pituitary in vitro. *Life sciences* **61,** 1783-1788.

Mohankumar P. S., Thyagarajan S. and Quadri S. K. (1997b) Tyrosine hydroxylase and DOPA decarboxylase activities in the medical preoptic area and arcuate nucleus during the estrous cycle: effects of aging. *Brain research bulletin* **42,** 265-271.

MohanKumar S. M. and MohanKumar P. S. (2002) Effects of interleukin-1 beta on the steroid-induced luteinizing hormone surge: role of norepinephrine in the medial preoptic area. *Brain research bulletin* **58,** 405-409.

MohanKumar S. M. and MohanKumar P. S. (2004) Aging alters norepinephrine release in the medial preoptic area in response to steroid priming in ovariectomized rats. *Brain Res* **1023,** 24-30.

MohanKumar S. M. and MohanKumar P. S. (2005) Systemic Interleukin-1beta stimulates the simultaneous release of norepinephrine in the paraventricular nucleus and the median eminence. *Brain Res Bull* **65,** 451-456.

MohanKumar S. M., MohanKumar P. S. and Quadri S. K. (1998) Specificity of interleukin-1beta-induced changes in monoamine concentrations in hypothalamic nuclei: blockade by interleukin-1 receptor antagonist. *Brain research bulletin* **47,** 29-34.

MohanKumar S. M., Smith C. L. and MohanKumar P. S. (2003) Central adaptation to chronic administration of interleukin-1beta (IL-1beta) in rats. *Brain research bulletin* **62,** 71-76.

Mueller E. and Nistico G. (1989a) *Brain Messengers and the Anterior Pituitary.* Academic Press, San Diego.

Mueller E. E. and Nistico G. (1989b) *In: Brain Messengers and the Pituitary*, pp 462-467. Academic Press, New York.

Murphy G. M., Jr., Yang L. and Cordell B. (1998) Macrophage colony-stimulating factor augments beta-amyloid-induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells. *The Journal of biological chemistry* **273,** 20967-20971.

Nadjar A., Bluthe R. M., May M. J., Dantzer R. and Parnet P. (2005) Inactivation of the cerebral NFkappaB pathway inhibits interleukin-1beta-induced sickness behavior and c-Fos expression in various brain nuclei. *Neuropsychopharmacology* **30,** 1492-1499.

Nadjar A., Combe C., Laye S., Tridon V., Dantzer R., Amedee T. and Parnet P. (2003) Nuclear factor kappaB nuclear translocation as a crucial marker of brain response to interleukin-1. A study in rat and interleukin-1 type I deficient mouse. *Journal of neurochemistry* **87,** 1024-1036.

Nagatani S., Tsukamura H. and Maeda K. (1994) Estrogen feedback needed at the paraventricular nucleus or A2 to suppress pulsatile luteinizing hormone release in fasting female rats. *Endocrinology* **135,** 870-875.

Nagatani S., Tsukamura H., Murahashi K., Bucholtz D. C., Foster D. L. and Maeda K. (1996) Paraventricular norepinephrine release mediates glucoprivic suppression of pulsatile luteinizing hormone secretion. *Endocrinology* **137,** 3183-3186.

Nagatsu T. (1991) Genes for human catecholamine-synthesizing enzymes. *Neuroscience research* **12,** 315-345.

Nagatsu T. (1995) Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology. *Essays Biochem* **30,** 15-35.

Nagatsu T., Levitt M. and Udenfriend S. (1964) Tyrosine Hydroxylase. the Initial Step in Norepinephrine Biosynthesis. *The Journal of biological chemistry* **239,** 2910-2917.

Nankova B., Kvetnansky R., McMahon A., Viskupic E., Hiremagalur B., Frankle G., Fukuhara K., Kopin I. J. and Sabban E. L. (1994) Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 5937-5941.

Neveu P. J. and Liege S. (2000) Mechanisms of behavioral and neuroendocrine effects of interleukin-1 in mice. *Annals of the New York Academy of Sciences* **917,** 175-185.

Nicholson G., Greeley G., Humm J., Youngblood W. and Kizer J. S. (1978) Lack of effect of noradrenergic denervation of the hypothalamus and medial preoptic area on the feedback regulation of gonadotropin secretion and the estrous cycle of the rat. *Endocrinology* **103,** 559-566.

Nie M., Pang L., Inoue H. and Knox A. J. (2003) Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. *Mol Cell Biol* **23,** 9233-9244.

Oh J. W., Schwiebert L. M. and Benveniste E. N. (1999) Cytokine regulation of CC and CXC chemokine expression by human astrocytes. *Journal of neurovirology* **5,** 82-94.

Ondo J., Mansky T. and Wuttke W. (1982) In vivo GABA release from the medial preoptic area of diestrous and ovariectomized rats. *Experimental brain research. Experimentelle Hirnforschung* **46,** 69-72.

Pacak K., McCarty R., Palkovits M., Kopin I. J. and Goldstein D. S. (1995) Effects of immobilization on in vivo release of norepinephrine in the bed nucleus of the stria terminalis in conscious rats. *Brain research* **688,** 242-246.

Parlanti I. A. and Monis B. (1980) Microscopic study of the mucification of vaginal epithelium in immature and prepuberal rats. Changes with age, castration and hormone replacement, and data on the ultrastructure of the luminal glycocalyx. *Acta anatomica* **106,** 370-377.

Pau K. Y. and Spies H. G. (1997) Neuroendocrine signals in the regulation of gonadotropin-releasing hormone secretion. *The Chinese journal of physiology* **40,** 181-196.

Plata-Salaman C. R. and Borkoski J. P. (1994) Chemokines/intercrines and central regulation of feeding. *The American journal of physiology* **266,** R1711-1715.

Plotkin S. R., Banks W. A. and Kastin A. J. (1996) Comparison of saturable transport and extracellular pathways in the passage of interleukin-1 alpha across the blood-brain barrier. *Journal of neuroimmunology* **67,** 41-47.

Poli V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *The Journal of biological chemistry* **273,** 29279-29282.

Qian L., Block M. L., Wei S. J., Lin C. F., Reece J., Pang H., Wilson B., Hong J. S. and Flood P. M. (2006) Interleukin-10 protects lipopolysaccharide-induced neurotoxicity in primary midbrain cultures by inhibiting the function of NADPH oxidase. *The Journal of pharmacology and experimental therapeutics* **319,** 44-52.

Quan N. and Banks W. A. (2007) Brain-immune communication pathways. *Brain, behavior, and immunity* **21,** 727-735.

Quirarte G. L., Galvez R., Roozendaal B. and McGaugh J. L. (1998) Norepinephrine release in the amygdala in response to footshock and opioid peptidergic drugs. *Brain research* **808,** 134-140.

Raber J., Koob G. F. and Bloom F. E. (1995) Interleukin-2 (IL-2) induces corticotropin-releasing factor (CRF) release from the amygdala and involves a nitric oxide-mediated signaling; comparison with the hypothalamic response. *The Journal of pharmacology and experimental therapeutics* **272,** 815-824.

Rance N., Wise P. M., Selmanoff M. K. and Barraclough C. A. (1981) Catecholamine turnover rates in discrete hypothalamic areas and associated changes in median eminence luteinizing hormone-releasing hormone and serum gonadotropins on proestrus and diestrous day 1. *Endocrinology* **108,** 1795-1802.

Rettori V., Gimeno M. F., Karara A., Gonzalez M. C. and McCann S. M. (1991) Interleukin 1 alpha inhibits prostaglandin E2 release to suppress pulsatile release of luteinizing hormone but not follicle-stimulating hormone. *Proc Natl Acad Sci U S A* **88,** 2763-2767.

Reyes T. M., Walker J. R., DeCino C., Hogenesch J. B. and Sawchenko P. E. (2003) Categorically distinct acute stressors elicit dissimilar transcriptional profiles in the paraventricular nucleus of the hypothalamus. *J Neurosci* **23,** 5607-5616.

Rivest S. and Rivier C. (1993a) Centrally injected interleukin-1 beta inhibits the hypothalamic LHRH secretion and circulating LH levels via prostaglandins in rats. *Journal of neuroendocrinology* **5,** 445-450.

Rivest S. and Rivier C. (1993b) Central mechanisms and sites of action involved in the inhibitory effects of CRF and cytokines on LHRH neuronal activity. *Annals of the New York Academy of Sciences* **697,** 117-141.

Rivest S. and Rivier C. (1993c) Interleukin-1 beta inhibits the endogenous expression of the early gene c-fos located within the nucleus of LH-RH neurons and interferes with hypothalamic LH-RH release during proestrus in the rat. *Brain research* **613,** 132-142.

Rivest S., Lee S., Attardi B. and Rivier C. (1993) The chronic intracerebroventricular infusion of interleukin-1 beta alters the activity of the hypothalamic-pituitary-gonadal axis of cycling rats. I. Effect on LHRH and gonadotropin biosynthesis and secretion. *Endocrinology* **133,** 2424-2430.

Rivier C. (1994) Stimulatory effect of interleukin-1 beta on the hypothalamic-pituitary-adrenal axis of the rat: influence of age, gender and circulating sex steroids. *The Journal of endocrinology* **140,** 365-372.

Rivier C. and Vale W. (1989) In the rat, interleukin-1 alpha acts at the level of the brain and the gonads to interfere with gonadotropin and sex steroid secretion. *Endocrinology* **124,** 2105-2109.

Rivier C. and Vale W. (1990) Cytokines act within the brain to inhibit luteinizing hormone secretion and ovulation in the rat. *Endocrinology* **127,** 849-856.

Rivier C. and Erickson G. (1993) The chronic intracerebroventricular infusion of interleukin-1 beta alters the activity of the hypothalamic-pituitary-gonadal axis of cycling rats. II. Induction of pseudopregnant-like corpora lutea. *Endocrinology* **133,** 2431-2436.

Rivier C. and Rivest S. (1993) Mechanisms mediating the effects of cytokines on neuroendocrine functions in the rat. *Ciba Foundation symposium* **172,** 204-220; discussion 220-205.

Rivier C., Vale W. and Brown M. (1989) In the rat, interleukin-1 alpha and -beta stimulate adrenocorticotropin and catecholamine release. *Endocrinology* **125,** 3096-3102.

Roozendaal M. M., De Kruijf H. F., Reuling R. J., Threels A., Swarts J. J., Wiegant V. M. and Mattheij J. A. (1997) Inhibition of the LH Surge by Restraint Stress in Cyclic Rats: Studies on the Role of GABAA and GABAB Receptors. *Stress (Amsterdam, Netherlands)* **1,** 241-248.

Rostene W., Kitabgi P. and Parsadaniantz S. M. (2007) Chemokines: a new class of neuromodulator? *Nat Rev Neurosci* **8,** 895-903.

Rotsztejn W., Besson J., Pattou E. and Drouva S. V. (1981) Hypothalamic neuropeptides (NP) can be neurotransmitters, neurohormones or neuromodulators. *Advanced Physiological Sciences* **14,** 217-229.

Sabban E. L., Hiremagalur B., Nankova B. and Kvetnansky R. (1995) Molecular biology of stress-elicited induction of catecholamine biosynthetic enzymes. *Annals of the New York Academy of Sciences* **771,** 327-338.

Saito H., Sherwood E. R., Varma T. K. and Evers B. M. (2003) Effects of aging on mortality, hypothermia, and cytokine induction in mice with endotoxemia or sepsis. *Mechanisms of ageing and development* **124,** 1047-1058.

Sajapitak S., Iwata K., Shahab M., Uenoyama Y., Yamada S., Kinoshita M., Bari F. Y., I'Anson H., Tsukamura H. and Maeda K. I. (2008) Central lipoprivation-induced suppression of LH pulses is mediated by paraventricular catecholaminergic inputs in female rats. *Endocrinology.*

Sakamaki K., Nomura M., Hatakenaka S., Miyakubo H. and Tanaka J. (2003) GABAergic modulation of noradrenaline release in the median preoptic nucleus area in the rat. *Neuroscience letters* **342,** 77-80.

Sapolsky R., Rivier C., Yamamoto G., Plotsky P. and Vale W. (1987) Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. *Science (New York, N.Y* **238,** 522-524.

Sarkar D. K., Chiappa S. A., Fink G. and Sherwood N. M. (1976) Gonadotropin-releasing hormone surge in pro-oestrous rats. *Nature* **264,** 461-463.

Sawchenko P. E. and Swanson L. W. (1982) The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain research* **257,** 275-325.

Sawyer C. H., Markee J. E. and Hollinshead W. H. (1947) Inhibition of ovulation in the rabbit by adrenergic blocking agent dibenamine. *Endocrinology* **41,** 395-399.

Sawyer C. H., Markee J. E. and Everett J. W. (1950) Further experiments on blocking pituitary activation in the rabbit and the rat. *J Exp Zool* **113,** 659-663.

Scammell T. E., Griffin J. D., Elmquist J. K. and Saper C. B. (1998) Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever. *Am J Physiol* **274,** R783-789.

Schneider J. E. (2004) Energy balance and reproduction. *Physiology & behavior* **81,** 289-317.

Schutze S., Machleidt T. and Kronke M. (1994) The role of diacylglycerol and ceramide in tumor necrosis factor and interleukin-1 signal transduction. *Journal of leukocyte biology* **56,** 533-541.

Schwanzel-Fukuda M. and Pfaff D. W. (1989) Origin of luteinizing hormone-releasing hormone neurons. *Nature* **338,** 161-164.

Scott C. J. and Clarke I. J. (1993) Inhibition of luteinizing hormone secretion in ovariectomized ewes during the breeding season by gamma-aminobutyric acid (GABA) is mediated by GABA-A receptors, but not GABA-B receptors. *Endocrinology* **132,** 1789-1796.

Serantes R., Arnalich F., Figueroa M., Salinas M., Andres-Mateos E., Codoceo R., Renart J., Matute C., Cavada C., Cuadrado A. and Montiel C. (2006) Interleukin-1beta enhances GABAA receptor cell-surface expression by a phosphatidylinositol 3-kinase/Akt pathway: relevance to sepsis-associated encephalopathy. *The Journal of biological chemistry* **281,** 14632-14643.

Serova L. I., Nankova B. B., Feng Z., Hong J. S., Hutt M. and Sabban E. L. (1999) Heightened transcription for enzymes involved in norepinephrine biosynthesis in the rat locus coeruleus by immobilization stress. *Biological psychiatry* **45,** 853-862.

Shaftel S. S., Carlson T. J., Olschowka J. A., Kyrkanides S., Matousek S. B. and O'Banion M. K. (2007) Chronic interleukin-1beta expression in mouse brain leads to leukocyte infiltration and neutrophil-independent blood brain barrier permeability without overt neurodegeneration. *J Neurosci* **27,** 9301-9309.

Shanks N., McCormick C. M. and Meaney M. J. (1994) Sex differences in hypothalamic-pituitary-adrenal responding to endotoxin challenge in the neonate: reversal by gonadectomy. *Brain Res Dev Brain Res* **79,** 260-266.

Simon C., Tsafriri A., Chun S. Y., Piquette G. N., Dang W. and Polan M. L. (1994) Interleukin-1 receptor antagonist suppresses human chorionic gonadotropin-induced ovulation in the rat. *Biology of reproduction* **51,** 662-667.

Simpkins J. W. and Kalra S. P. (1979) Blockade of progesterone-induced increase in hypothalamic luteinizing hormone-releasing hormone levels and serum gonadotropins by intrahypothalamic implantation of 6-hydroxydopamine. *Brain research* **170,** 475-484.

Simpkins J. W., Advis J. P., Hodson C. A. and Meites J. (1979) Blockade of steroid-induced leuteinizing hormone release by selective depletion of anterior hypothalamic norepinephrine activity. *Endocrinology* **104,** 506-509.

Singh S. R. and Briski K. P. (2005) Central GABAA but not GABAB receptors mediate suppressive effects of caudal hindbrain glucoprivation on the luteinizing hormone surge in steroid-primed, ovariectomized female rats. *Journal of neuroendocrinology* **17,** 407-412.

Singh S. R., Sylvester P. W. and Briski K. P. (2004) Caudal hindbrain glucoprivation enhances gamma-aminobutyric acid release in discrete septopreoptic structures in the steroid-primed ovariectomized rat brain: role of mu opioid receptors. *Neuroendocrinology* **80,** 201-209.

Sirivelu M. P., Shin A., C, MohanKumar P. S. and Mohankumar S. M. J. (2006) Does interleukin-1 beta affect norepinephrine biosynthesis to suppress luteinizing hormone surge? Mechanistic insights using NE precursor L-dopa, in *Society for Neuroscience 36th Annual meeting*, Atlanta,GA.

Slack J. L., Schooley K., Bonnert T. P., Mitcham J. L., Qwarnstrom E. E., Sims J. E. and Dower S. K. (2000) Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. *The Journal of biological chemistry* **275,** 4670-4678.

Smagin G. N., Swiergiel A. H. and Dunn A. J. (1996) Peripheral administration of interleukin-1 increases extracellular concentrations of norepinephrine in rat hypothalamus: comparison with plasma corticosterone. *Psychoneuroendocrinology* **21,** 83-93.

Smith M. J. and Jennes L. (2001) Neural signals that regulate GnRH neurones directly during the oestrous cycle. *Reproduction (Cambridge, England)* **122,** 1-10.

Spergel D. J., Kruth U., Hanley D. F., Sprengel R. and Seeburg P. H. (1999) GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. *J Neurosci* **19,** 2037-2050.

Spinedi E., Chisari A., Pralong F. and Gaillard R. C. (1997) Sexual dimorphism in the mouse hypothalamic-pituitary-adrenal axis function after endotoxin and insulin stresses during development. *Neuroimmunomodulation* **4,** 77-83.

Spinedi E., Suescun M. O., Hadid R., Daneva T. and Gaillard R. C. (1992) Effects of gonadectomy and sex hormone therapy on the endotoxin-stimulated hypothalamo-pituitary-adrenal axis: evidence for a neuroendocrine-immunological sexual dimorphism. *Endocrinology* **131,** 2430-2436.

Srinivasan D., Yen J. H., Joseph D. J. and Friedman W. (2004) Cell type-specific interleukin-1beta signaling in the CNS. *J Neurosci* **24,** 6482-6488.

Subramaniam S., Stansberg C. and Cunningham C. (2004) The interleukin 1 receptor family. *Developmental and comparative immunology* **28,** 415-428.

Szafarczyk A., Malaval F., Laurent A., Gibaud R. and Assenmacher I. (1987) Further evidence for a central stimulatory action of catecholamines on adrenocorticotropin release in the rat. *Endocrinology* **121,** 883-892.

Szawka R. E., Franci C. R. and Anselmo-Franci J. A. (2007) Noradrenaline release in the medial preoptic area during the rat oestrous cycle: temporal relationship with plasma secretory surges of prolactin and luteinising hormone. *Journal of neuroendocrinology* **19,** 374-382.

Tabarean I. V., Korn H. and Bartfai T. (2006) Interleukin-1beta induces hyperpolarization and modulates synaptic inhibition in preoptic and anterior hypothalamic neurons. *Neuroscience* **141,** 1685-1695.

Taleisnik S., Haymal B. and Caligaris L. (1993) Intraventricular injection of agents that enhance cyclic adenosine monophosphate formation leads to inhibition of proestrous luteinizing hormone surge in rats. *Acta endocrinologica* **129,** 273-278.

Tanebe K., Nishijo H., Muraguchi A. and Ono T. (2000) Effects of chronic stress on hypothalamic Interleukin-1beta, interleukin-2, and gonadotrophin-releasing hormone gene expression in ovariectomized rats. *Journal of neuroendocrinology* **12,** 13-21.

Temel S., Lin W., Lakhlani S. and Jennes L. (2002) Expression of estrogen receptor-alpha and cFos in norepinephrine and epinephrine neurons of young and middle-aged rats during the steroid-induced luteinizing hormone surge. *Endocrinology* **143,** 3974-3983.

Thibeault I., Laflamme N. and Rivest S. (2001) Regulation of the gene encoding the monocyte chemoattractant protein 1 (MCP-1) in the mouse and rat brain in response to circulating LPS and proinflammatory cytokines. *The Journal of comparative neurology* **434,** 461-477.

Thrasher J. D., Clark F. I. and Clarke D. R. (1967) Changes in the vaginal epithelial cell cyle in relation to events of the estrous cycle. *Experimental cell research* **45,** 232-236.

Tilbrook A. J., Turner A. I. and Clarke I. J. (2000) Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of reproduction* **5,** 105-113.

Tin Tin Win S., Mitsushima D., Shinohara K. and Kimura F. (2004) Sexual dimorphism of GABA release in the medial preoptic area and luteinizing hormone release in gonadectomized estrogen-primed rats. *Neuroscience* **127,** 243-250.

Tsagarakis S., Gillies G., Rees L. H., Besser M. and Grossman A. (1989) Interleukin-1 directly stimulates the release of corticotrophin releasing factor from rat hypothalamus. *Neuroendocrinology* **49,** 98-101.

Tsukahara S., Tsukamura H., Foster D. L. and Maeda K. I. (1999) Effect of corticotropin-releasing hormone antagonist on oestrogen-dependent glucoprivic suppression of luteinizing hormone secretion in female rats. *Journal of neuroendocrinology* **11,** 101-105.

Tsukamura H., Nagatani S., Cagampang F. R., Kawakami S. and Maeda K. (1994) Corticotropin-releasing hormone mediates suppression of pulsatile luteinizing hormone secretion induced by activation of alpha-adrenergic receptors in the paraventricular nucleus in female rats. *Endocrinology* **134,** 1460-1466.

Turrin N. P. and Rivest S. (2004) Unraveling the molecular details involved in the intimate link between the immune and neuroendocrine systems. *Exp Biol Med (Maywood)* **229,** 996-1006.

van der Meer M. J., Sweep C. G., Pesman G. J., Tilders F. J. and Hermus A. R. (1996) Chronic stimulation of the hypothalamus-pituitary-adrenal axis in rats by interleukin 1beta: central and peripheral mechanisms. *Cytokine* **8,** 910-919.

Viau V. and Meaney M. J. (1991) Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology* **129,** 2503-2511.

Watanobe H. and Takebe K. (1993) Intrahypothalamic perfusion with interleukin-1-beta stimulates the local release of corticotropin-releasing hormone and arginine vasopressin and the plasma adrenocorticotropin in freely moving rats: a comparative perfusion of the paraventricular nucleus and the median eminence. *Neuroendocrinology* **57,** 593-599.

Watanobe H. and Habu S. (2003) Adrenal glucocorticoids do not mediate impaired reproductive function induced by lipopolysaccharide in rats. *Neuroendocrinology* **78,** 23-28.

Watanobe H. and Hayakawa Y. (2003) Hypothalamic interleukin-1 beta and tumor necrosis factor-alpha, but not interleukin-6, mediate the endotoxin-induced suppression of the reproductive axis in rats. *Endocrinology* **144,** 4868-4875.

Watkins L. R., Wiertelak E. P., Goehler L. E., Mooney-Heiberger K., Martinez J., Furness L., Smith K. P. and Maier S. F. (1994) Neurocircuitry of illness-induced hyperalgesia. *Brain research* **639,** 283-299.

Webster J. I. and Sternberg E. M. (2004) Role of the hypothalamic-pituitary-adrenal axis, glucocorticoids and glucocorticoid receptors in toxic sequelae of exposure to bacterial and viral products. *The Journal of endocrinology* **181,** 207-221.

Weesner G. D., Krey L. C. and Pfaff D. W. (1993) Alpha 1 adrenergic regulation of estrogen-induced increases in luteinizing hormone-releasing hormone mRNA levels and release. *Brain Res Mol Brain Res* **17,** 77-82.

Weick R. F. (1978) Acute effects of adrenergic receptor blocking drugs and neuroleptic agents on pulsatile discharges of luteinizing hormone in the ovariectomized rat. *Neuroendocrinology* **26,** 108-117.

Weiner R. I., Shryne J. E., Gorski R. A. and Sawyer C. H. (1972) Changes in the catecholamine content of the rat hypothalamus following deafferentation. *Endocrinology* **90,** 867-873.

Weiss J. M., Downie S. A., Lyman W. D. and Berman J. W. (1998) Astrocyte-derived monocyte-chemoattractant protein-1 directs the transmigration of leukocytes across a model of the human blood-brain barrier. *J Immunol* **161,** 6896-6903.

Wetsel W. C., Valenca M. M., Merchenthaler I., Liposits Z., Lopez F. J., Weiner R. I., Mellon P. L. and Negro-Vilar A. (1992) Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 4149-4153.

Wise P. M. (1984) Estradiol-induced daily luteinizing hormone and prolactin surges in young and middle-aged rats: correlations with age-related changes in pituitary responsiveness and catecholamine turnover rates in microdissected brain areas. *Endocrinology* **115,** 801-809.

Wise P. M., Rance N. and Barraclough C. A. (1981) Effects of estradiol and progesterone on catecholamine turnover rates in discrete hypothalamic regions in ovariectomized rats. *Endocrinology* **108,** 2186-2193.

Witkin J. W., Paden C. M. and Silverman A. J. (1982) The luteinizing hormone-releasing hormone (LHRH) systems in the rat brain. *Neuroendocrinology* **35,** 429-438.

Wong M. L. and Licinio J. (1994) Localization of interleukin 1 type I receptor mRNA in rat brain. *Neuroimmunomodulation* **1,** 110-115.

Woulfe J. M., Flumerfelt B. A. and Hrycyshyn A. W. (1990) Efferent connections of the A1 noradrenergic cell group: a DBH immunohistochemical and PHA-L anterograde tracing study. *Exp Neurol* **109,** 308-322.

Wright D. E. and Jennes L. (1993) Origin of noradrenergic projections to GnRH perikarya-containing areas in the medial septum-diagonal band and preoptic area. *Brain research* **621,** 272-278.

Xu Y., Day T. A. and Buller K. M. (1999) The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta administration. *Neuroscience* **94,** 175-183.

Yabuuchi K., Minami M., Katsumata S. and Satoh M. (1994) Localization of type I interleukin-1 receptor mRNA in the rat brain. *Brain Res Mol Brain Res* **27,** 27-36.

Yirmiya R., Avitsur R., Donchin O. and Cohen E. (1995) Interleukin-1 inhibits sexual behavior in female but not in male rats. *Brain, behavior, and immunity* **9,** 220-233.

Zhang Y. H., Lu J., Elmquist J. K. and Saper C. B. (2003) Specific roles of cyclooxygenase-1 and cyclooxygenase-2 in lipopolysaccharide-induced fever and Fos expression in rat brain. *J Comp Neurol* **463,** 3-12.

Zheng H., Fletcher D., Kozak W., Jiang M., Hofmann K. J., Conn C. A., Soszynski D., Grabiec C., Trumbauer M. E., Shaw A. and et al. (1995) Resistance to fever induction and impaired acute-phase response in interleukin-1 beta-deficient mice. *Immunity* **3,** 9-19.