THE REGULATION OF C-FOS INDUCTION IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS

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- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
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- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
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- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
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Geotechnology 0428
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Plastics Technology 0795
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"My chapter defines the concept of stress. I am not certain whether one who undertakes this task has an enormous ego, is immeasurably stupid, or is totally mad."

Seymour Levine, in G.P. Moberg (ed.), Animal Stress, 1985.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Immunocytochemical detection of Fos protein was used to test the hypothesis that *c-fos* expression in the PVN is regulated by neural pathways which are modulated by adrenal and gonadal steroids.

Basal levels of Fos-like immunoreactivity (FLI) were very low in the PVN but rose following adrenalectomy (ADX), unilateral ADX (uADX) or sham ADX (30 minutes - 8 hours; ADX ≥ sham ADX > uADX). No circadian variation in basal or ADX-induced FLI was observed. Opposite effects were seen in two supposedly hyporesponsive models, i.e., Lewis rats displayed smaller responses to uADX or sham ADX compared to Sprague-Dawley rats, but adrenal surgery (AS) induced more FLI in 3 and 11 day old rats compared to adults. FLI response to AS was attenuated by exogenous corticosterone, but FLI levels were potentiated after similar surgeries (ovariectomy or laparotomy) in previously (7 days) ADX rats. Lactating females (LF; postpartum day 7-21) showed a profound suckling-dependent inhibition of the FLI response to AS. This attenuation was also noted in LFs treated with NMA (a glutamate agonist) or Metrazole (a convulsant).

The influence of sex steroids on *c-fos* gene expression in the PVN was revealed using the NMDA antagonist MK801. MK801 (1 mg/kg) induced FLI in the PVN of males, females, and lactating females (M>F>LF). At lower doses, (0.1 or 0.3 mg./kg.) sex differences were reversed (F>M). Effects of gonadectomy and androgenization were inconclusive but suggest that observed sex differences might be androgen dependent. At all doses tested, behavioural anomalies were greater in females.

These results emphasize the difficulty of isolating the specific effects of experimental manipulations from those of non-specific stressors. Data presented suggest that quantification of FLI in the PVN is a useful approach to study the influence of adrenal and gonadal steroids on PVN function, and that the lactating female rat and Lewis rat should be useful models for further investigations in this area.

LIST OF ABBREVIATIONS

ACTH - adrenocorticotropic hormone (corticotropin)

ADH - antidiuretic hormone

ADX - adrenalectomy

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANDR - androgenized

ANOVA - analysis of variance

ANS - autonomic nervous system

AP-1 - activator protein-1

AR - androgen receptors

AS - adrenal surgery

BNST - bed nucleus of the stria terminalis

CBG - corticosterone binding globulin

CCK - cholecystokinin

CNS - central nervous system

CORT - corticosterone

CRB - Cambridge Research Biochemicals

CRH - corticotropin releasing hormone

DPBS - Dulbecco's phosphate buffered saline

F - female

EB - estradiol benzoate

ER - estrogen receptors

FLI - Fos-like immunoreactivity

Fra - Fos related antigen

GABA - gamma-amino-butyric acid

GC - glucocorticoid

GH - growth hormone (somatotropin)

GHRH - growth hormone releasing hormone

GnRH - gonadotropin releasing hormone

gonadX - gonadectomy

GR - glucocorticoid receptor

HC - hippocampus

HCG - human chorionic gonadotropin

HPA - hypothalamo-pituitary-adrenal

HPG - hypothalamo-pituitary-gonadal

HPT - hypothalamo-pituitary-thyroid

i.c.v. - intracerebroventricular

IEG - immediate-early gene

IL-6 - interleukin-6

i.p. - intraperitoneal

K - kilobyte

KA - kainic acid

LC - locus coeruleus

LHRH - luteinizing hormone releasing hormone

LSHRP - stress-hyporesponsive period during lactation

LUT - look-up-table

M - male

Mb - megabyte

ME - median eminence

MK801 - dizocilpine maleate

MR - mineralocorticoid receptor

mRNA - messenger ribonucleic acid

NANDR - non-androgenized

NE - norepinephrine

NIH - National Institutes of Health

NMA - N-methyl-$_{D,L}$-aspartate

NMDA - N-methyl-$_{D}$-aspartate

NPY - neuropeptide-Y

NTS - nucleus tractus solitarius

oil - sesame oil

OVX - ovariectomy

PBS - phosphate buffered saline

pd - postpartum day

POA - preoptic area

POMC - pro-opiomelanocortin

PVN - paraventricular nucleus of the hypothalamus

RAM - random access memory

RAS - reticular activating system

s.c. - subcutaneous

SCN - suprachiasmatic nucleus

S-D - Sprague-Dawley

SFO - subfornical organ

SHRP - stress hyporesponsive period

SON - supraoptic nucleus

TIFF - Tag Image File Format

TP - testosterone propionate

TRH - thyrotropin releasing hormone

TSH - thyroid stimulating hormone

Tx - Triton X-100

uADX - unilateral adrenalectomy

uOVX - unilateral ovariectomy

VMH - ventromedial nucleus of the hypothalamus

VP - arginine vasopressin (antidiuretic hormone; ADH)

2-DG - 2-deoxyglucose

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INTRODUCTION

All animals, from unicellular organisms to mammals, possess the ability to modify their activity in response to changes in their external environment. Higher animals, when faced with real or anticipated physiological challenge, exhibit a collection of behavioural, neural and humoral adjustments intended to maintain or restore homeostasis. Generally, these adjustments, which are collectively referred to as the stress response, are adaptive. Without them, organisms suffer illness or death, and species become depleted or extinct. In some instances however, activation, particularly chronic activation, of the stress response is maladaptive. Stress, or an inability to manage stress, is believed to play a significant role in the etiology of some diseases (e.g., gastric ulcers, melancholic depression), and is known to exacerbate many others (e.g., rheumatoid arthritis, inflammatory bowel disease). Unchecked, the stress response can inflict damage to the nervous system, suppress growth and reproductive function, and depress immunocompetence.

Selye (1973) defined stress as a non-specific response of the body to any demand made upon it. More recently, it has become apparent that organisms do not respond to stress in a non-specific manner. Rather, the nature of the stress response is determined by the strength and duration of the stressor, and the constitution and state of the organism. For example, in rats, hypoglycemia induces arginine vasopressin (VP) secretion but does not effect plasma oxytocin levels; restraint stress increases oxytocin release without effecting VP secretion; and hemorrhage induces secretion of both oxytocin and VP. Qualitative and quantitative differences in the stress-response exist across species (e.g., primates versus rodents), and within some species (e.g., Lewis rats are reported to be

stress-hyporesponsive compared to other rat strains), but a number of cellular responses (e.g., the production of heat shock proteins; Blake et al. 1990) are highly conserved across phylogenic boundaries.

The stress system represents an excellent model for studying short- and long-term intracellular changes which occur in neurons as a result of extracellular stimulation. Although the principal physiological processes involved in the stress response are well documented, the cellular mechanisms underlying these processes are less well understood. As a first step towards determining the nature of the response of stress system neurons to various forms of stress, experiments in this thesis seek to localize individual neurons activated during the stress response, and to identify factors which determine or regulate the level of activation of these neurons. It is anticipated the the results of this thesis will contribute to understanding the processes which control stress system operation. Also, determining factors involved in stimulating or blocking the activation of specific central nervous system (CNS) neurons during the stress response is critical to developing effective clinical strategies for management of stress-related disorders.

This Introduction outlines the basic physiology of the stress response (particularly the neuroendocrine stress response), then focusses specifically on the phenomenon of hyporesponsiveness of the hypothalamo-pituitary-adrenal (HPA) system. This is followed by a description of the use of early onset gene expression as a mapping tool to probe cellular responses to stressful stimuli.

I. NEUROBIOLOGY OF THE STRESS SYSTEM

Contemporary theory holds that stress-induced changes in activity are the result of activation of an integrated stress system. The principal components of this system include the corticotropin releasing hormone (CRH), and the locus coeruleus/norepinephrine (LC/NE; sympathetic) nervous systems. These systems are interrelated in a positive, reverberatory feedback loop (see Chrousos and Gold 1992). The CRH system extends throughout the brain but is particularly well developed in the paraventricular nucleus of the hypothalamus (PVN). Activation of the CRH system activates both the HPA axis, and the LC/NE nervous system. The LC/NE system is centered in the rostral pons. Activation of LC/NE neurons results in the release of NE from a dense network of neurons which extends throughout the CNS. Its actions include excitation of peripheral sympathetic neurons and inhibition of peripheral parasympathethetic neurons, and stimulation of catecholamine secretion from cells of the adrenal medulla. Stimulation of the CRH and LC/NE stress systems induces activity in dopaminergic and noradrenergic circuits which project to brain regions implicated in motivation, emotion, and cognitive function (the prefrontal cortex, nucleus accumbens, and amygdala/hippocampal complex), and, via CRH neurons in the PVN, activates pro-opiomelanocortin (POMC) producing neurons in the arcuate nucleus (see Chrousos and Gold 1992). While all aspects of the integrated stress system exert significant effects, the balance of this thesis will concentrate upon the role of the PVN in stress-induced activation of the HPA axis.

The Hypothalamo-Pituitary-Adrenal (HPA) axis

Activation of the HPA axis, which results in elevated plasma glucocorticoid (GC) titers, is a hallmark of the generalized stress response. GC are produced and released from cells of the zona glomerulosa of the adrenal cortex when these cells are stimulated by adrenocorticotropic hormone (ACTH; also known as corticotropin). ACTH is produced and secreted by cells (corticotropes) in the anterior lobe of the pituitary gland under the control of CRH and other hormones (VP, oxytocin, and epinephrine; Plotsky 1985) secreted from the PVN, and by negative feedback by circulating GC. Production and secretion of CRH is also regulated by GC negative feedback, and by a complex combination of factors outlined below. Stimuli which influence or regulate components of the HPA axis are illustrated schematically in Figure 1.

The Paraventricular Nucleus of the Hypothalamus

During the early part of this century, the PVN was known variously as the nucleus of Malone, filiform nucleus, and subventricular nucleus, and initially, was thought to consist exclusively of magnocellular neurons. More recently, distinct magnocellular and parvocellular divisions have been described and a detailed picture of its composition has evolved. In the rat, the PVN is a densely-packed, wing-shaped conglomeration of approximately 10,000 neurons. It lies on either side of the third ventricle, occupying about 0.5 mm^3 of tissue in the rostral portion of the hypothalamus (Swanson and Sawchenko 1983; Figure 2). It is composed of topographically segregated populations of neurons that project direct efferents to the posterior pituitary, neurohemal zone of the median eminence (ME), and brainstem and spinal cord.

On the basis of cytoarchitecture, neural circuitry, and neurochemistry, Swanson and Kuypers (1980) divided the PVN into a total of eight subnuclei

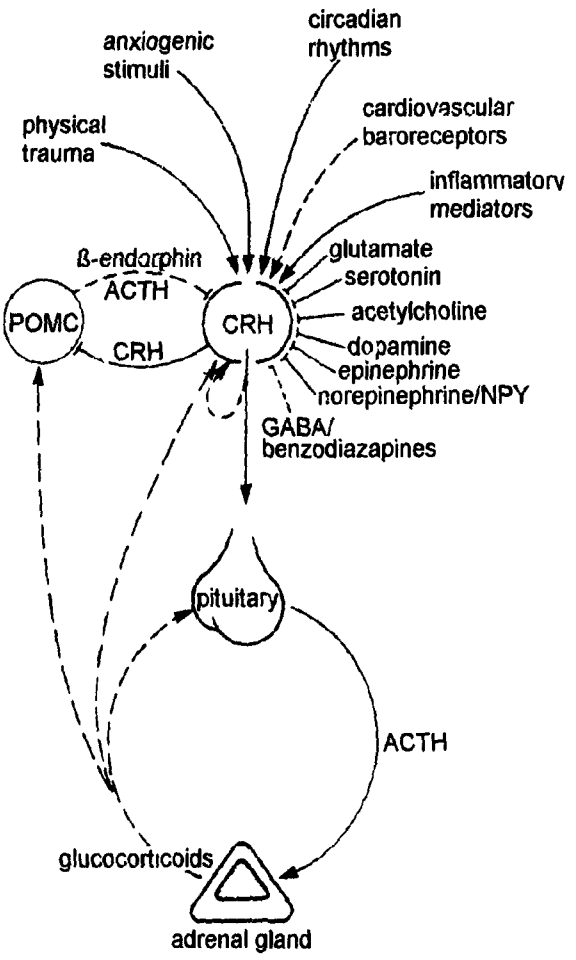
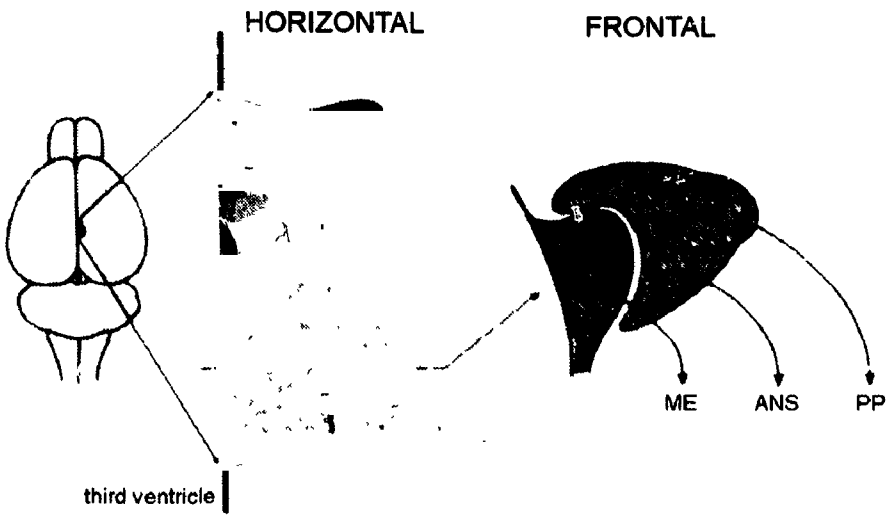


Figure 1 Schematic representation of the putative regulation of the HPA axis. (solid lines = excitatory influences; broken lines = inhibitory influences). ACTH = adrenocorticotropic hormone; CRH = corticotropin releasing hormone; GABA = gamma-amino-butyric acid; NPY = neuropeptide-Y; POMC = proopiomelanocortin (adapted from Johnson et al 1992).



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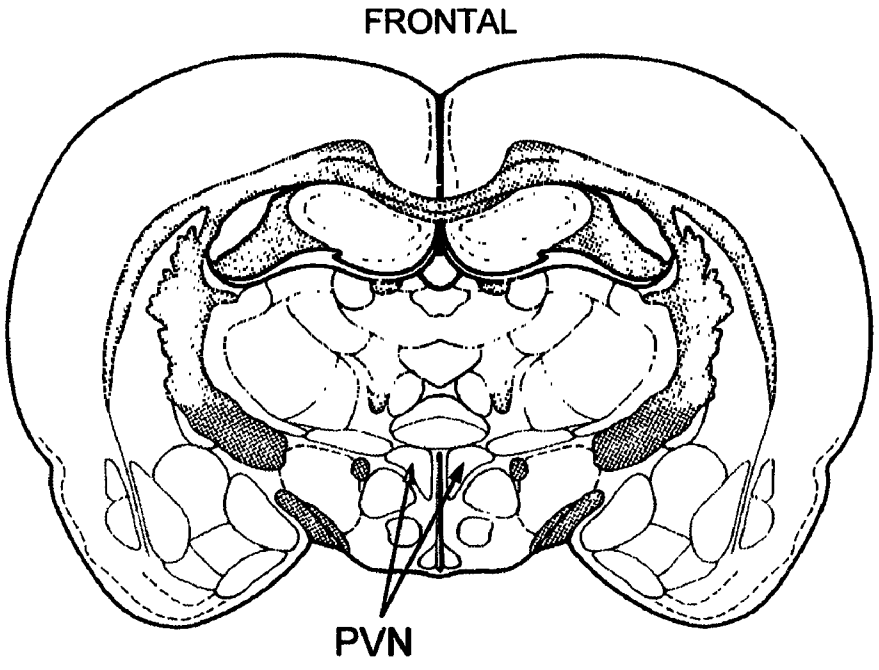


b

Figure 2 Schematic representation of the PVN illustrating its location in the rat brain and general topography (a, Swanson et al 1986; b. adapted from Palkovits and Brownstein 1988).

(Figure 3a). More recently, largely on the basis of evidence gleaned from immunocytochemical and retrograde tracer studies, the PVN has been partitioned, on the basis of distinct efferent projections, into three functional zones (medial, intermediate, and lateral) (Swanson et al. 1986; see Figure 3b). The medial, parvocellular zone consists of partially overlapping subpopulations of neurosecretory cells with cell bodies concentrated in the dorsal medial parvocellular and periventricular regions of the PVN. These cells project to the external lamina of the ME and release CRH and other members of the family of neurohormones commonly known as hypothalamic releasing factors/hormones to the hypophyseal portal blood. Geographically distinct neurochemical regions have been identified within the parvocellular zone (Swanson et al. 1986; Figure 3c). Cells containing somatostatin are concentrated in the most medial region, adjacent to the third ventricle; cells containing TRH are most plentiful in the intermediate region of this zone; and cells containing CRH are most densely concentrated in the more lateral portions of the parvocellular zone. Small numbers of neurosecretory dopamine and GHRH cells are also found in the medial parvocellular zone (see Swanson 1991).

The intermediate, mediocellular zone of the PVN consists of neurons which occupy the dorsal, ventral medial, and lateral parvocellular regions of the PVN and project to other parts of the hypothalamus, and to the brainstem and spinal cord. Evidence suggests that these neurons, referred to as the "descending group" by Swanson and Simmons (1989), can be further partitioned into a dorsal portion in which 90% of the cells project to sympathetic preganglionic neurons in the spinal cord, and a ventral medial portion which consists of an intermixture of cells projecting to parasympathetic neurons in the dorsal vagal complex, and to sympathetic preganglionics in the spinal cord (Swanson and Kuypers 1980). Thus, efferents from the intermediate mediocellular zone can influence output to

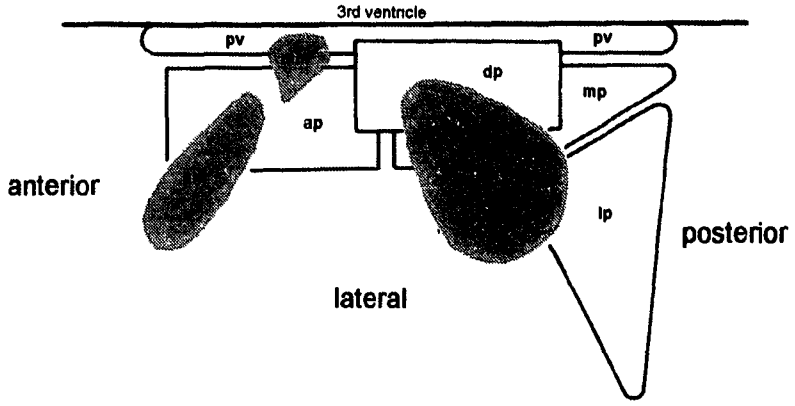


Figure 3a Schematic representation of a the rat PVN, as viewed from above, showing the 8 major sub nuclei. pv-periventricular, ap-anterior parvocellular, dp-dorsal parvocellular, mp-medial parvocellular, lp-lateral parvocellular, am-anterior magnocellular, mm-medial magnocellular, pm-posterior magnocellular (redrawn from Swanson et al 1981).

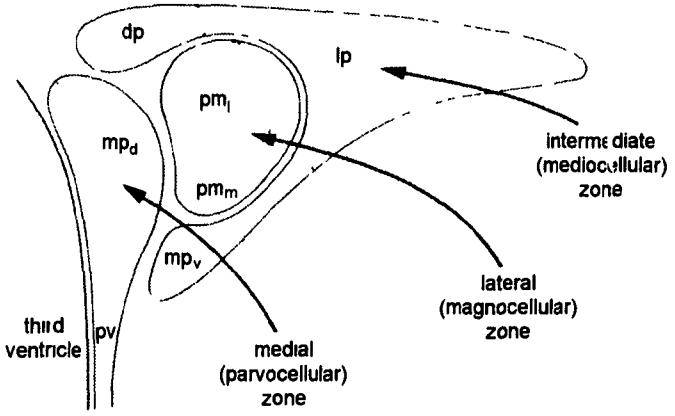


Figure 3b Schematic representation of the relationship between the cytoarchitecture, and 3 functional zones of the PVN (the medial, parvocellular zone which projects to the median eminence, the intermediate, mediocellular zone which projects to autonomic centers in the brainstem and spinal cord, and the lateral magnocellular zone which projects to the posterior pituitary). The caudal half of the nucleus is shown, collapsed upon itself, in the frontal plane. pv-periventricular, mp_d-dorsal medial parvocellular, mp_v-ventral medial parvocellular, lp-lateral parvocellular, dp-dorsal parvocellular, pm_l-lateral posterior magnocellular, pm_v-medial posterior magnocellular (adapted from Sawchenko and Swanson 1983).

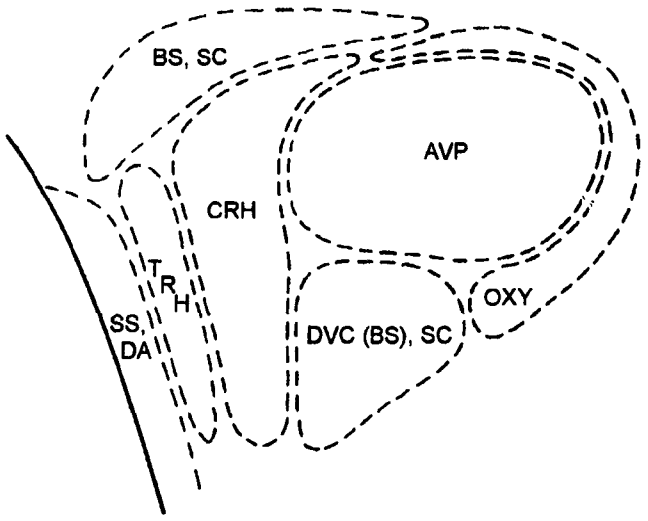


Figure 3c Schematic representation of the neurochemical topography of the PVN illustrating the location of the highest concentrations of various populations of neurons. AVP - vasopressin, BS - brainstem, CRH - corticotropin releasing hormone, DA - dopamine, DVC - dorsal vagal complex, OXY - oxytocin, SC - spinal cord, SS - somatostatin, TRH - thyrotropin releasing hormone (adapted from Swanson et al 1986).

the central gray and reticular formation, the central relays for visceral and nociceptive afferents, and both branches of the autonomic nervous system (ANS).

The lateral, magnocellular zone consists of large VP- and oxytocin-synthesizing neurons with cell bodies located primarily in the anterior, medial, and posterior magnocellular regions of the PVN. The axons of these cells extend to the posterior pituitary as part of the hypophyseal tract. Immunocytochemical studies indicate this zone is partitioned neurochemically into anterior and medial portions which are almost exclusively oxytocinergic, and a posterior portion which consists of an anteromedial oxytocinergic region and a posterolateral vasopressinergic region (see Swanson et al. 1986).

In summary, placed in the context of the cytoarchitectural partitions of the PVN, the anterior and medial magnocellular regions are almost exclusively oxytocinergic, whereas the posterior magnocellular region comprises an anteromedial oxytocinergic and posterolateral vasopressinergic portion. Within the parvocellular PVN, the majority of somatostatin cells are located in the periventricular region, while CRH and thyrotropin releasing hormone (TRH) neurons are concentrated in the dorsal part of the medial parvocellular region. Efferents to the dorsal vagal complex and spinal cord originate from the ventral medial, and lateral parvocellular subnuclei, with and the majority of efferents from the dorsal parvocellular region projecting to the spinal cord (Swanson and Kuypers 1980; Swanson et al. 1986). The efferent connections of the PVN are summarized schematically in Figure 4.

The PVN receives extensive input from numerous different cell populations (see Figure 4). Many of these innervate more than one cell group within the PVN. These are most appropriately considered in terms of the three functional divisions outlined above. Swanson et al. (1986) described four sources of afferents to parvocellular CRH neurons: 1) catecholaminergic and

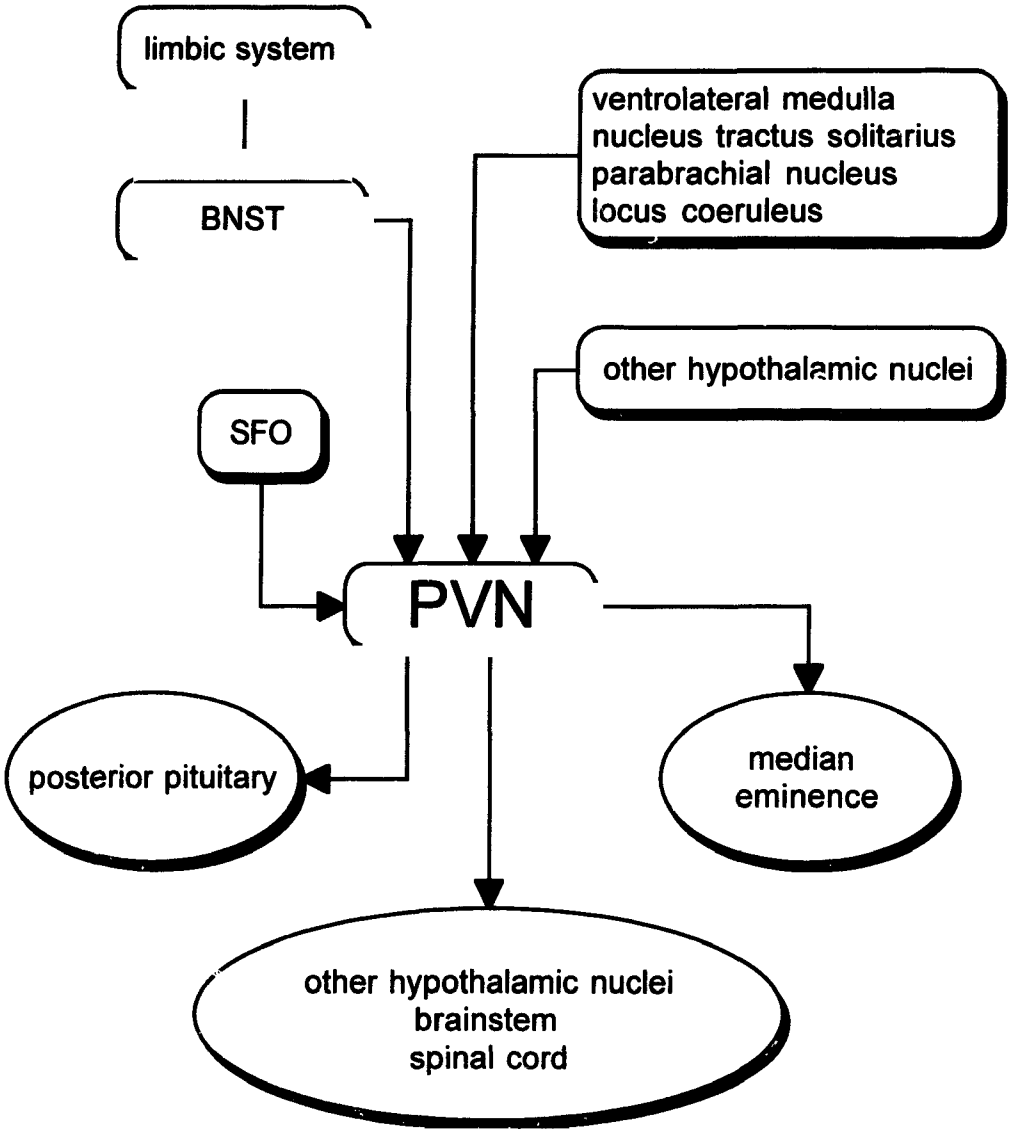


Figure 4 Schematic representation of the major afferent and efferent connections of the rat PVN (BNST = bed nucleus of the stria terminalis, SFO = subfornical organ).

neuropeptide-Y (NPY) fibers ascending from the ventrolateral medulla, nucleus tractus solitarius (NTS), parabrachial nucleus, and LC [many of these are likely relaying visceral sensory information from the vagus and glossopharyngeal nerves to the dorsal medial parvocellular region of the PVN (see Swanson and Sawchenko 1983; Swanson et al. 1986; Swanson 1991)]; 2) anti-angiotensin II-immunoreactive neurons projecting from the subfornical organ (SFO) (which mediates the effects of plasma angiotensin II on fluid balance, and projects to all parts of the PVN); 3) axons arising from the ventral area of the BNST (which receives direct afferents from regions of the ventral hippocampus (HC) and amygdala, and likely transmits cognitive information which influences secretions of the HPA axis); and 4) afferents from other areas of the hypothalamus including the substantia innominata, suprachiasmatic nucleus (SCN), arcuate nucleus, and various regions of the preoptic area (POA) (Swanson 1991).

Magnocellular regions of the PVN receive relatively few direct afferents from forebrain structures, but may be influenced indirectly via axons from parvocellular neurons (Sawchenko and Swanson 1983). Direct afferent connections from the zona incerta and paraventricular nucleus of the thalamus have also been reported (see Swanson et al. 1987). Vasopressinergic magnocellular areas of the PVN receive excitatory noradrenergic projections from the A_1 region of the caudal ventrolateral medulla. Monosynaptic pathways projecting from the SFO and medial POA, and sparse afferents from the NTS, parabrachial nucleus, and medullary adrenergic neurons, have also been identified (Sawchenko and Swanson 1983). These authors also localized a direct afferent connection from the SFO to the preautonomic (mediocellular) cell group, and several authors (see Sawchenko et al. 1986) have reported evidence which suggests that the preautonomic regions of the PVN receive input from all of the brainstem areas which project to the parvocellular region.

Genes for over a dozen neuropeptides, along with a number of nonpeptidergic compounds (see Swanson 1991), are expressed by neurons in all three functional groups in the PVN. Swanson and Simmons (1989) suggest two general principles concerning peptide distribution within the PVN. First, some peptides, including CRH, are expressed by neurons in all three groups; and second, under appropriate conditions, several peptides may be co-expressed within subpopulations of neurons. Small populations of oxytocin-, VP-, dopamine-, somatostatin-, enkephalin-, neurotensin-, CRH-, and angiotensin-containing neurons, that project to the brainstem and spinal cord, have been identified (see Swanson et al. 1986). Changes in gene expression induced by disrupted glucocorticoid feedback (Kiss et al. 1984; Wolfson et al. 1985; Swanson and Simmons 1989), or altered ascending adrenergic stimulation (Sawchenko 1988) demonstrate that the ratios of neuropeptides in specific neurons within the PVN can be altered by neural or hormonal stimulation.

Corticotropin Releasing Hormone

CRH is the putative mediator that links the CNS to the endocrine response to stress. Swanson et al. (1983) identified three functionally distinct systems of CRH neurons within the rat brain. One system projects from the PVN to the neurohemal zone of the ME where axons terminate on capillaries of the hypophyseal portal system. Lesions of the PVN eliminate virtually all CRH immunoreactivity from the ME (Antoni et al. 1983). The cell bodies of these neurons are located in all eight subnuclei of the PVN but the largest group is located in the dorsal medial parvocellular regions of the nucleus (Swanson et al. 1983). CRH secreted from these neurons enters the hypophyseal circulation, is carried to the anterior lobe of the pituitary, and stimulates pituitary corticotropes to synthesize and secrete ACTH. This pathway contains the only CRH neurons

which exhibit increased immunostaining for CRH following adrenalectomy (ADX) (Swanson et al. 1983). A variety of other peptides including oxytocin, VP, neurotensin, enkephalin, and cholecystokinin (CCK) have been identified in CRH-containing neurons in the PVN (see Swanson et al. 1986). In addition to the CRH neurons projecting to the ME, a small number of CRH-containing PVN neurons which project to the brainstem and spinal cord have also been identified (Swanson et al. 1986).

A second group of CRH neurons is located in regions of the basal forebrain, hypothalamus, and brainstem that are reported to play central roles in the mediation of ANS function. High concentrations of CRH neurons are located in the central nucleus of the amygdala, substantia innominata, BNST, areas of the medial POA, lateral hypothalamus, periaqueductal gray area, laterodorsal tegmentum, locus coeruleus, parabrachial nucleus, dorsal vagal nucleus, and ventrolateral parts of the medullary reticular formation. These areas are interconnected by fibers in the medial forebrain bundle and its dorsal extension in the reticular activating system, and by a periventricular system in the thalamus and central gray area.

A third group of CRH neurons is scattered throughout nearly all regions of the cerebral cortex. Most of these appear to be bipolar interneurons running perpendicular to the surface of the cortex. These are found in higher concentrations in limbic regions such as the prefrontal areas and cingulate gyrus (Swanson et al. 1983).

CRH neurons have also been located in the spinal cord, sympathetic ganglia and adrenal glands (Suda et al. 1984) and CRH has been detected in organs of the immune (Ritchie et al. 1986), endocrine (Petrusz et al. 1983) and digestive (Petrusz et al. 1984) systems. High concentrations of CRH receptors have been identified on the corticotropes of the anterior pituitary and in the brain, adrenal

medulla and sympathetic ganglia (Cummings et al. 1983; De Sousa et al. 1985; Valentino et al. 1988). In rats, the distribution of CRH receptors is closely related to known pharmacological sites of action of CRH and areas known to receive CRH innervation (De Sousa et al. 1985)

Secretion of CRH from PVN neurons is stimulated by emotional arousal (likely by way of afferent pathways from limbic structures), noxious stimulation [via afferent projections from the lateral spinothalamic tracts by way of the reticular activating system (RAS)], and blood pressure changes (via afferents from the NTS).

In humans, maternal plasma CRH levels (from the placenta) are dramatically increased in the third trimester of pregnancy, and rise at the onset of parturition. Surprisingly, no further increase in plasma CRH is seen during the stress of labour, and no changes in circulating ACTH or cortisol levels are seen at any time during pregnancy (Goland et al. 1986; Campbell et al. 1987).

The Pituitary Gland

The pituitary gland (hypophysis) lies inferior to the median eminence of the hypothalamus, and rests in a concavity in the sphenoid bone called the sella turcica. It consists of 2 major lobes separated by a less well developed intermediate lobe. The posterior lobe, also known as the neurohypophysis or pars nervosa, is connected to the hypothalamus by the hypophyseal tract which contains the axons of oxytocinergic and vasopressinergic magnocellular neurons of the SON and PVN. The neurohormones oxytocin and VP [also known as antidiuretic hormone (ADH)] are secreted to the circulation from the axon terminals of these neurons The anterior lobe, also known as the adenohypophysis or pars distalis, is derived embryologically from glandular epithelial tissue and consists of a heterogeneous population of cells which

produce and secrete a family of tropic hormones which include ACTH. Secretion of each of these hormones is regulated by releasing/inhibiting hormones produced in the hypothalamus and carried to the anterior lobe by the blood via the hypophyseal portal circulation.

Adrenocorticotropic Hormone

ACTH is produced by corticotropes of the anterior pituitary from POMC synthesized in the brain, gut and some endocrine organs. Within the corticotropes, POMC is split into ACTH and ß-lipotropin (further processing of ß-lipotropin results in the formation of ß-endorphin). From its site of formation in the anterior pituitary, ACTH is transported to the adrenal glands by the systemic circulation. There, it stimulates the synthesis and secretion of the corticosteroid hormones including the GC. Besides stimulating hormone synthesis, CRH is the primary stimulator of ACTH secretion. However, under stress, peptides other than CRH also influence ACTH levels. These include VP, oxytocin, angiotensin-II, vasoactive intestinal polypeptide, serotonin and (in the rat) epinephrine and NE. When VP is secreted from parvocellular neurons in the PVN, it acts synergistically with CRH in stimulating ACTH release. Glucocorticoids inhibit ACTH release directly by inhibiting the corticotropes of the anterior pituitary, and indirectly by inhibiting CRH neurons in the PVN.

The Adrenal Glands

The paired adrenal (suprarenal) glands are located at the superior poles of the kidneys. Each gland consists of an inner adrenal medulla, which produces and secretes catecholamines in response to direct neural stimulation by sympathetic pre-ganglionic neurons, surrounded by an outer adrenal cortex which produces a variety of steroid hormones in response to humoral stimulation. The adrenal

cortex consists of 3 anatomically and neurochemically distinct layers. The outer zona glomerulosa produces and secretes mineralocorticoid hormones. The zona fasciculata, which lies beneath the zona glomerulosa, produces and secretes the GC hormones, and the innermost zona reticularis, which lies adjacent to the adrenal medulla, produces GC and small amounts of androgens and estrogens. All hormones produced by the adrenal glands are implicated in the stress response. In this thesis, however, discussion will be restricted to the role of GC in feedback regulation of the HPA axis.

Glucocorticoids

Glucocorticoids are produced by cells in the zona glomerulosa of the adrenal cortex. At normal circulating levels, GC perform a largely permissive role (enabling other hormones to function) which is essential for maintaining homeostasis. At elevated levels, GC assume an additional regulatory role, suppressing inflammatory and immune responses, and inhibiting the production or secretion of other hormones.

In mature animals, a large percentage of circulating GC (95% in humans) is bound to corticosteroid-binding globulin (CBG) and is physiologically inactive. The residual free fraction is available to bind to GC receptors which are found throughout the brain. Sapolsky and Meaney (1986) divided GC receptors into 2 functional categories i.e., "bioactive" and "auxiliary". When GC binds to bioactive GC receptors, the resulting hormone-receptor complex is able to pass through the nuclear membrane, bind to chromatin, and thereby influence genomic events (Fuxe et al. 1987; Liposits et al. 1987; Evans and Ariza 1989). This is believed to be the mechanism by which GC influence activity of target cells. Complexes formed by attachment of GC to auxiliary receptors, are unable to enter cell nuclei and are thereby unable to influence genomic events.

Actions of GC on the CNS are mediated by two distinct types of bioactive intracellular receptors. Type I (mineralocorticoid) receptors (MR) are localized in relatively few brain areas. The highest concentrations are found in limbic structures, and relatively few are found in the anterior pituitary (see Jacobson and Sapolsky 1991). MR bind corticosterone (CORT) and aldosterone with high affinity. Eighty to ninety percent of these receptors are occupied at normal circulating corticosteroid levels (Reul and De Kloet 1985; Reul et al. 1987a,b). MR in limbic structures are believed to mediate a tonic action of CORT which regulates the threshold for activation of the stress response. Type II (glucocorticoid) receptors (GR) are widely distributed in the brain. High concentrations of GR are located in the hypothalamus (particularly in CRH neurons) and in brain areas that contain POMC (HC, lateral septal area, amygdala, NTS, LC), and in the anterior pituitary. GR bind CORT with a tenfold lower affinity than MR. In unstressed conditions, only 10-15% of GC receptors are occupied. This rises to 75% following immobilization stress or CORT injection (see Meaney et al. 1991). GR are believed to be involved in terminating stress-induced activation of neuroendocrine pathways (see Joëls and de Kloet 1992). Depletion of GR, such as occurs with age, impairs the negative feedback control of GC secretion resulting in chronically elevated plasma GC levels. This can result in degeneration of hippocampal neurons (Gould and McEwen 1993).

In addition to the GC receptors described above, transcortin receptors, which are physically and chemically identical to CBG, have been identified in pituicytes, and hippocampal neurons. Transcortin receptors bind CORT with high affinity. Unlike GC attached to GR or MR receptors, GC molecules attached to transcortin receptors, like those bound to circulating CBG, are rendered physiologically inactive. This enables these molecules to perform an important role in regulating the stress response.

Finally, GR and MR receptors have recently been identified in the plasma membrane of hippocampal and hypothalamic (PVN) neurons. Liposits and Bohn (1993) suggest that whereas bioactive intracellular GC receptors are implicated in signal transduction at the level of the genome, membrane receptors might participate in GC-regulated changes in membrane excitability as these changes occur too rapidly to be attributed to genome-related events.

Contribution of other Factors/Hormones

Along with the secretion products of the HPA axis, several other endogenous factors participate in the stress response. These include the catecholamines, opioid peptides, secretion products of the hypothalamo-pituitary-thyroid (HPT) and hypothalamo-pituitary-gonadal (HPG) axis, somatostatin and growth hormone, prolactin, VP, oxytocin, angiotensin-II, NPY and the peptides of the immune system (interleukins, tumour necrosis factor, interferons and transforming growth factors).

Cytokines and inflammatory mediators activate the HPA axis inducing increased ACTH and GC secretion (Brown et al. 1990). GC in turn, suppress cytokine production (Perretti et al. 1989) and function, inhibit leukocyte migration, disrupt cell-mediated immunity and enhance suppressor T-cell function. Sustained exposure to elevated levels of GC results in leukopenia and a loss of thymic, splenic, and lymph node tissue mass. Although GC possess potent immunosuppressive and anti-inflammatory qualities, not all stress-induced modulation of immune function is HPA axis dependent. Stress-induced suppression of lymphocyte function has been observed in adrenalectomized and hypophysectomized rats (Keller et al. 1983, 1988). Surprisingly, CORT secretion sometimes occurs in the apparent absence of pituitary corticotrope involvement. Evidence suggests that the immune system can directly stimulate the secretion of GC during antigenic challenge. Smith et al. (1982) reported that Newcastle disease virus induced an increase in plasma CORT titers in hypophysectomized mice and suggested the possibility of a lymphoid-adrenal axis.

II. STRESS-HYPORESPONSIVENESS

Some animals reportedly fail to mount a typical response to some or all stressors. In the rat, at least three models of hyporesponsiveness have been identified. Newborn rat pups, lactating female rats, and Lewis rats are all reported to exhibit an attenuated HPA response to stress.

Neonatal HPA Function

CRH or its messenger ribonucleic acid (mRNA) is detectable in rat fetal tissue from embryological day 17 (Grino et al. 1989a; Baram and Lerner 1991). During the latter third of fetal life, circulating CORT titers are in or near the normal adult range (Martin et al. 1977; Henning 1978; Meaney et al. 1985) and the HPA axis responds to stress and negative feedback control (Dupouy and Chatelain 1984). Also, the fetal adrenal cortex contains an additional "fetal zone" of steroid secreting tissue.

At birth, rat pituitary and adrenal tissues are functionally mature but the hypothalamic-hypophyseal portal system does not develop fully until 2 weeks after birth (Glydon 1957). Neonatal pituitary tissue contains adult levels of CRH receptors (Walker et al. 1986a) and double the adult concentration of corticotropes (Childs et al. 1982), and produces ACTH when stimulated by CRH (Guillet and Michaelson 1978; Walker et al. 1986a). In vitro studies reveal that throughout the postnatal period, adrenocortical tissue is capable of synthesizing and secreting CORT at roughly the same rate as adult tissue (Levine et al. 1972).

Towards the end of the prenatal period, hypothalamic CRH mRNA and anterior pituitary POMC mRNA levels drop sharply (Grino et al. 1989a). By postnatal day 1, basal hypothalamic CRH peptide content is about 1/10 of the adult value (Rundle and Funder 1988; Walker et al. 1986a). During the immediate postnatal period, circulating CORT levels remain high, and for the

first two days of postnatal life, rat pups demonstrate adult-like CORT responses to many stressors (Haltmeyer et al. 1966; Butte et al. 1973; Arai and Widmaier 1991; Walker et al. 1991). During this time, however, the "fetal zone" of the adrenal involutes (Van Dorp and Deane 1950), circulating CORT levels drop sharply (Martin et al. 1977; Meaney et al. 1985), and around the fourth day of postnatal life, rat pups enter a stress-hypo-responsive period (SHRP) which persists until the end of the second postnatal week. (Levine 1970; Gray 1971; D'Agostino and Henning 1981; Meaney et al. 1985; Walker et al. 1986a, 1990; Grino et al. 1989a; Muret et al. 1992).

The first reference to stress-hyporesponsiveness in the neonatal HPA system was by Jailer (1950). Initially, this stage of development was believed to be a "stress-non-responsive-period" (Schapiro et al. 1962) but its status was later revised to hyporesponsive when several stressors were shown to induce increased HPA activity during this period. To date, the precise mechanisms underlying this stress-hyporesponsiveness are not fully understood but alterations of structure and function at the level of the brain, pituitary and adrenal glands have been implicated.

Throughout the SHRP, the concentrations of CRH binding sites in the striatum, HC and frontal cortex are well above adult levels (Pihoker et al. 1992), but hypothalamic CRH mRNA (Grino et al. 1989a) and peptide (Walker et al. 1986a; Rundle and Funder 1988) levels are below adult values. Anterior pituitary corticotrope (Childs et al. 1982), and POMC mRNA (Grino et al. 1989a; Vazquez and Akil 1992) and peptide (Vazquez and Akil 1992) levels are also depressed during the SHRP, but ß-endorphin levels are high and respond to stress (Iny et al. 1987). Although the animal is growing rapidly, no change in adrenal weight occurs between postnatal day 5 and postnatal day 11 (Schoenfeld et al. 1980), and adrenal (Levine 1970) and circulating (Walker and Vrana 1993) CORT

concentrations remain below adult levels. Circulating CORT concentrations do not reach adult levels until postnatal day 15 (Henning 1978; Guillet et al. 1980; Meaney et al. 1985; Vazquez and Akil 1992), the age at which the SHRP is reported to end.

The GC receptor system also undergoes extensive modification during the neonatal period. In adult animals, GC receptor concentrations are regulated by plasma CORT titers but this is not the case during the first 3-4 weeks of life (Meaney et al. 1985). At birth, rat brain and pituitary tissue contain adult levels of GC receptors. Pituitary GC receptor concentrations remain at adult levels throughout the SHRP (Olpe and McEwen 1976), but brain GC receptor concentrations drop to 20% of the adult basal level by the onset of the SHRP, then rise slowly, returning to adult values by the end of the SHRP (Meaney et al. 1985). This pattern is paralleled by pituitary transcortin receptor concentrations (Sakly and Koch 1981, 1983), and circulating CBG (Henning 1978; D'Agostino and Henning 1981) and CORT levels. Leeper et al. (1988) suggest that, since there is no evidence of increased CORT production at the end of the SHRP (Henning et al. 1986), the observed increase in circulating CORT is at least partly due to increased retention in the blood. This interpretation is consistent with the observation that given a fixed supply of CORT (via ADX and CORT pellet implantation), 12-20 day old rats display age-dependent increases in plasma CORT levels which parallel concurrent increases in plasma CBG levels (Schroeder and Henning 1989). Sapolsky and Meaney (1986) suggest that the timing of the neonatal SHRP correlates well with the ontogeny of pituitary transcortin receptors which damp pituitary sensitivity to CORT negative feedback by reducing nuclear uptake of CORT (see Meaney et al. 1991).

Hippocampal GC receptor levels are also well below adult levels at the onset of the SHRP (Meaney et al. 1985). Sapolsky and Meaney (1986) suggest that until

adult hippocampal GC receptor levels are achieved (around day 9), the HC is unable to bind sufficient CORT to successfully inhibit the HPA axis. The observation that in adult rats, hippocampal ablation or deafferentation results in increased CRH mRNA in the medial parvocellular PVN, and elevated basal and stress-induced circulating CORT levels (Sapolsky and Meaney 1986; Herman et al. 1989) provides support for this argument.

During the SHRP, the rat HPA system displays an attenuated CORT response to exogenous CRH in vivo (Guillet and Michaelson 1978; Guillet et al. 1980; Walker et al. 1986a), and in vitro (Guillet et al. 1980), and to ACTH (Cote and Yasumara 1975; Rosenfeld et al. 1991; Suchecki et al. 1993a,b). Plasma CORT-response to recognized stressors such as ether (Walker et al. 1986a, 1991; Iny et al. 1987; Eck and Kuhn 1992), bacterial endotoxin (Witek-Janusek 1988), histamine (Butte et al. 1973; Cote and Yasumara 1975), interleukin-1 (Levine et al. 1994), and insulin-induced hypoglycemia (Widmaier 1989; Arai and Widmaier 1991; Muret et al. 1992) is also attenuated during the SHRP.

In contrast, CORT response to other stressors, including heat stress (63°C for 3 min.), cold stress (Walker et al. 1991; Joyce and Barr 1992; Avishai-Eliner et al. 1995), hypoxia (Walker et al. 1986a), electric shock (Haltmeyer et al. 1966; Zarrow et al. 1966), and maternal separation (Stanton and Levine 1990; Walker et al. 1991), though somewhat lower than adult levels, remains robust throughout the SHRP. A significant CORT response to histamine administration on postnatal day 10 (Walker et al. 1991), and intraperitoneal (i.p.) injection of excitatory amino acids on postnatal day 7 (Chautard et al. 1993) has also been reported. Thus, evidence indicates that throughout the SHRP, the adrenal gland of the neonatal rat is capable of mounting a CORT response to selected forms of stress. This suggests that the mechanisms responsible for the attenuated response to some

stressors lies above the level of the adrenal glands i.e., in the pituitary or the brain.

Throughout the SHRP, the rat anterior pituitary secretes ACTH in response to direct stimulation by exogenous CRH in vivo (Guillet and Michaelson 1978; Walker et al. 1986a; Walker and Dallman 1993) and in vitro (Hary et al. 1993; Walker and Vrana 1993). Walker and Vrana (1993) noted a potentiated ACTH response to CRH in pituitary cells from 6 and 11 day old rats compared to tissue from younger or older animals. During the SHRP, rats are also able to mount an adequate ACTH response to a variety of stressful stimuli including urethane (Walker et al. 1986a), ADX (Walker et al. 1990; Levine et al. 1994), ether (Walker et al. 1991; Walker and Dallman 1993), cold temperature, histamine, electric shock (Walker et al. 1991), bacterial endotoxin (Witek-Janusek 1988), and several excitatory amino acids (Chautard et al. 1993). Other stressors such as insulin-induced hypoglycemia (Widmaier 1989; Muret et al. 1992) and interleukin-1 injection (Levine et al. 1994) provoke, at best, an attenuated ACTH response during the SHRP. Surprisingly, hypoxia does not induce a measurable increase in plasma ACTH during the SHRP, but does elevate plasma CORT (Walker et al. 1986a).

Taken together, the above evidence indicates that the rat pituitary is able to mount an ACTH response to CRH throughout the SHRP, so the altered response to some stressors which is observed during this period likely involves central mechanisms which either modulate CRH release or inhibit pituitary responsiveness to CRH. Further support for this hypothesis is offered by the observation that the excitatory amino acids N-methyl-$_{D,L}$-aspartate (NMA), quisqualate, and kainate provoke significant ACTH responses in 7 day old rats, but fail to elicit ACTH secretion from 7 day old rat pituitary tissue incubated in vitro (Chautard et al. 1993).

During the SHRP, hypothalamic CRH appears to be somewhat refractory to perturbation. Both basal and stressed levels of hypothalamic and circulating CRH differ from adult values. In vitro, the rate at which hypoglycemia induced CRH secretion from hypothalami from rats 9-24 days of age was less than 30% of the adult rate (Widmaier et al. 1989). In vivo, insulin-induced hypoglycemia does not increase plasma CRH in rats under 35 days of age (Widmaier 1989), and during the SHRP, ADX does not increase CRH mRNA in the PVN (Grino et al. 1989b).

A recent report indicates that the expression of interleukin-6 (IL-6; which promotes neuronal survival and regeneration; Gadient and Otto 1993) and its receptor molecule is elevated in rat hypothalamus during the neonatal SHRP (Gadient and Otto 1993). In addition, the time course of expression of IL-6 and its receptor IL-6R correlates well with the maturation of the HPA axis (Gadient and Otto 1993) suggesting a possible role in its development. During the SHRP, plasma CORT response to bacterial endotoxin is significantly attenuated (although ACTH response is not; Witek-Janusek 1988).

The neonatal SHRP is also modulated by external factors. Maternal contact appears to exert a complex, multifactorial inhibitory influence over the stress-response of nursing pups. Pups separated from their mothers for 24 hours during the SHRP display increased stress-induced secretion of ACTH, increased adrenal responsiveness to ACTH, and elevated basal levels of plasma ACTH and CORT (Kuhn et al. 1990; Levine et al. 1991; Rosenfeld et al. 1991; Cirulli 1992; Suchecki et al. 1993b; Avishai-Eliner et al. 1995). Similar changes in HPA activity were observed in pups not separated from their mothers but deprived of physical contact with them (Stanton et al. 1987). Some aspects of maternal contact are essential to maintaining the SHRP while others are not. For example, pup CORT response to a novel environment is attenuated by contact with an

anaesthetized lactating female, but not by contact with an anaesthetized littermate or adult male (Stanton and Levine 1990). Different aspects of mother-pup interaction appear to act at different levels of the HPA axis. During periods of maternal-separation, feeding (via oral cannula) suppresses CORT increases but does not inhibit ACTH release whereas anogenital stroking (to induce urination/defecation) suppresses ACTH release but does not inhibit CORT secretion (Suchecki et al. 1993b; Rosenfeld et al. 1992).

The effect of maternal deprivation during the SHRP on HPA activity is dependent upon the timing and duration of isolation. For example, repeated intermittent maternal deprivation (1 hour a day on 3 consecutive days) does not elevate anterior pituitary POMC mRNA or peptide, plasma ACTH, or plasma CORT in animals younger than 14 days (Vazquez and Akil 1992). Rosenfeld et al. (1992) suggest that while maternal reunification might eradicate the effects of short periods of deprivation, the effects of deprivation periods of 8-24 hours on HPA axis responsiveness persist for at least 4 days. The critical role of maternal contact in normal development of the HPA axis is illustrated by the observation that adult rats subjected to 3-4 hours of maternal isolation a day for the first 2-3 weeks of life exhibit elevated hypothalamic CRH mRNA (Plotsky and Meaney 1993), and enhanced negative feedback sensitivity to GC (Muneoka et al. 1994).

Neonatal handling, particularly during the first 7 postnatal days, is also reported to induce permanent changes in the HPA axis (Meaney et al. 1985, 1989; Plotsky and Meaney 1993). Adult rats handled in infancy display increased hippocampal GR binding, and attenuated CRH, ACTH, and CORT response to a variety of stressors (see Meaney et al. 1991). Pituitary transcortin receptor levels are reduced in males only (see Meaney et al. 1991). Neonatal handling induces a mild, transient decrease in pup body temperature (Hutchings 1969; Schaeffer 1969). The effects of neonatal handling are obliterated if body temperature is

maintained throughout the handling procedure. In neonatal rats, thyroid hormone increases serotonin metabolism (J.M. Mitchell et al. 1990), and in vitro, hippocampal MR binding capacity is increased by 4 days exposure to serotonin (J.B. Mitchell et al. 1990). It has been suggested that the changes in stress-responsiveness observed in handled animals are, in fact, triggered by changes in thyroid hormone activation induced by declining body temperature (see Meaney et al. 1991).

In contrast to the blunted sensitivity to many stressors, sensitivity to the effects of excitatory amino acids is elevated during early postnatal life. Large increases in circulating ACTH and corticosterone concentrations are seen in 7 day old rats injected systemically with kainate, NMA, or quisqualate (Chautard et al. 1993). Kainate stimulates the greatest increase in ACTH release followed by NMA, then quisqualate. Excitatory amino acids which act at different subtypes of glutamate receptors display different ontogenic profiles of neurotoxicity. Seven day old rats are more susceptible to the neurotoxic effects of N-methyl-D-aspartate (NMDA) than adult rats, whereas adult rats are more sensitive to the effects of kainic acid than 7 day old rats (McDonald et al. 1988; McDonald and Johnston 1990). The convulsant qualities of kainate, NMDA, quisqualate, and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) are also elevated in 7-11 day old rats compared to older animals (Schoepp et al. 1990; Mares and Vilesek 1992). Seizure-related mortality rates are much higher in immature rats compared to adults (Albala et al. 1984; Okada et al. 1984; Brooks et al 1994) but, unlike older animals, young rats (5-10 days old) do not develop spontaneous recurrent seizures following convulsant doses of kainic acid (Stafstrom et al. 1992).

In summary, during the neonatal SHRP, circulating CORT and CBG; pituitary ACTH, transcortin, and MR; and hypothalamic CRH peptide and mRNA levels

are below adult levels. Despite its low level in the blood, CORT negative feedback on the pituitary is amplified due to the paucity of pituitary transcortin receptors, and the SHRP appears to be largely due to an inability of many stressors to overcome this amplified signal (see De Kloet et al. 1988). It has been suggested that since both basal and stress-induced levels of ß-endorphin are high during the neonatal SHRP, ß-endorphin might perform some of the protective role assumed by CORT in later life (Iny et al 1987).

HPA Function in Lactating Animals

Lactation induces major behavioural and neuroendocrine adaptations in the rat. During lactation, aggressive behaviour is suppressed and maternal behaviour is initiated. Circulating CBG titers are depressed during lactation. This, combined with increased suckling-induced ACTH release, results in increased circulating CORT levels (Voogt et al. 1969; Stern and Voogt 1973/74; Tucker 1994). There is no loss of circadian rhythmicity, but morning trough levels of CORT and ACTH are elevated, significantly diminishing the amplitude of normal daily rhythms (Stern et al. 1973). The extent to which the amplitude of CORT and ACTH rhythms are dampened is influenced by litter size (Walker et al. 1992). Basal circulating levels of epinephrine and NE (Clapp et al. 1985), growth hormone (GH) (Chen et al. 1974), prolactin (Stern and Voogt 1973/74; Blake 1974; Higuchi et al. 1989), oxytocin (Higuchi et al. 1988), and ACTH (Blake 1974) are also elevated during lactation. The secretion of thyroxine and its concentration in the blood, is suppressed in lactating rats (Lorscheider and Reineke 1971). Many of these changes are suckling-dependent. For example, within 12 hours of pup removal, circulating prolactin levels decrease and approach those observed in non-lactating animals (Stern and Voogt 1973/74), and within 14-24 hours CORT concentrations are within the non-lactating range

(Stern et al. 1973; Walker et al. 1992). Curiously, given the observed changes in blood TSH and thyroxine levels during lactation, TSH secretion rate is unaffected by suckling (Sar and Meites 1969).

Oxytocin and VP mRNA levels in the SON and PVN of lactating rats drop abruptly immediately following parturition and, apart from transient increases in SON oxytocin mRNA on postpartum day (pd) 4, and VP mRNA on pd 4 and pd 7, these levels remain low until pd 10 (Crowley et al. 1993). Interestingly, suckling is necessary to maintain depressed levels of oxytocin and VP mRNA in the SON during the first week following parturition, but PVN levels of these substances are not suckling-dependent (Crowley et al. 1993).

Pituitary content of hormones or their mRNAs is also altered during lactation. Anterior lobe prolactin levels drop within 1-2 minutes of the onset of suckling. The extent of this drop is dependent upon the number of pups and frequency of suckling (see Tucker 1994). Pituitary oxytocin and VP mRNA levels drop on pd 1 and remain depressed until pd 10 (Crowley et al. 1993). Brief bursts of oxytocin are released from the posterior pituitary in response to the suckling stimulus (Lincoln et al. 1973; Wakerly and Lincoln 1973).

The endocrine response to a variety of stressors is significantly depressed in lactating rats. Compared to non-lactating rats, females nursing pups display an attenuated CORT response to ether exposure (Stern et al. 1973; Stern and Voogt 1973/74; Schlein et al. 1974; Myers et al. 1975; Walker et al. 1992), electric shock (Thoman et al. 1970), and osmotic stress (Lightman and Young 1989). Ether-induced ACTH response is also depressed in lactating animals (Stern et al. 1973; Walker et al. 1992) and CRH mRNA increases, which are normally observed in the PVN following osmotic stress, are completely abolished (Schlein et al. 1974; Myers et al. 1975; Walker et al. 1992). The hypothalamic CRH response to ADX, however, is not attenuated during lactation (Lightman and Young 1989).

In rats, in addition to its effect on smooth muscle during parturition and milk ejection, oxytocin participates in the endocrine stress response (Lang et al. 1983; Carter and Lightman 1987). In non-lactating animals, oxytocin secretion is increased by central administration of CRH (Bruhn et al. 1986) and a variety of stressors including immobilization and osmotic stress (Lang et al. 1983; Carter and Lightman 1987; Higuchi et al. 1988). The magnitude of oxytocin response is greater in (non-lactating) females than in males (Williams et al. 1985). During lactation, the oxytocin response to immobilization is completely abolished (Carter and Lightman 1987; Higuchi et al. 1988) and the increase induced by osmotic stress is significantly reduced (Lightman and Young 1989; Higuchi et al. 1988).

In lactating animals, the magnitude of the prolactin response to ether (Terkel at al 1972; Stern and Voogt 1973/74), immobilization (Higuchi et al. 1989) and opiates (Callahan et al. 1988) is attenuated or abolished, and plasma catecholamine increases following immobilization stress are significantly reduced (Higuchi et al. 1989).

HPA Response in Lewis Rats

Lewis rats are an inbred strain of arthritis susceptible, stress-hyporesponsive animals. Glowa et al. (1992) found no differences in basal circulating CORT levels of Lewis rats compared to histocompatible Fischer rats, or Sprague-Dawley (S-D) animals. Other authors, however, have reported that Lewis rats exhibit lower basal circulating CORT levels than Fischer rats (Griffin and Whitacre 1991; Smith et al. 1994). Dhabhar et al. (1993) detected no differences between Lewis and S-D rat morning CORT levels, but noted that evening CORT levels were

lower in Lewis rats. Also, circulating CORT levels are lower in male Lewis rats compared to Lewis females (Griffin and Whitacre 1991).

Lewis rats are also reported to exhibit a blunted plasma CORT and ACTH response, and a blunted PVN CRH mRNA response to inflammatory agents and other stressful stimuli (Sternberg et al. 1989a,b; Aksentijevich et al. 1992; Glowa et al. 1992; Rivest and Rivier 1994). Rivest and Rivier (1994) discounted the possibility that the hyporesponsiveness of the HPA axis of Lewis rats might be due to a defect in the afferent signal reaching CRH neurons, and, based upon the observation that hypothalamic explants from Lewis rats secreted less CRH in response to stimulation with acetylcholine, epinephrine, and serotonin compared to explants from Fischer rats, Calogero et al. (1992) suggested that the defect lies in the hypothalamic CRH neurons themselves. Sternberg et al. (1989b) noted that, in addition to defective hypothalamic synthesis and secretion of CRH, Lewis rats exhibit deficient expression of the hypothalamic enkephalin gene which is coordinately regulated with the CRH gene in response to stress. On this basis, they concluded that the hyporesponsiveness of the HPA axis of Lewis rats is primarily due to inappropriate regulation of the CRH gene.

Aksentijevich et al. (1992) reported that for the first 2 weeks of life, the blunted stress response observed in Lewis rats is no different to that observed in other strains. Around postnatal day 14, however, other strains develop the capability to mount a robust stress response whereas the stress system of Lewis rats remains unchanged. Consequently, these animals have a heightened susceptibility to inflammatory joint disease.

Behavioural differences in stress-response have also been reported between Lewis rats and other strains. Compared to Fischer and Harlan Sprague-Dawley rats, Lewis rats exhibit a greater "startle response" (reflex contraction of skeletal muscles) to acoustic or tactile stimuli (Glowa et al. 1992). Also, blood CORT

titers increase in startled Fischer and S-D rats compared to non-startled cohorts, whereas blood CORT levels of startled Lewis rats do not differ from those of non-startled Lewis rats (Glowa et al. 1992).

III. EXPERIMENTAL STRATEGY

Clearly, the stress system is immensely complex and exceedingly sensitive to perturbation. One of the greatest obstacles facing neuroanatomists studying stress systems has been an inability to collect data without introducing additional stressors which cannot be excluded from subsequent data analysis. The objective of this thesis, is to localize specific neuronal groups which are activated by specific stressors. To accomplish this, neural tissue must be collected. This involves biopsy or autopsy, both of which are highly stressful. In order to distinguish neurons activated by experimental manipulations from neurons activated by more recent stressors associated with sacrifice, it is necessary to utilize a measuring technique which is sensitive enough to detect subtle changes in neural metabolism, but is relatively insensitive to more recent events (i.e., those immediately preceding death). Immunocytochemical detection of the protein product of the *c-fos* proto-oncogene should fulfill these requirements. The accumulation of Fos protein within a neuron is widely accepted as a sensitive indicator of neuronal activation, and the latency between activation of the *c-fos* gene and synthesis of its protein product enables the investigator to discount the potential effects of stressors surrounding sacrifice. In the following section, the role of *c-fos* and other immediate early genes in intracellular signal transduction is described and a comprehensive literature review is presented documenting the circumstances under which the *c-fos* gene is activated.

IV. NEURAL RESPONSES AND THE ROLE OF PROTO-ONCOGENES (Immediate-Early Genes)

Until recently, techniques for measuring neural activation were largely limited to two general approaches, electrophysiological recording from neuronal populations or single units, and monitoring cellular uptake of labeled 2-deoxyglucose. While studies utilizing these approaches have yielded a plethora of information, neither is practical for identifying active neurons within a heterogeneous population such as the PVN. Metabolic uptake and gross activity recording techniques do not allow identification of individual neurons, and single unit recording from a large number of small scattered neurons is impractical (see Hoffman et al. 1992). Immunocytochemical techniques developed over the past ten years have added a new dimension to neurobiological research. Immunocytochemical techniques using antibodies raised against the protein product of *c-fos* have proven particularly useful for mapping changes in metabolic activity in neuronal populations.

The response of a neuron to stimulation consists of transient, transcription-independent, and sustained, transcription-dependent, changes. The molecules and mechanisms involved in short-term, transcription-independent events such as synaptic transmission or membrane depolarization are fairly well understood. The molecular mechanisms involved in long-term, transcription-dependent changes underlying phenomena such as neural plasticity or memory acquisition remain unclear.

At the cellular level, extrinsic stimulation results in the mobilization of intracellular transducer molecules (second messengers). Second messengers initiate short-term responses directly, and elicit long-term responses indirectly by modifying levels of expression of adaptive regulator molecules (see Figure 5). The molecules considered most likely to serve as adaptive regulators are the

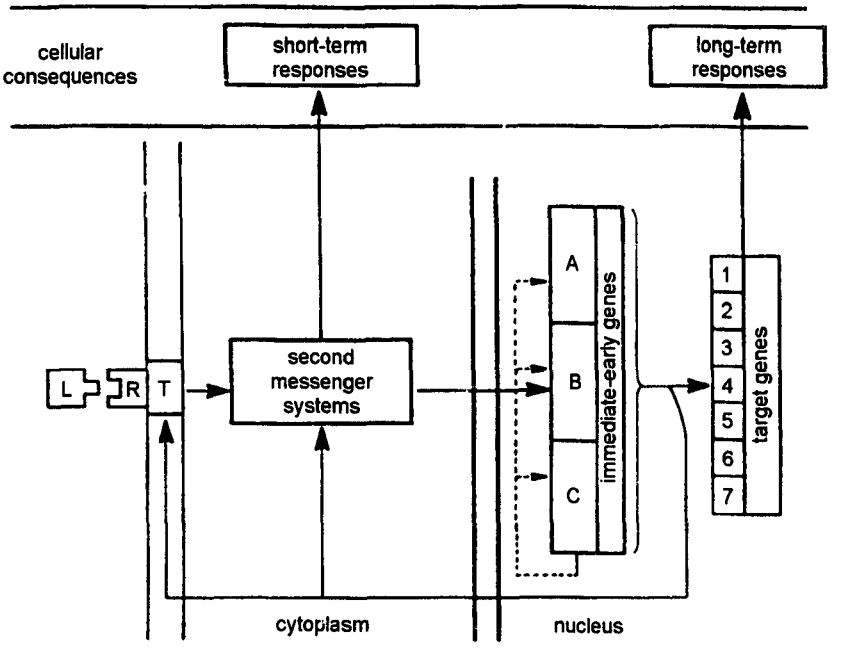


Figure 5 Proposed model for the role of cellular immediate early genes (proto-oncogenes) in stimulus-response coupling. L-extracellular ligand; R-receptor; T-membrane transducer molecule (redrawn from Curran and Morgan 1987).

immediate early genes (IEGs). In neurons of the CNS, and a variety of other cell types, transcription of IEGs is activated rapidly and transiently following stimulation. It is believed that IEGs encode regulatory proteins which function as intra- and inter-cellular third messenger molecules and modulate the expression of late response genes involved in long-term cellular changes (Curran and Morgan 1987; Carbone and Levine 1990; Morgan and Curran 1991).

Oncogenes and their Actions

Originally, the term "oncogene" was used to describe the genetic material which enables retroviruses to induce tumours. It is believed that retroviruses acquire viral oncogenes from the normal cellular genome during infection of a host cell. Cellular oncogenes belong to a family of genes variously known as immediate early genes, early-onset genes, or, as progenitors of viral oncogenes, proto-oncogenes. The genome of normal cells contains a variety of IEGs. Ordinarily, IEGs encode proteins involved in the normal cell growth processes of proliferation and differentiation but if cellular communication processes are disrupted, IEGs may be converted to transforming genes resulting in oncogenesis.

Approximately 50 oncogenes have been identified to date (Carbone and Levine 1990). In most tissues, basal levels of expression are low or undetectable, but transient increases in transcription activity, and mRNA and protein synthesis are stimulated by a variety of extracellular signals (Sheng and Greenberg 1990; Morgan and Curran 1991; Krukoff 1994).

The *c-fos* Proto-oncogene

The *c-fos* proto-oncogene is the normal cellular counterpart of two acutely oncogenic retroviruses. *c-fos* gene transcription is activated within 5 minutes of

the onset of stimulation, continues for 15-20 minutes, then decreases sharply (Morgan and Curran 1991). Accumulations of *c-fos* mRNA peak 30-45 minutes following the onset of stimulation, then decline rapidly, with a half life of approximately 12 minutes (Morgan and Curran 1991, Cullinan et al. 1995). Elevated levels of Fos, the protein product of *c-fos*, have been detected from 20 minutes to 7 days following the onset of stimulation (Morgan et al. 1987; Le Gal La Salle 1988; Menetry et al. 1989; Mugnaini et al. 1989; Jacobson et al. 1990; Elmquist et al. 1993; Rivest and Rivier 1995).

Following synthesis, Fos protein undergoes extensive post-translational modification and is translocated to the nucleus. There it forms a heterodimeric transcription factor complex with the protein product of a member of the Jun family of oncogenes (Morgan and Curran 1989; Curran and Morgan 1995; Figure 6). The Fos/Jun heterodimer binds with high affinity to the activator-protein-1 (AP-1) binding site of DNA (Sheng and Greenberg 1990; Morgan and Curran 1991). Thus, Fos can up- or down-regulate the transcription of genes containing this site. The precise nature of the cellular response to *c-fos* activation depends upon the target genes within the host cell which are selected for regulation.

Fos immunocytochemistry

Fos protein is comprised of 380 amino acids. Fos antibodies used in immunocytochemistry are generated against subsets of the complete Fos amino acid sequence. Because much of this sequence is common to both Fos and Fos-related antigens (Fra), some Fos antibodies lack specificity ie., form immune complexes with both Fos protein and Fra (Sonnenberg et al. 1989a,b; Sharp et al. 1991). For example, antibodies raised against *c-fos* amino acids 127-152 demonstrate cross-reactivity with a set of Fra, and several other Fos-associated proteins (see Morgan and Curran 1991). In general, Fos antibodies generated

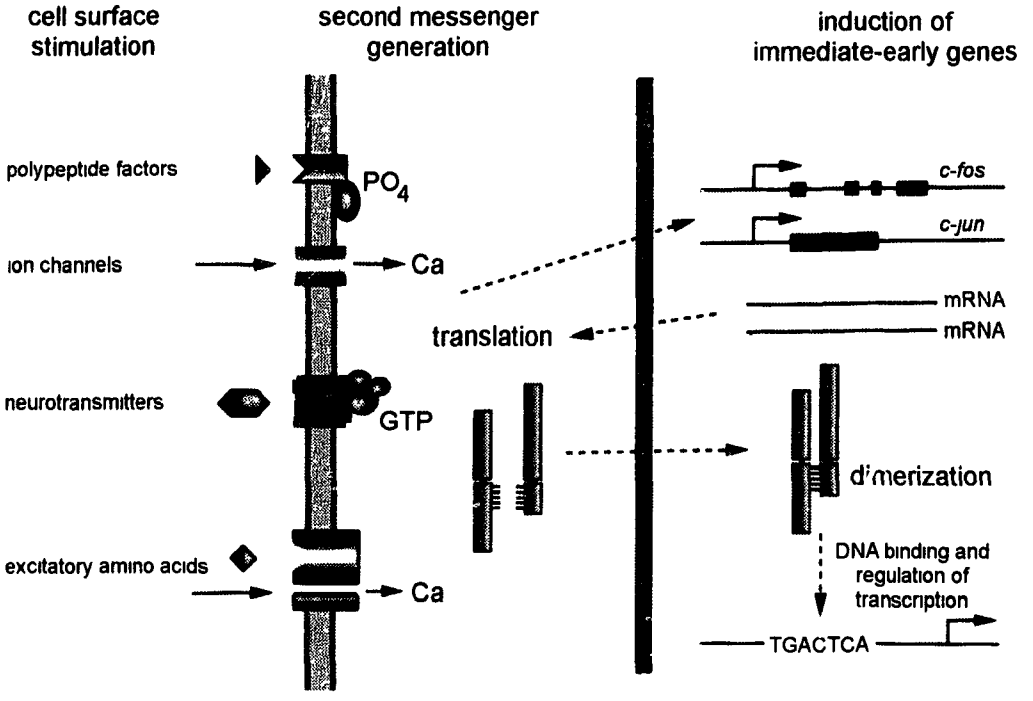


Figure 6 The role of *c-fos* in signal transduction. Exogenous stimuli impinging upon the cell surface activate second messenger systems which alter transcriptional activity of *c-fos, c-jun* and other immediate early genes. The resulting mRNA strands accumulate in the cytoplasm and protein products are translated. Fos protein is translocated to the nucleus where it forms a heterodimer with the protein product of *c-jun* or another member of the Jun family. The Fos-Jun heterodimer binds to DNA at the AP-1 binding site (TGACTCA) thereby regulating expression of target genes (redrawn from Curran and Morgan 1995).

against a portion of Fos protein that includes the leucine zipper (which is common to Fos and Fra) are likely to be less specific than antibodies generated against amino acid sequences from the N-terminal region of Fos (see Hoffman et al. 1993). As a variety of extracellular stimuli induce both Fos and Fra, the protein product identified by Fos immunocytochemistry protocols is correctly referred to as Fos-like immunoreactivity (FLI).

c-fos and metabolic mapping in the CNS

Several lines of evidence support the contention that expression of *c-fos* in CNS neurons is a reliable indicator of recent activation. Basal expression of *c-fos* is very low in adult CNS neurons (Dragunow et al. 1987; Dragunow and Robertson 1988a) but dramatic increases occur in a variety of circumstances. Many exogenous and endogenous stimuli have been identified which elicit rapid, transient increases in *c-fos* mRNA and Fos protein in CNS neurons (Morgan et al. 1987; Kononen et al. 1990; Sheng and Greenberg 1990; Morgan and Curran 1991). The literature review which follows, though by no means exhaustive, illustrates the broad range of situations in which *c-fos* gene activation occurs.

Circadian rhythm studies

Circadian studies afford a natural, non-invasive scenario in which to examine the involvement of IEGs in neural activation. In mammals, daily rhythms in characteristics such as activity level and body temperature, are controlled by an internal clock or pacemaker. This rhythm is influenced by environmental lighting changes but in the absence of light-dark variation, a cyclical rhythm is maintained with a periodicity which is very close to 24 hours. (Rusak et al. 1990). Endogenous diurnal fluctuations in *c-fos* gene expression occur in the SCN, cortex, cerebellum, HC, putamen, and pineal gland (Kononen et al. 1990; Kilduff

et al. 1992; Rea 1992; Sutin and Kilduff 1992; Carter 1993; Grassi-Zucconi 1993, 1994; Schwartz et al. 1994). The SCN is believed to be a primary centre for the control of mammalian circadian rhythms. The firing rate and metabolic rate of SCN neurons fluctuate spontaneously throughout the day with peaks during the light period of the light-dark cycle (Schwartz and Gainer 1977; Green and Gillette 1982; Gillette and Prosser 1988). Kononen et al. (1990) determined that the level of FLI in the SCN also fluctuates, following a pattern which corresponds quite closely with electrophysiological and metabolic circadian rhythms.

Further support for the contention that changes in the level of *c-fos* gene activation reflect changes in levels of neural activity is obtained from studies in which the normal light-dark cycle is interrupted. Photic stimulation during the dark phase of the light-dark cycle, is followed by a dramatic increase in metabolic uptake of (^{14}C)-2-deoxyglucose (2-DG) by neurons in the SCN (Schwartz and Gainer 1977), and a dramatic increase in *c-fos* mRNA and FLI in the region of the SCN which receives retinal input (Rusak et al. 1990). Taken together, these results suggest a temporal relationship between increases in neural activity and increased *c-fos* activation.

Seizures

Seizures induced by pentylenetetrazole (Morgan et al. 1987; Dragunow and Robertson 1987a, 1988a; Douglas et al. 1988; Sonnenberg et al. 1989a,b; Gibbs et al. 1990; Shehab et al. 1992; Yount et al. 1994), kainic acid (KA) (Le Gal La Salle 1988; Popovici et al. 1988, 1990; Sonnenberg et al. 1989a; Robertson et al. 1991), domoic acid (Robertson et al. 1992), picrotoxin (Sonnenberg et al. 1989a), bicuculline (Gass et al. 1992), electroconvulsive shock (Daval et al. 1989; Cole et al. 1990; Zawia and Bondy 1990; Shehab et al. 1992), and direct electrical brain stimulation (Dragunow and Robertson 1987b; Dragunow et al. 1988; Chiasson et

al 1995; Ebert and Löscher 1995) are reported to induce FLI in the HC, amygdala, cortex and other brain regions.

Following kindled seizures, FLI appears sequentially in cohorts of neurons known to be recruited during kindling (Goddard et al. 1969; Engel et al. 1978; Prince 1978; Dragunow et al. 1988; Chiasson et al. 1995). This relationship is not unique to kindled seizures. The time course and distribution of FLI following KA seizures is highly correlated with electrophysiological and behavioural manifestations of KA seizure activity (Prince 1978; Ben-Ari et al. 1981; Lothman and Collins 1981; Le Gal La Salle 1988).

Disruption of fluid balance/ blood pressure

Water deprivation, experimentally induced hypotension or hypovolemia, or intracerebroventricular (i.c.v.) infusion of hypertonic saline, induce transient increases in *c-fos* gene expression in brain areas involved in fluid or blood pressure regulation. These include the supraoptic nucleus (SON), paraventricular nucleus of the hypothalamus (PVN), lamina terminalis, subfornical organ, medial preoptic area, and ventrolateral medulla (Sagar et al. 1988; Ceccatelli et al. 1989; Carter and Murphy 1990; Gibbs et al. 1990; Giovannelli et al. 1990; Hamamura et al. 1990, 1991a, 1992; Oldfield et al. 1991; Sharp et al. 1991; Badoer et al. 1992; Giovannelli and Bloom 1992; Shen et al. 1992a,b; Chan et al. 1993; Dun et al. 1993; Hoffman et al. 1993; Roberts et al. 1993; Li and Dampney 1994; Solano-Flores et al. 1993; Berghorn et al. 1994). Brattleboro rats, which are chronically dehydrated due to a genetic deficiency of vasopressin, display higher basal levels of FLI in these regions than other strains (Guldenaar et al. 1992).

Experimentally-induced hypertension is also reported to induce FLI in the PVN, SON, bed nucleus of the stria terminalis (BNST), and central nucleus of the

amygdala (McKinley et al. 1992). All of these areas contain vasopressinergic neurons (De Vries et al. 1992).

Stimulation of nociceptors

Several investigators have reported elevated levels of *c-fos* mRNA or Fos protein in CNS neurons following stimulation of somatic or visceral nociceptors. Application of noxious heat (Hunt et al. 1987; Bullitt 1990; Wisden et al. 1990; Williams et al. 1990a; Naranjo et al. 1991; Pan et al. 1994) or cold (Abbadie et al. 1994); electric shock (Pezzone et al. 1992; Smith et al. 1992; Wan et al. 1993), or mustard oil (Hunt et al. 1987; Pretel and Piekut 1991; Cintra et al. 1993) to the foot or footpad; application of histamine (Yao et al. 1992) or noxious heat (Lima and Avelino 1994) to the lower limb; subcutaneous (s.c.) application of capsaicin (Ceccatelli et al. 1989); or injection of carrageenan (Draisci and Iadarola 1989; Noguchi et al. 1991), formalin (Presley et al. 1990; Williams et al. 1990a; Gogas et al. 1991; Abbadie et al. 1992), or Freund's complete adjuvant (Menétrey et al. 1989) into the footpad induced increases in FLI in neurons in the dorsal horn of the spinal cord. Similar results were observed following electrical stimulation of the sciatic nerve (Cintra et al. 1993), periarticular injection of urate acid crystals (Menétrey et al. 1989), or mechanical tissue damage to the foot (Bullitt 1990). Application of mustard oil to the nasal mucosa (Anton et al. 1991), intestinal distension (DeLeo et al. 1991; Traub et al. 1993) or manipulation (Zittel et al. 1993; Bonaz et al. 1994), and i.p. infusion of acetic acid (Menétrey et al. 1989; DeLeo et al. 1991; Hammond et al. 1992), also induce FLI in central pain pathway neurons. In general, the pattern of FLI observed following peripheral noxious thermal, mechanical (Bullitt 1990), or chemical (Menetry et al. 1989; Presley et al. 1990) stimulation is consistent with data obtained from pathway tracing and electrophysiological studies (see Menétrey 1987).

Invasive Procedures

Focal brain injury or hypoxia/ischemia induces *c-fos* mRNA and protein in affected brain areas (Dragunow and Robertson 1988b; Herrera and Robertson 1989; Onodera et al. 1989; Dragunow et al 1990; Popovici et al. 1990; Sharp et al. 1989a, 1990; Wessel et al. 1991; An et al. 1993; Herrera et al. 1993; Ruzdijic et al. 1993; Amir et al. 1994; Munell et al. 1994; Shimazu et al. 1994). Invasive procedures such as direct electrical brain stimulation (Krukoff et al. 1992, 1994; Sandner et al. 1992; Petrov et al. 1994), electroacupuncture (Pan et al. 1994), trigeminal nerve section (Sharp et al. 1989b), thyroidectomy (Koibuchi et al. 1991), and bilateral ADX (Jacobson and Sharp 1989; Jacobson et al. 1990; Chan et al. 1993) induce FLI in the PVN and other brain regions implicated in the generalized stress response. Some of this FLI is no doubt due to stimulation of cutaneous and visceral nociceptors during surgery. Following direct electrical stimulation of the sensory or motor cortex both 2-DG uptake and FLI is elevated in cerebellar neurons (Sharp 1984; Sharp and Ryan 1984; Sagar et al. 1988; Sharp et al. 1988; Sharp et al. 1989c).

Analgesic interventions such as morphine injection (Presley et al. 1990; Yao et al. 1992) and electroacupuncture (Lee and Beitz 1992) suppress noxious stimulus-induced FLI in spinal cord neurons. Paradoxically, both morphine (Chang et al. 1988; Liu et al. 1994), and electroacupuncture (Pan et al. 1994) are also reported to elicit FLI in rat CNS neurons.

Neurochemical activation

Neurotransmitters, hormones, and drugs which participate in neurotransmitter pathways, also induce *c-fos* mRNA and Fos protein in the CNS. Intracerebroventricular infusion of CRH, VP, NE or histamine (Andreae and

Herbert 1993; Parkes et al. 1993; Tsujino et al. 1992; Stone et al. 1993; Kjær et al. 1994) elicits FLI in CNS neurons, as does systemic injection of VP (Giri et al. 1990), CCK (Hamamura et al. 1990, 1991b; Verbalis et al. 1991; Luckman et al. 1993: Rinaman et al. 1994), insulin (Kjær et al 1993; Bahjaoui-Bouhaddie et al. 1994), angiotensin II (McKinley et al. 1992), or glutamate (Kaczmarek et al. 1988; Krukoff et al. 1994). Other compounds which act at glutamate receptors, including NMDA (MacDonald et al. 1990,1993; Vendrell et al. 1992; Dave and Tortella 1994), kainic acid (Le Gal La Salle 1988; Popovici 1988,1990; Schreiber et al. 1992,1993), domoic acid (Robertson et al. 1991), and quinolinic acid (Massieu et al. 1992; Trescher et al. 1994) also activate *c-fos* gene activity.

c-fos and FLI induction is also detected following systemic or intra-cerebral injection of dizocilpine maleate (MK801) (a non-competitive NMDA receptor antagonist) (Dragunow and Faull 1990; Gass et al. 1993; Hughes et al. 1993); yohimbine (an α2-adrenoreceptor antagonist which modulates endogenous NE release) (Gubits et al. 1989; Stone et al. 1991; Tsujino et al. 1992); haloperidol and YM 09151-2 (D_2 dopamine receptor antagonists) (Dragunow et al. 1990; Nguyen et al. 1992); thioperimide (an H_2-receptor antagonist which enhances endogenous histamine release) (Vizuete et al. 1995); tetrodotoxin (a sodium channel antagonist) (Cole et al. 1990), and L-DOPA (a dopamine precursor) (Robertson et al. 1989). Amphetamine or cocaine injection (which stimulates central monoaminergic neurons) (Graybiel et al. 1990; Young et al. 1991; Hope et al. 1992; Torres and Rivier 1992, 1994; Moratalla et al. 1993; Rosen et al. 1994), fenfluramine injection (which stimulates release and inhibits reuptake of serotonin) (Torres and Rivier 1994), nicotine injection (Kiba and Jayaraman 1994), caffeine injection (Nakajima et al. 1988), morphine injection (Chang et al. 1988; Chang and Harlan 1990) or withdrawal (Hayward et al. 1990; Stornetta et al. 1993), and ethanol withdrawal (Dave et al. 1990) also induce *c-fos* and FLI in CNS

neurons. Several anaesthetics including urethane, halothane, and sodium pentobarbital induce FLI in the CNS (Krukoff et al. 1992; Takayama et al. 1994a).

Neuroimmune activation

Interleukins are reported to induce FLI in the CNS (Chan et al. 1993; Hamba et al. 1994; Rivest and Rivier 1994), as are endotoxins and other agents which challenge the immune system (Elmquist et al. 1993; Wan et al. 1993, 1994). Wan et al. (1993) noted that the pattern of FLI induced by i.c.v. infusion of endotoxin differed from that observed following i.p. injection of the same substance.

Activation by non-noxious stressors/stimuli

In rats, immobilization (Ceccatelli et al. 1989; Imaki et al. 1992; Kononen et al. 1992; Coveñas et al. 1993; Harbuz et al. 1993; Senba et al. 1994; Watanabe et al. 1994, Chen and Herbert 1995a,b; Cullinan et al. 1995), rough handling (Asanuma et al. 1992; Watanabe et al. 1994), treadmill running (Rivest and Rivier 1995); water-avoidance stress (Bonaz and Taché 1994), and removal to a novel environment (Koibuchi et al. 1991; Handa et al. 1993; Papa et al. 1993) all induce *c-fos* mRNA and FLI in brain regions known to be involved in the stress response. In some instances, the *c-fos* gene is sensitive to quite subtle cues as illustrated by its activation by odor (Brennan et al. 1992; Schellinck et al. 1993), or a single i.p. injection of water (Gubits et al. 1989).

Activation by reproductive-related activity

Several investigators have noted elevated levels of FLI in rat brain following vaginal stimulation or copulatory activity (Robertson et al. 1991; Bialy et al. 1992; Baum and Everitt 1992; Wu et al. 1992; Baum and Wersinger 1993; Erskine 1993; Flanagan et al. 1993; Pfaus et al. 1993, 1994). This activation appears to be

relatively independent of gonadal steroid secretion. Orchidectomized male rats, with or without hormone replacement, display similar patterns of mating-induced FLI to intact male rats (Baum and Wersinger 1993). Also, although ovarian hormone replacement potentiates copulation- or vaginocervical stimulation-induced FLI in ovariectomized (OVX) female rats, hormone replacement alone has little effect (Pfaus et al. 1994).

Effect of experience

Previous experience influences the efficacy of some stressors. Immobilization stress induced far less FLI in the PVN and LSA of rats subjected to prior immobilization stress compared to rats with no prior exposure to immobilization (Umemoto et al. 1994; Watanabe et al. 1994; Chen and Herbert 1995a,b). Bialy et al. (1992) noted that intromission/ejaculation-induced increases in c-fos mRNA in the sensory cortex of male rats was dependent upon prior copulatory experience. Several investigators (Asanuma et al. 1992; Pezzone et al. 1992; Smith et al. 1992; Swank and Berstein 1994) have noted that prior association of a non-stressful stimulus with a stressful one (Pavlovian-conditioning) alters the pattern of FLI induced by the non-stressful stimulus. The influence of prior exposure on FLI induction is not limited to rats. Beaver et al. (1993) detected higher levels of light-induced FLI in the visual pathways of kittens with prior light exposure compared to those with no prior light exposure.

Factors effecting intensity of *c-fos* induction

Studies of spinal nociceptive pathways have revealed that the pattern of FLI observed following stimulation is influenced by the route and mode of stimulation. Thermal, mechanical, and chemical stimulation of peripheral nociceptors induces similar general patterns of FLI but the intensity of FLI differs

(Menetry et al. 1989; Bullitt 1990; Presley et al. 1990). FLI induction is also influenced by anaesthetics, analgesics, anticonvulsants, and sedatives. Menétrey et al. (1989) noted that levels of FLI observed following stimulation of peripheral nociceptors were lower in anaesthetized animals than in unanaesthetized animals. Bullitt (1990) reported that levels of thalamic FLI induced by noxious stimulation varied inversely with depth of anesthesia but noted that prolonged inhalation anesthesia induced elevated levels of FLI in several brainstem locations. Presley et al. (1990) observed that chemical irritant-induced FLI was suppressed by systemic morphine in a dose dependent manner and Morgan et al. (1987) noted that seizure-induced FLI can be blocked completely by administration of benzodiazapines and barbiturates. FLI can also be inhibited by infusion into the brain of anti-sense oligonucleotides to *c-fos* mRNA. For example, prior intracerebral infusion of a *c-fos* antisense oligonucleotide into the striatum profoundly inhibits amphetamine-induced FLI in ipsilateral striatal neurons (Chiasson et al. 1992; Dragunow et al. 1993; Hooper et al. 1994). Also, i.c.v. infusion of a *c-fos* antisense oligonucleotide markedly attenuates subsequent hemorrhage-induced FLI in the the PVN and SON (Chiu et al. 1994).

c-fos in the Developing Nervous System

Several investigators have detected transient elevated levels of *c-fos* gene expression in specific regions of the developing nervous system. Gubits et al. (1988) noted elevated levels of c-fos mRNA in rat brain during the time of most rapid growth (embryological day 16 to postnatal day 13), myelinization and synaptogenesis (pd 13-30), and functional synaptic activity (pd 13-60). Gonzalez-Martin et al. (1991) reported that from embryological day 20 until postnatal day 1, elevated levels of FLI were seen in neurons in cortical layer VI. At specific times during the first 2 weeks of postnatal life, FLI is transiently elevated in the cerebral cortex, striatum, HC and cerebellum in a sequence that closely parallels ontogenic development of these structures (Alcantara et al. 1993). During the neonatal SHRP (which extends from postnatal day 3 until the end of the second postnatal week), the level of *c-fos* induction elicited by many stimuli is attenuated compared to levels seen in like-treated older animals. Schreiber et al. (1992) noted that, in contrast to results seen in older animals, kainic acid-induced seizures produced little evidence of *c-fos* gene activation in limbic structures of rats younger than 13 days. Similar results were reported by Pennypacker et al. (1994). Mechanical cortical insult, which elicits a significant local increase in FLI in older animals, failed to induce FLI in the cortex of 10 and 15 day old rats (Herrera et al. 1993). Similarly, one hour of exposure to cold ambient temperature or shaking on a constantly moving table induced little FLI in the POA or PVN of rats younger than 10 days of age (Joyce and Barr 1992).

Although evidence suggests that *c-fos* gene response in the CNS fluctuates during development, *c-fos* gene activation has been demonstrated in animals of all ages. Cholecystokinin injection induced FLI in the medulla of 2 day old rats (Rinaman et al. 1994), and cerebral hypoxia-ischemia increased *c-fos* mRNA in various brain areas of 7 day old animals (Munell et al. 1994). FLI has also been

detected in lumbar spinal cord neurons of 1-3 day old rats following noxious stimulation of a hindpaw (Williams et al. 1990b).

Perhaps most attention has been focused on glutamate-induced activation of *c-fos* in young rats. For example, young rats injected with kainic acid develop seizures, but no evidence of *c-fos* gene activation is seen in limbic structures of animals younger than 13 days of age (Schreiber et al. 1992). Also, NMDA-induces higher levels of FLI in the arcuate nucleus of 5 and 10 day old rats compared to older animals (MacDonald et al. 1993).

c-fos in lactating animals

Surprisingly, in spite of the persistent fluid losses to suckling litters, lactating rats display extremely low levels of FLI in the PVN and SON (Smith and Lee 1990; Fenelon et al. 1993, 1994). Maternal interaction with pups does, however, induce transient increases in FLI in the MPOA, BNST, and medial and cortical amygdala (Fleming and Walsh 1994; Numan and Numan 1994, 1995) which are contingent upon sensory cues from pups. Observed increases in FLI in the MPOA and BNST persists in animals subjected to olfactory desensitization or bulbectomy, and ventral analgesia (blockade of sensory feedback from the nipples) or thelectomy (surgical excision of the nipples) (Fleming and Walsh 1994; Numan and Numan 1994, 1995), but is eliminated in intact animals when mother-pup interactions are prevented by sequestering pups in a mesh bag (Numan and Numan 1995). Increases in amygdala FLI are unaffected by ventral analgesia but are attenuated by olfactory desensitization (Fleming and Walsh 1994).

Conflicting results are reported on the effect of removing, then restoring the suckling stimulus. Smith and Lee (1990) observed profoundly elevated FLI in the PVN, SON, MPOA and LC of rats allowed to suckle their litters after an 8 hour period of non-suckling, whereas Fenelon et al. (1993) noted very little suckling-induced FLI in the PVN and SON of lactating animals deprived of suckling for 18-72 hours.

Lactating rats also display attenuated levels of *c-fos* activation (compared to like-treated non-lactating females) following exposure to specific stressful stimuli. Fenelon et al. (1994) noted that hemorrhage and dehydration induced smaller increases in FLI in oxytocin and VP neurons in the PVN and SON of lactating rats (compared to like-treated virgin females). Lower levels of NMA-induced FLI have also been noted in the HC, cortex (Smith and Lee 1990; Abbud

et al. 1992, 1993), locus coeruleus, and dorsal raphe nucleus (Abbud et al. 1994) of lactating rats compared to like-treated non-lactating animals. Differences in cortical and hippocampal FLI disappear 24 hours after pup removal (Abbud et al. 1992).

Although some stimuli exhibit a diminished capacity to induce *c-fos* gene activation during lactation, the efficacy of others is unaltered. For example, unlike NMA, KA induced similar patterns of FLI in lactating and non-lactating animals (Abbud et al. 1992, 1994).

Value as an indicator of neuronal activation

Most of the evidence accumulated to date supports the contention that in most instances, induction of FLI is a reliable indicator of neuronal activity and, in several neuronal models, the value of *c-fos* immunocytochemistry as a mapping tool is well established. In many cases, the results obtained using *c-fos* immunocytochemistry have proven to be consistent with data obtained from electrophysiological stimulating/recording, neuroanatomical tract tracing, in situ hybridization, and/or autoradiography experiments. Under some circumstances, however, there may not be a good correlation between FLI and neuronal activity. The absence of FLI should not be interpreted as indicative of lack of activity. Some neurons may express IEGs which are not members of the Fos family in reponse to stimulation, and others which express *c-fos* in response to some stimuli may fail to do so in response to others. For example, dosages of N-methyl-D,L-aspartate which activated luteinizing hormone releasing hormone (LHRH) neurons, as evidenced by significant increases in luteinizing hormone secretion from their target cells, failed to induce *c-fos* in those neurons (Hoffman et al. 1993).

Changes in FLI expression do not coincide with changes in 2-DG uptake in all instances. For example, electrical stimulation of the sensory/motor cortex is followed by increases in 2-DG uptake in neurons of the caudate/putamen but no accompanying changes in FLI have been detected in these cells (Sagar et al. 1988; Sharp et al. 1989c). Sagar et al. (1988) suggest that if any focal changes in FLI occur in the caudate/putamen, they might be obscured by the high basal levels of FLI present in the striatum.

In some instances, FLI is detected in the absence of elevated 2-DG utilization. Dehydration (water deprivation for 24 hours) induces elevated levels of FLI in neurons of the SON and PVN (Sagar et al. 1988) but mild dehydration

(replacement of drinking water with 2% saline for 5 days) does not increase 2-DG utilization in these cells (Schwartz et al. 1979; Lightman et al. 1982; Gross et al. 1985). In evaluating this finding it should be noted that severe dehydration (5 days of water deprivation) increases 2-DG utilization in neurons of the SON and PVN (Gross et al. 1985) and previous electrophysiological studies have determined that dehydration or intraventricular infusion of angiotensin II increases electrical activity in these nuclei (Akaishi et al. 1980). Inconsistencies between FLI and 2-DG results have also been reported by other investigators (Jorgenson et al. 1989). Some of these inconsistencies may reflect the fact that both neuronal excitation and neuronal inhibition increase regional metabolic activity (Ackermann et al. 1984; Kadekaro et al. 1985) and thus also increase regional uptake of 2-DG.

Although Dragunow and Robertson (1987) saw no evidence of FLI in glial cells following pentylenetetrazole-induced seizures, glial cells have been shown to express *c-fos* following focal brain injury (Dragunow and Robertson 1988b; Dragunow et al. 1990) or light exposure during the dark period of the circadian cycle (Bennett and Schwartz 1994). Glial expression of *c-fos* is, however, easily distinguished from neuronal expression using double labeling techniques utilizing neuron and glial specific antibodies such as those raised against neuron-specific enolase and nonneuronal enolase (Schmechel et al. 1978; Leslie et al 1993), or against the neuron specific nuclear protein NeuN (Mullen et al 1992).

In summary, induction of FLI cannot be interpreted as absolute proof of elevated neuronal activity, however, when results are interpreted cautiously, and are consistent with data gleaned from other techniques, *c-fos* immunocytochemistry is a reliable and powerful tool for probing neural activation at the cellular level.

V. THESIS OUTLINE

In this thesis, *c-fos* immunocytochemistry is used to examine the role of the PVN in mediating the stress response in rats. As mentioned previously, activation of the stress system by removal of the animal from its home cage, exposure to odors from previously killed animals, administration of anaesthetic, loss of motor control and consciousness, and exposure to other stressful circumstances immediately preceding perfusion, represents a major obstacle to studying stress system activation. Given the time delay between *c-fos* gene activation and Fos protein synthesis it is reasonable to assume that changes in levels of FLI detected in neurons in the PVN reflect the effect of experimental manipulation on activation of these neurons, and do not detect activation by stressors encountered by the animal immediately prior to death (see Hoffman et al. 1992).

The first chapter examines the effect of disrupting the integrity of the HPA axis (by removing one or both of the adrenal glands) on *c-fos* gene response in the PVN of adult male and female S-D rats, and 3 stress-hyporesponsive rat models (i.e., newborn and lactating S-D rats, and genetically stress-hyporesponsive Lewis rats). In the second chapter, a computer-assisted quantitative image analysis method is developed and used to analyze the effect of lactation on *c-fos* gene response in the PVN of S-D rats following a variety of stressful challenges. In the third chapter, which also utilizes computer assisted image analysis, the non-competitive NMDA antagonist MK801 is used to characterize the role of glutamatergic neurons in activating *c-fos* gene expression in the PVN, and to determine the role of gonadal steroids in mediating this response. A short introduction is included in each chapter.

MATERIALS AND METHODS

Animals

Rats were obtained from Charles River Breeding Farms, Montreal, Quebec, Canada. All animals were housed in plastic cages under controlled lighting conditions (lights on from 0700-2100 h) with free access to food and water. Lactating rats were housed with their litters in individual cages, and all other rats were housed 2-4 per cage.

Anaesthesia

For surgery, adult animals were anaesthetized by injection of sodium pentobarbital (Somnotol; i.p.; 55 mg/kg). To ensure maximal survival among neonates and juvenile animals, 3-day-old pups were anaesthetized by cooling in ice water (cryogenic anaesthesia; see Kolb and Whishaw 1981; Cairncross et al. 1989; Levine et al. 1994), and 11 day and 18 day old pups by ether inhalation (Ethyl Ether, Fisher Scientific, Fair Lawn, NJ).

Surgical Protocols

Adrenal Surgeries

Bilateral incisions were made in the dorsolateral flanks; both adrenal glands were exposed; and both (ADX), one (uADX), or neither (sham-ADX) adrenal gland was removed. Following surgery, the muscle layer of animals aged 7 days or older was sutured closed and the skin closed with wound clips. The muscle layer of 3 day old animals was closed with a single suture, and the skin closed with a drop of cyanoacrylate cement (Instant Krazy Glue). Following closure,

animals were returned to their home cages. Throughout the post-surgical period, all animals had free access to food and water, and animals with post-surgical survival times greater than 4 hours, had access to food, water, and isotonic saline (ADX animals require salt replacement as, in the absence of the adrenal mineralocorticoid aldosterone, they suffer excessive renal losses of fluid and sodium due to an inability to concentrate their urine).

Ovariectomies

Bilateral incisions were made in the dorsolateral flanks; both ovaries were exposed; and both (OVX), one (uOVX), or neither (sham-OVX) ovary was removed. Following surgery, the muscle layer was sutured closed and the skin closed with wound clips, and animals were returned to their home cages.

Perfusion/Sectioning

Animals were deeply anaesthetized (Somnotol; i.p.; 65 mg/kg) and perfused via the left ventricle with ice-cold Dulbecco's phosphate-buffered saline (DPBS; pH 7.4, 4°C; Gibco Laboratories, Grand Island, NY) followed by 4% buffered paraformaldehyde (pH 7.4, 4°C). Brains were removed and post-fixed in 4% buffered paraformaldehyde at 4°C for at least 24 hours. Prior to sectioning, brains were placed, ventral side up, in a Jacobowitz Rat Brain Slicer (ZIVIC-MILLER Laboratories Inc., Allison Park, PA) and sliced coronally using a single edged industrial razor blade (VWR Scientific, Media, PA) to yield a block of brain tissue extending from the rostral margin of the optic chiasm to the caudal margin of the mamillary nuclei. The caudal surface of the block was glued to an aluminum stage using cyanoacrylate cement (Instant Krazy Glue, The Borden Co. Ltd., Willowdale, Ontario) and mounted in a vibrating microtome (Vibratome 1000, TPI, Inc., St. Louis, MO.). Fifty μm coronal sections were cut and floated in DPBS. For immunocytochemistry, sections from the caudal margin of the anterior commissure to the rostral region of the ME were

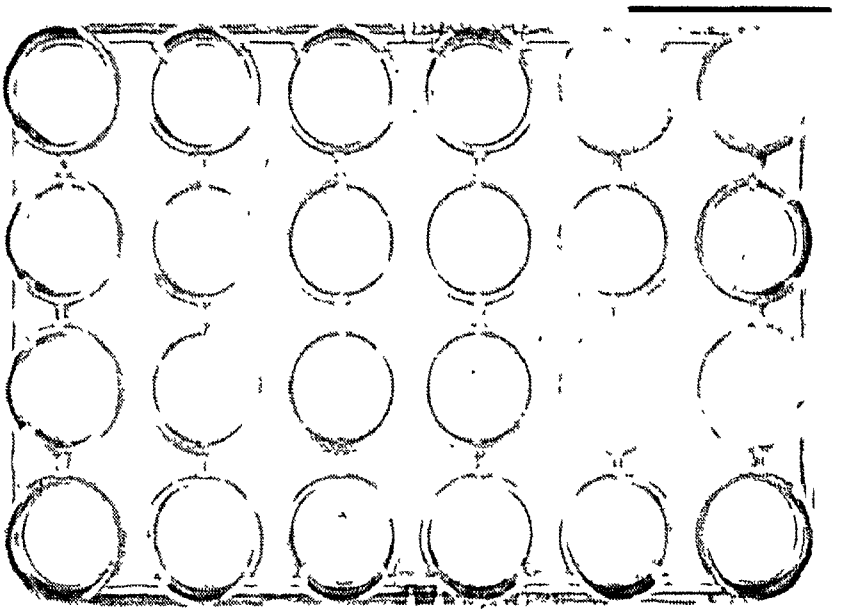


Figure 7a Top view of 24 well tissue culture plate used for immunocytochemistry procedure.

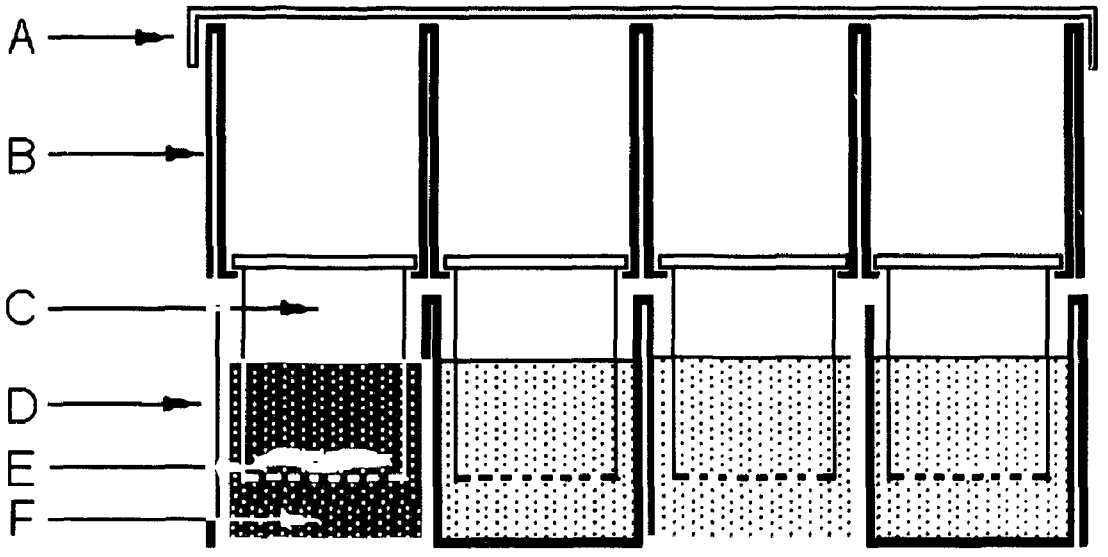


Figure 7b Schematic cross-section through tissue culture plates used for immunocytochemistry: A - lid; B - 24 well tissue culture plate with bottoms cut from wells; C - mesh-bottomed basket; D - 24 well tissue culture plate; E - tissue section in basket; F - 1.25 ml. of solution in each well (covering tissue). To move the contents of all 24 baskets simultaneously from one solution to the next, the upper tissue culture plate holding the mesh-bottomed baskets is lifted from the lower plate to the plate containing the next solution (1.25 ml./well).

transferred to mesh bottomed baskets suspended in 24 well tissue culture plates (Becton Dickinson, Lincoln Park, NJ; see Figure 7) containing .01M phosphate buffered saline (pH 7.4, 4°C) with 0.2% Triton X-100 (Sigma, St. Louis, MO) (PBS/Tx). Brains of 3 day old pups proved too fragile (immature) to section at 50 µm so these brains were cut into 75 or 100 µm thick sections.

Immunocytochemistry

Fos Immunocytochemistry (Nickel Intensified DAB Method)

Brain sections in mesh bottomed baskets in 24 well tissue culture plates containing .01M PBS/Tx (pH 7.4, 4°C; 1.25 ml. per well), were placed on a Janke and Kunkel IKA-VIBRAX-VXR orbital shaker. Following three 10 minute rinses in PBS/Tx, sections were rinsed for 20 minutes in 0.4% hydrogen peroxide (1.25 ml./well), rinsed in PBS/Tx three more times (10 min./rinse), and rinsed in 3% NRS in PBS/Tx containing 0.1% sodium azide (1.25 ml./well) for 60 minutes. All of the above rinses were performed at room temperature. Sheep polyclonal anti-Fos antibody (lot #823 and #824, Cambridge Research Biochemicals Ltd., UK) was diluted 1:20,000 (lot #823) or 1:10,000 (lot #824) in 0.01M PBS containing 3% normal rabbit serum (NRS) (Gibco Laboratories, Grand Island, NY) and 0.1% sodium azide. Sections were immersed in the primary antibody solution (1.25 ml./well). The plates were then sealed in plastic bags with moist paper towels to prevent dehydration, and incubated, with shaking, for at least 48 hours at 4 degrees centigrade. Sections were then returned to room temperature, rinsed three times in PBS/Tx (10 min./rinse), reacted for 60 minutes in a solution containing biotinylated rabbit anti-sheep immunoglobulin (Vector, Burlingame, CA) diluted 1:500 in PBS containing 1% normal rabbit serum and 1% Tx (1.25 ml/well), and again rinsed three times in PBS/Tx (10 min./rinse). Next, sections were transferred to ABC solution (*Vectastain Elite kit*, Vector, Burlingame, CA; 3µl of A plus 3µl of B per ml of 0.01M PBS, 1.25 ml./well) for 60-90 minutes, and rinsed three more times in PBS/Tx (10 min/rinse). Finally, sections were transferred to a freshly prepared solution consisting of one 10 mg tablet of DAB (Sigma, St. Louis MO) and 300 mg of Ammonium nickel (II) sulphate hexahydrate (Aldrich, Milwaukee, WI) per 50 ml of 0.05M TRIS buffer (pH 7.4; 1.25 ml/well). Five minutes later, 25µl of 0.3% hydrogen peroxide solution was

added to each well giving a final concentration of 0.006% . Once sufficient blue-black reaction product was visible in the positive control sections (5-20 minutes), the reaction was stopped by rinsing three more times in PBS/Tx. Throughout the above procedures, the tissue was continuously agitated (orbital shaker) except when removed to change immersion solutions. Upon completion of the immunocytochemical reaction, sections were floated onto glass microscope slides, dehydrated, and coverslipped using Entellan coverslipping medium. Experimental and control tissue was processed together. Sections of hippocampal tissue from rats subjected to kainic acid-induced seizures were always included as FLI-positive controls (Le Gal La Salle 1988; Robertson et al. 1992). Some sections were also processed without primary antibody to determine non-specific staining.

Digital Imaging for Figure Preparation

A schematic representation of the equipment used for digital imaging is presented in Figure 8, and a digital image of this equipment scanned from a colour 35 mm. photograph using an Apple OneScanner and Power Macintosh 6100/60AV computer (Apple Computer Inc., Cupertino, CA) is presented in Figure 9.

Step 1: Image Capturing

Composite colour images of brain sections were transmitted to a Macintosh IIci computer (Apple Computer Inc., Cupertino, CA) equipped with 8 megabytes (Mb) of random-access memory (RAM), and a 2 Mb NuVista+ frame grabber (Truevision Inc., Indianapolis, IN) using a Javelin model JE3662RGB video camera (Javelin Electronics, Torrance, CA) mounted on a Leitz Laborlux-S microscope. A JVC model TM-122 monochrome video monitor was used to frame and focus images. Using Adobe Photoshop (version 2.5, Adobe Systems Inc., Mountain View, CA) with NuVista "capture" image acquisition software (Truevision Inc., Indianapolis, IN), images were captured and converted to greyscale.

Step 2: Size and Resolution Adjustment, and Image Storage

Greyscale images were then adjusted to 2-3 inches wide by 1.5-2 inches high with a resolution of 150 pixels per inch, using the "Image Size" command from the Adobe Photoshop "Image" pull down menu. The resulting image was stored to a 88 Mb SyQuest removable hard drive cartridge (Dynatek Automation Systems Inc., Bedford, NS) as an uncompressed Tag Image File Format (TIFF) file. Each greyscale image TIFF file of the above dimensions and resolution required less

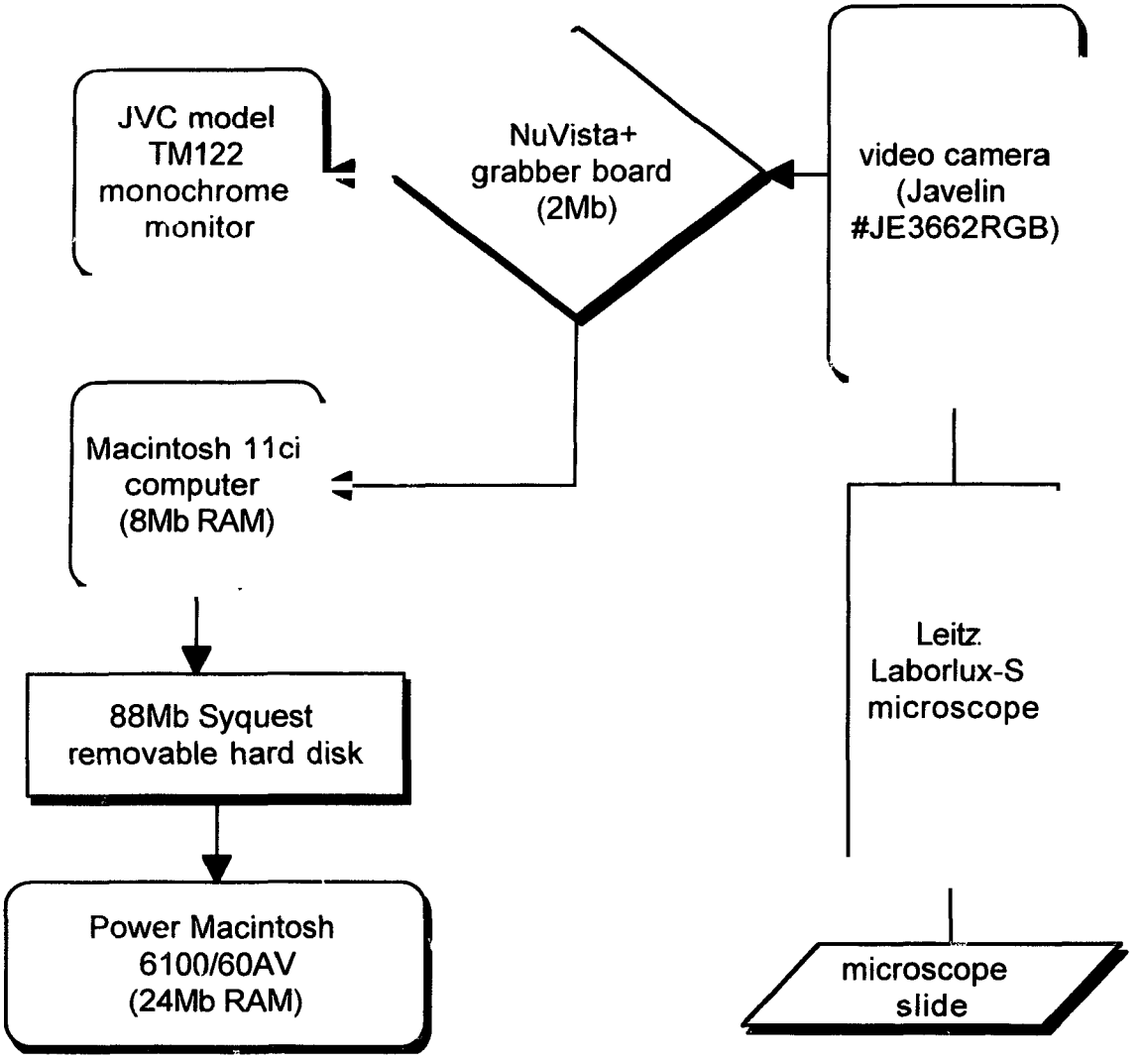


Figure 8 Schematic representation of the apparatus used for image capturing, enhancement and storage.

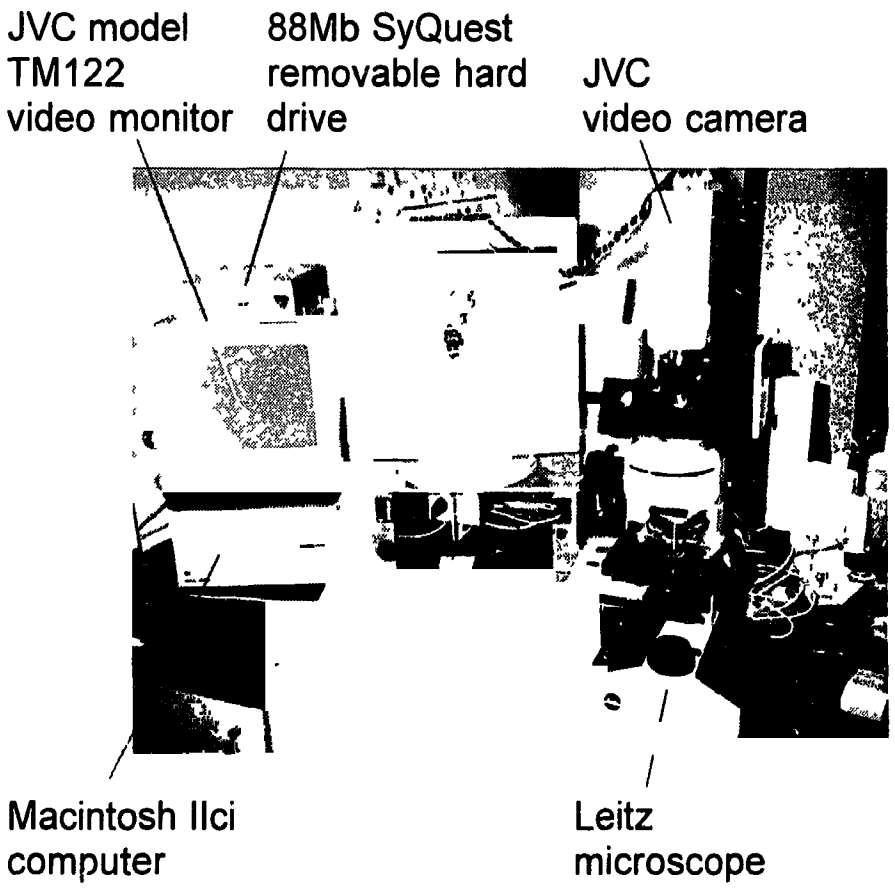


Figure 9 Apparatus used for image capturing, enhancement, and storage

than 200 kilobytes (K) of disk space for storage. The decision to use a resolution of 150 pixels per inch was based on empirical observation that lower resolutions resulted in noticeable loss of acuity in the final printed image, and higher levels of resolution did not result in any noticeable improvement in the printed image. Also, higher resolution images, when pasted into a collage with up to eleven other images of the same size and resolution, exceeded the memory capacity of the Power Macintosh computer system described below.

Step 3: Image Enhancement and Labeling

For further image adjustment and preparation of Figures, the SyQuest cartridge was transferred to a second SyQuest drive attached to a Power Macintosh 6100/60AV computer (Apple Computer Inc., Cupertino, CA) equipped with 24 Mb of RAM. Using Adobe Photoshop, the brightness and contrast of images were adjusted to achieve optimal clarity, and to balance the overall background luminescence of groups of images which were ultimately to form a collage in a single Figure. Individual images were pasted to a 6 inch by 9 inch "canvas", and positioned and cropped as desired. A 1 pixel wide black border was added to each image as required using the Adobe Photoshop text tool, and appropriate labeling was added to the bottom right corner. A 100 µm. scale bar was added to at least one image per figure.

Step 4: Figure Composition and Printing

Final Figures were composed by importing image files to Microsoft Word (version 5.1a, Microsoft Corporation, Redmond, WA) documents using the "paste" or "Picture" command from the "Insert" pull down menu. Figure captions were added, and the completed Figure was saved on the computer hard disk. Figures were printed on gloss-finish paper (Jenson Gloss 160; Unisource Canada

Inc., Dartmouth, NS) at 600 dpi, using a LaserWriter Pro laser printer (Apple Computer Inc., Cupertino, CA).

Using the equipment described above it is possible to produce high quality laser prints from scanned images of 35 mm. photographs (an excellent example of an image produced by this process is found in Figure 9 of this thesis). To the naked eye, scanned images of photographs show little or no loss of information compared to the original photographic prints. In Figure 10, a scanned, enlarged image of a 35 mm. photograph of a coronal section through the rat PVN is presented alongside a digital image of the same brain section before, and after image adjustment and labeling. The digital image was captured directly from a JVC video camera to a Macintosh IIci computer. A visual comparison of the scanned photographic image and the adjusted digital image (see Figure 10) illustrates that captured digital images, such as those presented in the results sections of this thesis, exhibit high fidelity, and equal or superior clarity compared to images produced by conventional photography.

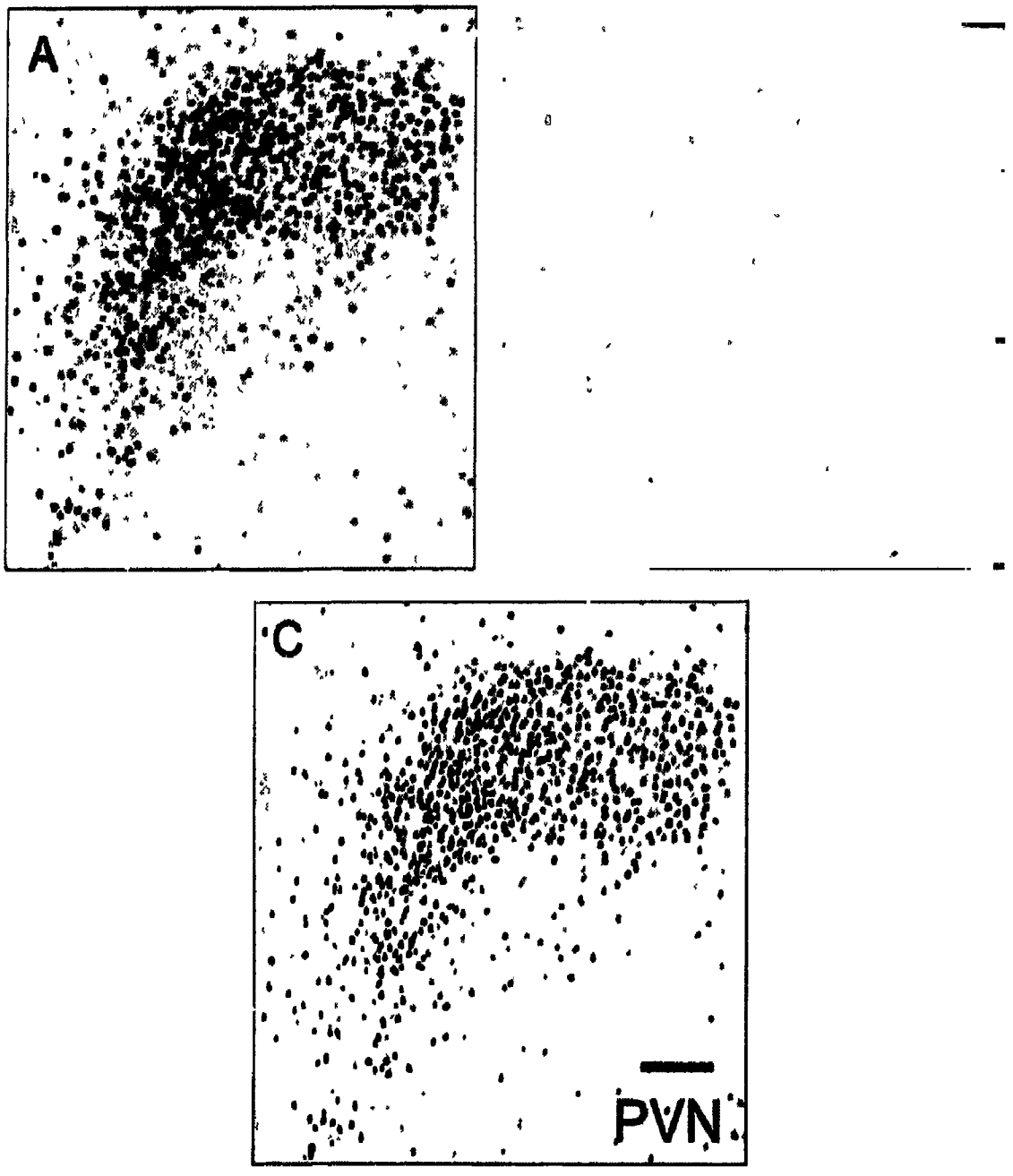


Figure 10 A scanned, enlarged image of a 35 mm. photograph of a coronal section through the PVN of the rat (A) presented alongside a digital image of the same section captured directly from a JVC video camera to a Macintosh IIci computer before (B), and after (C) image adjustment and labeling. Scale bar = 100 μm.

CHAPTER 1: ADRENALECTOMY AND THE INDUCTION OF FLI IN THE PVN OF THE RAT.

Introduction

Bilateral ADX, which eliminates normal levels of circulating corticosteroids, induces a dramatic increase in CRH levels in neurons of the medial parvocellular PVN (Paull and Gibbs 1983: Swanson et al. 1983; Sawchenko 1987). Hypothalamic CRH content rises to a peak 2-3 minutes following ADX, returns to near basal levels by 20 minutes, then rises again at around 40 minutes reaching a second, higher peak at 80 minutes following surgery (Sato et al. 1975). Changes in hypothalamic CRH are accompanied by an increase in the synthesis and secretion of ACTH which persists for 2 hours (Dallman et al. 1972; Sato et al 1975). Following ADX, levels of CRH and VP immunoreactivity are elevated, and a large percentage of CRH immunoreactive neurons display concurrent immunoreactivity to VP (Kiss et al. 1984; Wolfson et al. 1985). Glucose utilization in the PVN (and LC, HC, ME, and anterior pituitary) is also reported to increase following ADX (Kadekaro et al 1988). In short, evidence suggests that ADX alters the level of activity of at least some (CRH) neurons in the PVN.

Unilateral ADX induces compensatory hypertrophy in the remaining adrenal gland. Initially, this phenomenon was attributed exclusively to the removal of steroid negative feedback, but more recent evidence indicates that a neural mechanism, independent of ACTH regulation, is also involved (see Gerendai et al. 1981). Mary Dallman and assorted coworkers have contributed greatly to knowledge in this area. Dallman et al. (1977) reported that blood ACTH and CORT levels, which rise immediately following uADX, return to the normal range within 2 hours, whereas compensatory adrenal growth continues for (at least) several days. Dallman's group also noted that uADX-induced

compensatory adrenal hypertrophy is blocked by application of lidocaine to the adrenal gland prior to its removal; spinal cord hemisection at T_2 contralateral (but not ipsilateral) to the site of uADX; or unilateral hypothalamic lesion or hemi-island creation ipsilateral (but not contralateral) to the site of uADX (Engeland and Dallman 1975; Dallman et al. 1977; Holzwarth and Dallman 1979). These phenomena are incompatible with the hypothesis that compensatory adrenal growth is a consequence of exclusively humoral feedback control. More recent work has revealed that systemic administration of diazepam, which potentiates the action of GABA at all levels of the neuraxis, also inhibits uADX-induced compensatory adrenal growth (Zieleniewski et al. 1990).

Like uADX, sham ADX induces an immediate increase in plasma CORT levels which persists for about 2 hours (Dallman et al. 1972; Sato et al. 1975). Hypothalamic CRH content, which increases 4-6 fold within 2 minutes of the onset of sham ADX surgery, returns to near basal values by 40 minutes (Sato et al. 1975).

In this chapter, *c-fos* immunocytochemistry is used to examine the effects of adrenal surgery on activation of neurons in the PVN. Jacobson et al. (1990) detected elevated levels of FLI in the PVN of male rats killed 1 to 7 days following ADX. As noted earlier in the general introduction to this thesis, other authors have detected FLI in various brain regions from 20 minutes to 24 hours following the onset of a variety of stimuli. This chapter surveys the contribution of various factors which influence *c-fos* gene expression in the PVN. Specifically, experiments in this chapter are designed to identify a model in which the unique effects of ADX on neurons in the PVN can be isolated from the contributions of other stimuli (i.e., activation of nociceptors, circadian influences, survival time, developmental changes, strain differences, etc.).

In the initial experiment in this chapter, the effect of ADX, uADX, and sham ADX on FLI in the PVN is examined in animals killed 10 minutes to 7 days following surgery. It is designed to: 1) test the hypothesis that *c-fos* immunocytochemistry is an appropriate technique for isolating the effects of adrenal surgery on neurons in the PVN from the effects of more recent general stressors; 2) map the time course of induction of FLI in the PVN following adrenal surgery, and localize the neurons activated by this procedure; and 3) determine the effect of uADX, which has not been previously reported, on FLI in the PVN.

Circadian rhythms are known to alter basal blood CORT levels (see Dallman 1993) and FLI in specific brain regions (Kononen et al. 1990; Schwartz et al. 1994). In the second experiment in this chapter, the influence of these rhythms upon ADX-induced FLI in the PVN is investigated to determine the optimal time in the light-dark cycle for future experiments.

During the neonatal SHRP, the HPA axis is relatively refractory to perturbation. ADX during the neonatal SHRP elicits an ACTH response (Walker et al 1990), but does not increase CRH mRNA in the PVN (Grino et al. 1989b). In experiment 3, the influence of the neonatal SHRP on ADX-induced FLI in the PVN is investigated. This experiment is designed to: 1) test the hypothesis that the suppression of ADX-induced CRH mRNA observed in the PVN during the neonatal SHRP is accompanied by a parallel change in the magnitude of the FLI response to ADX; 2) map the ontogeny of ADX-induced FLI in the PVN at selected points before, during, and after the neonatal SHRP; and 3) determine whether stress-hyporesponsive neonatal rats represent a useful model in which to isolate the effects of ADX on the expression of FLI in the PVN from the influences of other stressors.

Lewis rats are unable to mount an adequate CORT and ACTH response, and also display a blunted stress-induced PVN CRH mRNA response, to a variety of stressors. It has been suggested that this inadequacy might be due to a defect in hypothalamic CRH neurons (Calogero et al. 1992). Experiment 4 compares the effect of ADX on FLI in the PVN of male Lewis and S-D rats to establish whether the reported strain differences in HPA function are reflected in differing patterns of adrenal surgery-induced FLI in the PVN.

The final 2 experiments in this chapter are designed to evaluate the role of CORT feedback inhibition in the regulation of adrenal surgery-induced FLI in the PVN. Activation of CRH neurons in the PVN is mediated in part, by direct CORT negative feedback inhibition of hypothalamic CRH neurons (see Figure 11). Administration of pharmacological doses of CORT are reported to attenuate or abolish the CRH response of PVN neurons to a variety of stressors by directly inhibiting CRH neurons (see Keller-Wood and Dallman 1984; Owens and Nemeroff 1991). Experiment 5 examines whether ADX-induced FLI in the PVN is altered or abolished by injection of CORT prior to surgery.

Laparotomy-induced increases in hypothalamic CRH content (which rises 4-6 fold in intact rats) are almost completely absent in ADX animals (Sato et al. 1975). Experiment 6 examines whether similar changes might be seen in the pattern of FLI seen in the PVN of previously adrenalectomized (7 days) rats killed 4 hours following OVX, laparotomy, or anaesthesia.

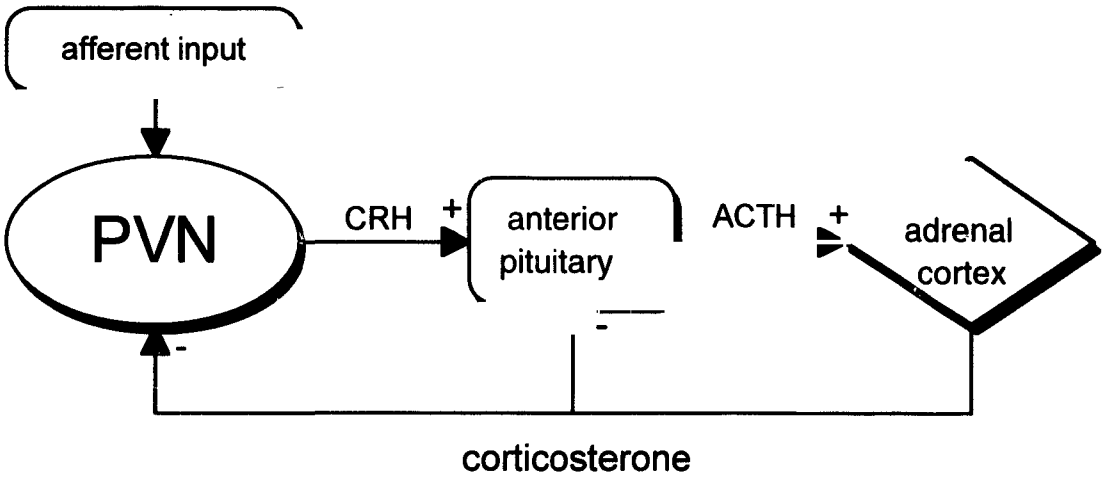


Figure 11 Schematic illustrating the mechanism of CORT negative feedback regulation of the HPA axis of the rat.

Experiment 1.1 Time course of ADX-induced FLI in the PVN of adult S-D rats.

1.1 Methods

Adult male S-D rats (approx. 250 g.) were anaesthetized, and bilaterally, unilaterally, or sham adrenalectomized (see Materials and Methods, page 55). At 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours, 4 days, or 7 days after surgery, these animals, along with "untouched" animals (i.e., animals taken from the same room as other animals used in this experiment) and animals given anaesthetic without surgery (3-5 animals/treatment at each survival time except 10 minutes), were deeply anaesthetized and perfused. Brains were removed, postfixed, and sectioned, and alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

1.1 Results

Digital images of brain sections from animals in this experiment are presented in Figures 1.1.1, 1.1.2, 1.1.3, and 1.1.4. Levels of FLI in the PVN were low or undetectable in "untouched" animals, and animals which received anaesthetic without surgery (Figure 1.1.1). Compared to these animals, no detectable increase in FLI was seen in the parvocellular regions of the PVN of animals killed 10 minutes following adrenal surgery. In one animal killed 10 minutes following ADX, scattered FLI was seen in brain areas outside of the PVN and a few faintly labeled magnocellular neurons were visible in one hemisphere of the PVN. A few faintly immunoreactive parvocellular neurons were seen in the PVN of animals killed 30 minutes following ADX, and in one each of the animals killed 30 minutes following uADX (Figure 1.1.1).

Detectable increases in FLI were seen in the PVN of animals killed at 1, 2, 4, or 8 hours following ADX, uADX, or sham ADX. The magnitude of FLI in the PVN

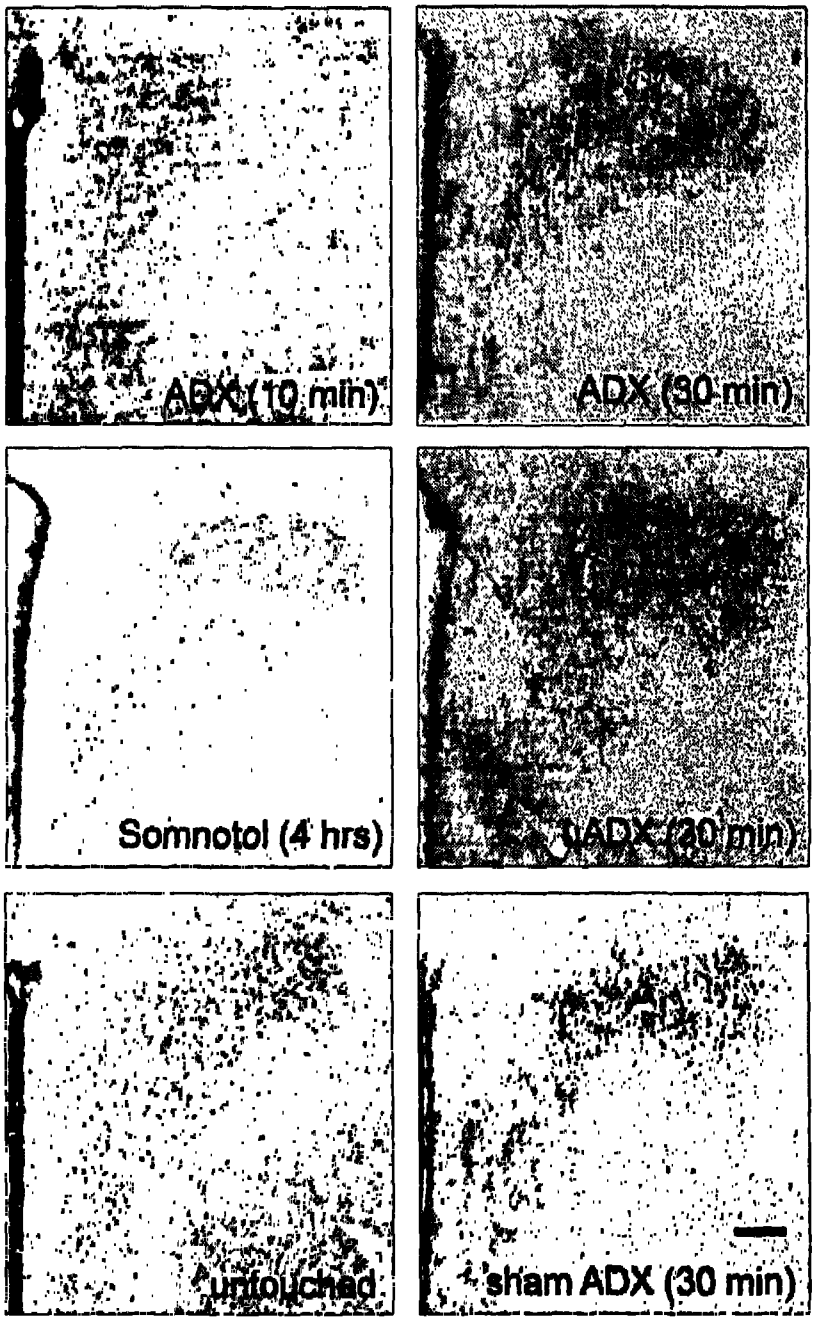


Figure 1.1.1 FLI in the PVN of untouched adult male Sprague-Dawley rats, and rats killed 4 hours following anaesthetic without surgery, 10 minutes following ADX, or 30 minutes following ADX, uADX, or sham ADX. Scale bar = 100 μm.

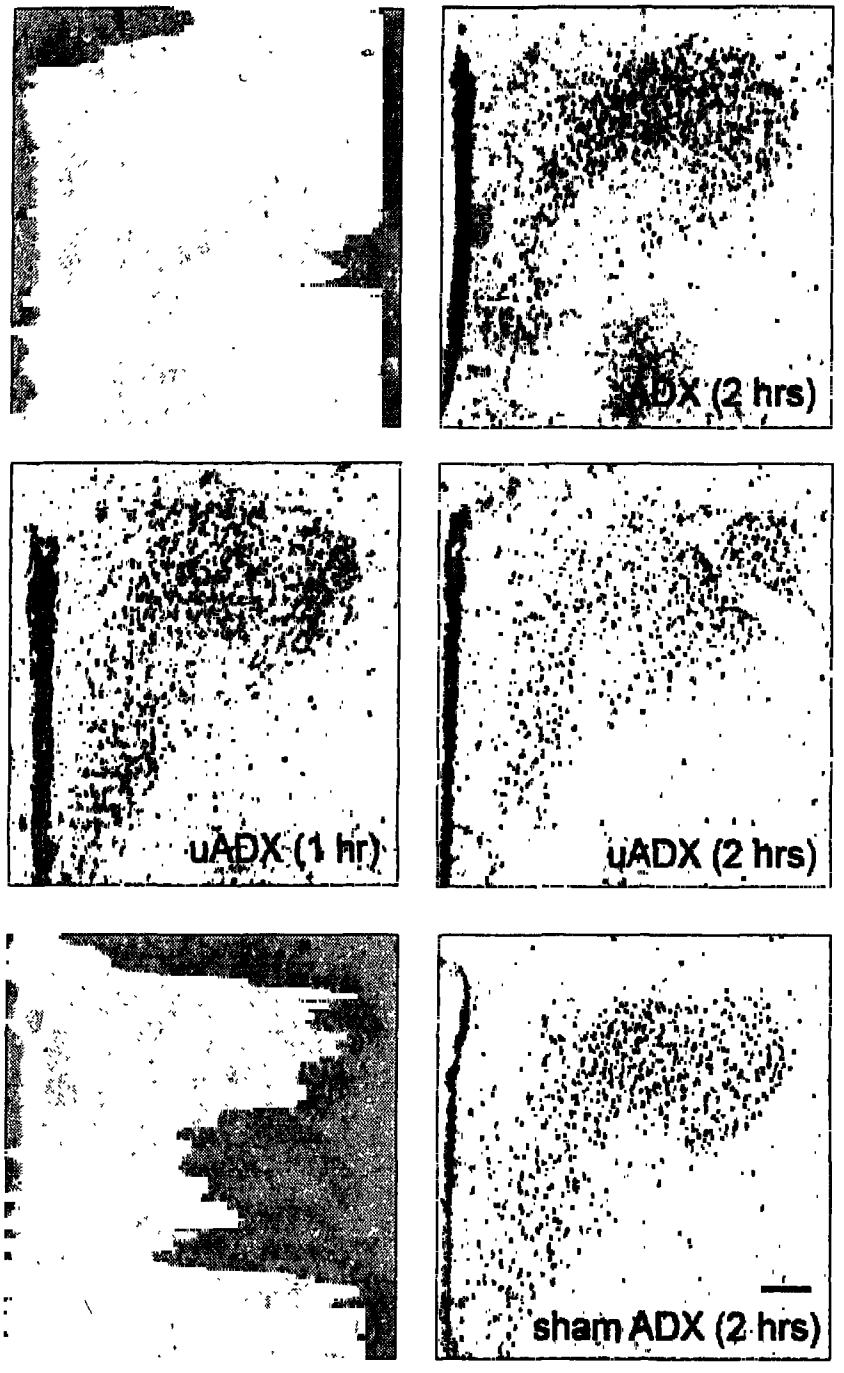


Figure 1.1.2 FLI in the PVN of adult male Sprague-Dawley rats killed 1 hour or 2 hours following ADX, uADX, or sham ADX. Scale bar = 100 μm.

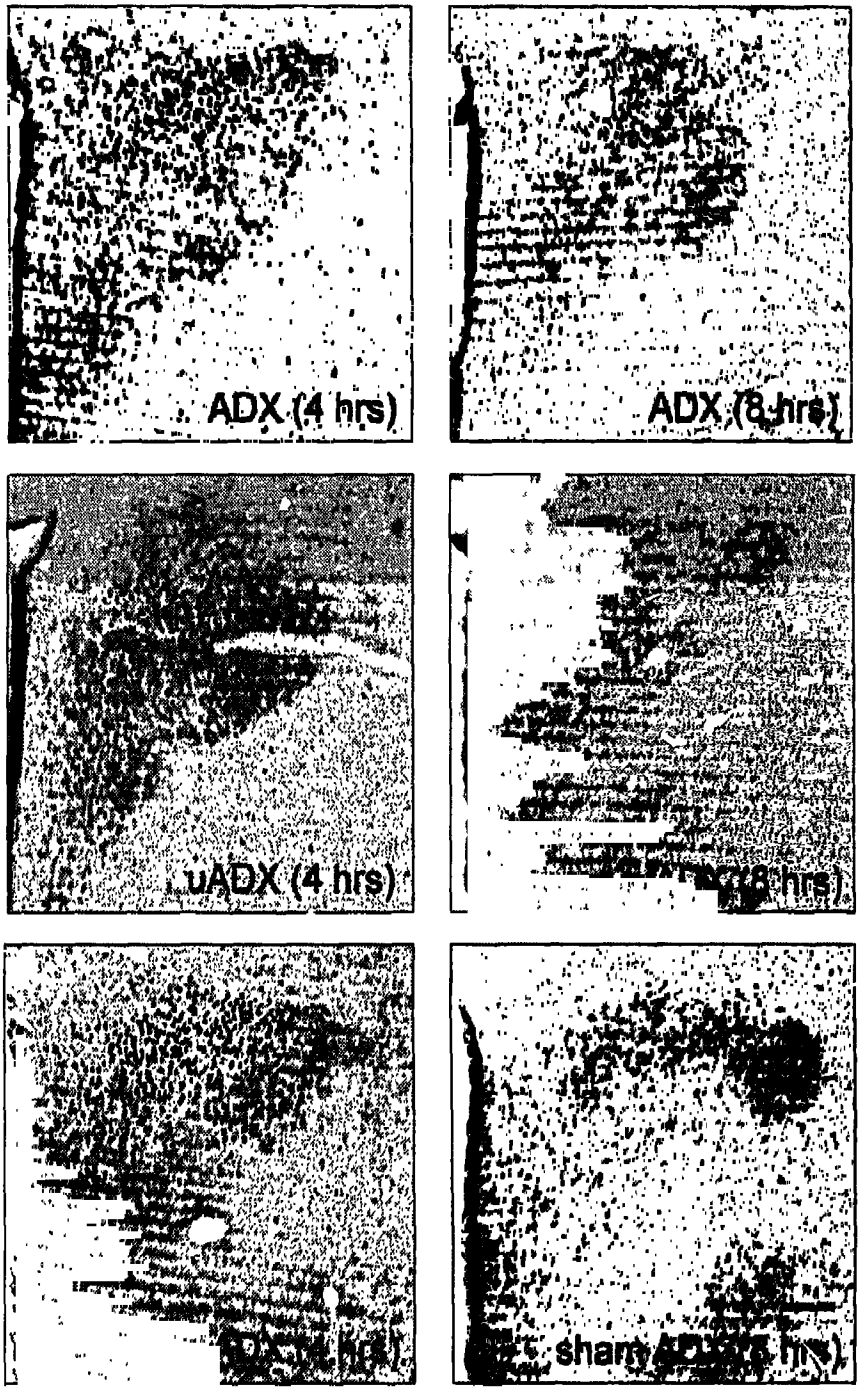


Figure 1.1.3 FLI in the PVN of adult male Sprague-Dawley rats killed 4 hours or 8 hours following ADX, uADX, or sham ADX. Scale bar = 100 μm.

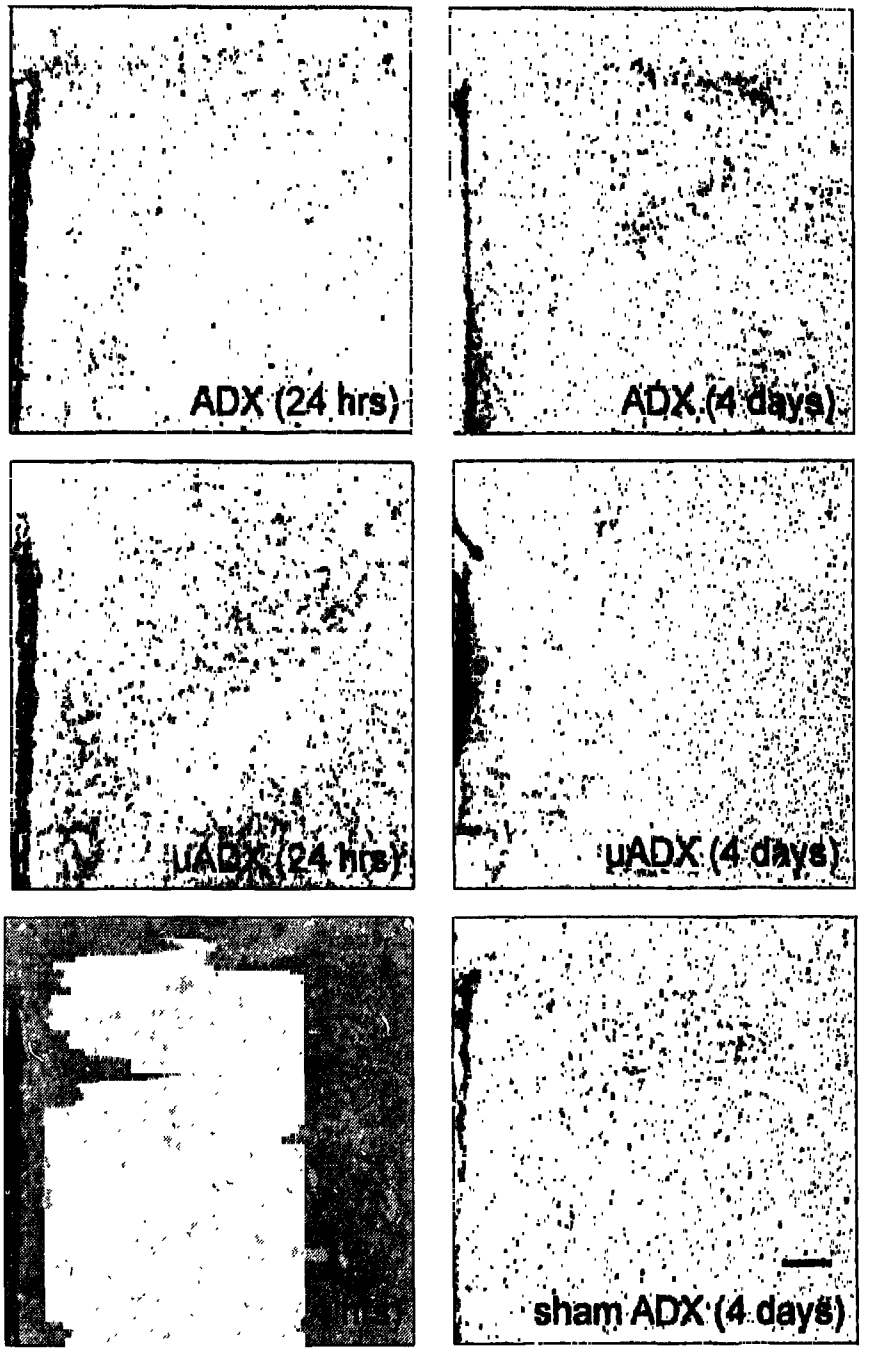


Figure 1.1.4 FLI in the PVN of adult male Sprague-Dawley rats killed 24 hours or 4 days following ADX, uADX, or sham ADX. Scale bar = 100 μm.

increased steadily between 1 hour and 4 hours after surgery, but by 8 hours, an overall decrease was noted (Figures 1.1.2, 1.1.3, and 1.1.4).

At 1, 2, 4, and 8 hours following surgery, ADX appeared to induce a greater increase in FLI in the PVN than did uADX or sham ADX. Surprisingly, at 1, 2, and 4 hours after surgery, less FLI seemed to be present in the PVN of uADX animals compared to sham ADX cohorts (Figures 1.1.2 and 1.1.3).

FLI in the PVN of animals killed 24 hours or longer after surgery did not differ appreciably from basal levels observed in untouched animals (Figure 1.1.4; 7 day survival results not shown).

Experiment 1.2 Circadian variation of basal and ADX-induced FLI in the PVN.

1.2 Methods

At 2-3 hours after "lights on" (1030-1130 AM) or 1-0 hours before "lights off" (9-10 PM), adult male S-D rats (150-250 g.; 3 animals per group) were bilaterally adrenalectomized as previously described. Four hours following surgery, adrenalectomized animals, along with "untouched" control animals, were killed and perfused. Brains were removed, postfixed, and sectioned; and alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

1.2 Results

Digital images of brain sections from these animals are presented in Figure 1.2.1. No FLI was seen in the PVN of untouched control animals killed in the AM or PM, but appreciable accumulations of FLI were seen in the PVN of animals killed 4 hours following ADX. No noticeable AM-PM differences were detected in basal or ADX-induced FLI in the PVN.

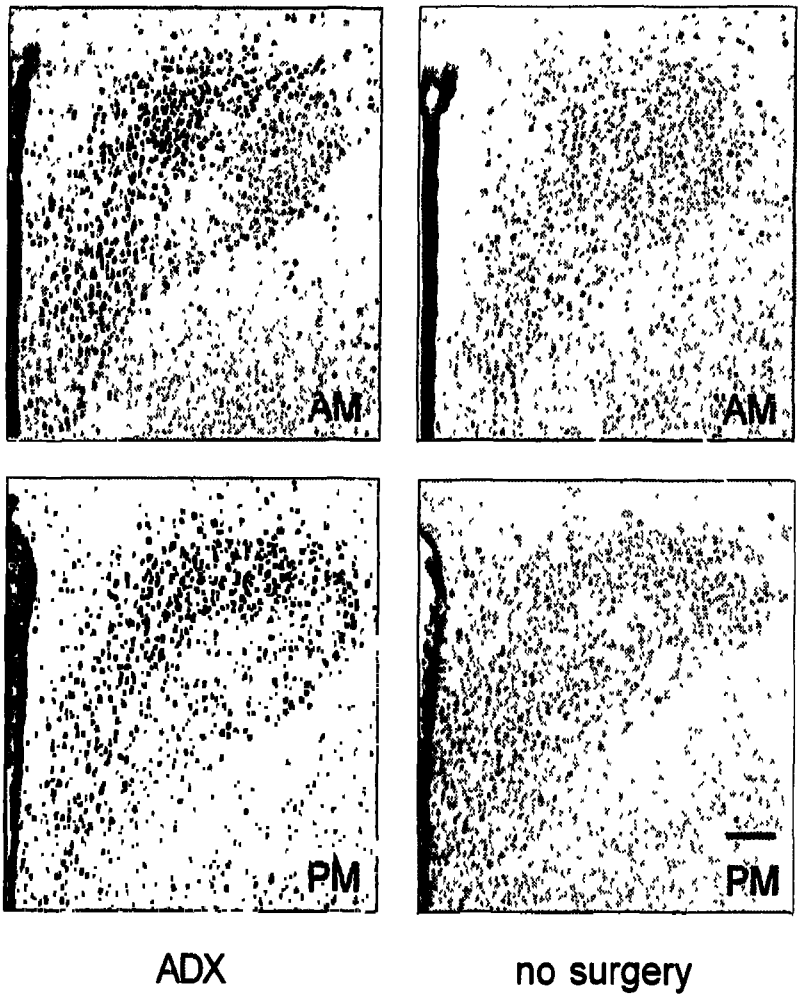


Figure 1.2.1 FLI in the PVN of adult male Sprague-Dawley rats killed 4 hours after ADX or no surgery at 1030-1130 h. (AM) or 2100-2200 h. (PM). Scale bar = 100 μm.

Experiment 1.3. Ontogeny of ADX-induced FLI in the PVN.

1.3 Methods

Male S-D rat pups aged 3, 11, and 18 days, and females aged 3, and 12 days, were removed from their mothers, immediately anaesthetized by cooling (pd 3) or ether inhalation (pd 11, 12 and 18), and bilaterally, unilaterally, or sham adrenalectomized. Following surgery, pups were housed together under warming lights along with untouched pups, and pups which had been anaesthetized but had not received surgery. To prevent maternal cannibalism (which was occasionally observed in pilot studies), pups were housed separated from their mothers throughout the post-surgical period. Two hours (n = 1-2 per treatment) or 4 hours (for numbers per treatment see Table 1.3.1) following surgery or anaesthesia without surgery, animals were killed and perfused; and brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

Table 1.3.1 *Number of rat pups per surgical treatment group divided by sex and age (survival time = 4 hours).*

sex/age	ADX	uADX	sham ADX
male/3 days	6	4	6
male/11 days	9	5	10
male/18 days	2	2	2
female/3 days	3	3	2
female/12 days	2	2	2

1.3 Results

Elevated levels of FLI were seen in the PVN of 3 day old male rats killed 4 hours after ADX, and somewhat lower levels were seen in 2 of 4 animals killed 4

hours after uADX, and in 5 of 6 animals killed 4 hours after sham ADX (Figure 1.3.1). Two animals showed no FLI in the PVN after uADX, and 1 animal showed no FLI after sham ADX. No FLI was seen in the PVN of untouched 3 day old males (n=2; not shown).

Little or no FLI was seen in the PVN of 11 day old male pups killed after 4 hours of separation from their mothers (n=2), and only a few, faintly labeled nuclei were detected in the PVN of 11 day old males killed 4 hours after ether anaesthesia without surgery (n=2; Figure 1.3.2). Elevated levels of FLI were seen in the PVN of 11 day old male rats killed 4 hours after ADX, uADX, or sham ADX (Figure 1.3.1). The general pattern of FLI observed in the PVN of adult animals (i.e., ADX ≥ sham ADX > uADX) was not apparent at this age.

A small increase in FLI was seen in the PVN of 18 day old male rats killed 4 hours after separation from their mothers (n=2), or ether anaesthesia (10 min.) without surgery (n=2; Figure 1.3.2). Increases in FLI seen in the PVN of 18 day old male rats killed 4 hours after ADX, uADX, or sham ADX appeared to be slightly lower than those seen in like-treated younger animals (Figure 1.3.1).

Digital images from 3 day old and 12 day old <u>female</u> rats are presented in Figure 1.3.3. The results seen in female rats were slightly different to those seen in age-matched males. For example, marked increases in FLI were seen in the PVN of 3 day old females killed 4 hours after ADX, whereas very little FLI was seen in the PVN of females killed 4 hours following uADX or sham ADX (see Figure 1.3.3). Levels of FLI in the PVN of 12 day old ADX, uADX, and sham ADX female rats were comparable to those seen in 11 day old males (see Figures 1.3.1 and 1.3.3).

Levels of FLI in the PVN of 11 day old male rats killed <u>2 hours</u> after ADX, uADX, sham ADX, or anaesthetic without surgery were markedly higher than those seen in like-treated age-matched males killed at 4 hours after treatment

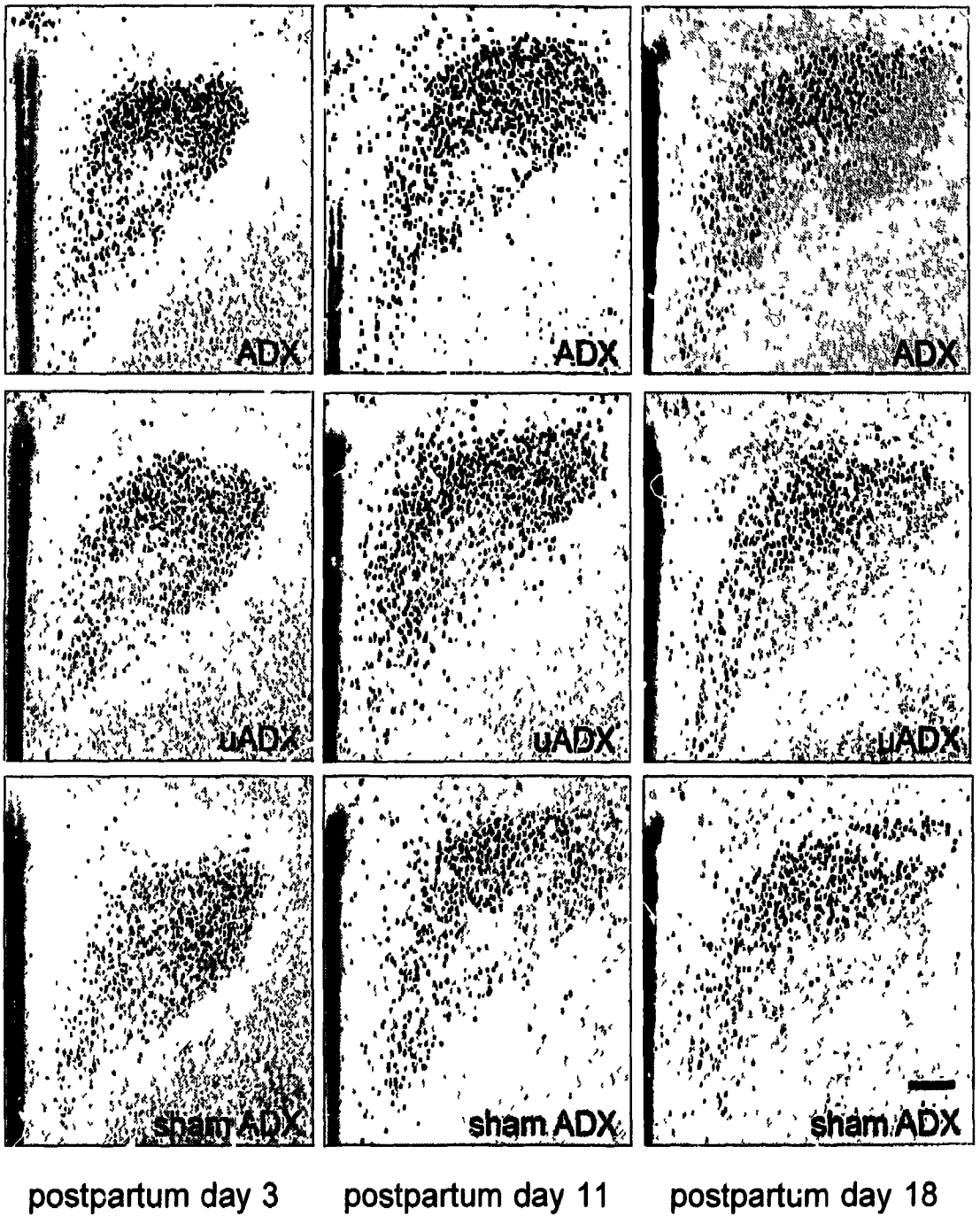


Figure 1.3.1 FLI in the PVN of 3 day, 11 day, and 18 day old male Sprague-Dawley rats killed 4 hours after ADX, uADX, or sham ADX. Sections from 3 day old rats were cut at 100 µm. Sections from 11 and 18 day old rats were cut at 50 µm. Scale bar = 100 µm.

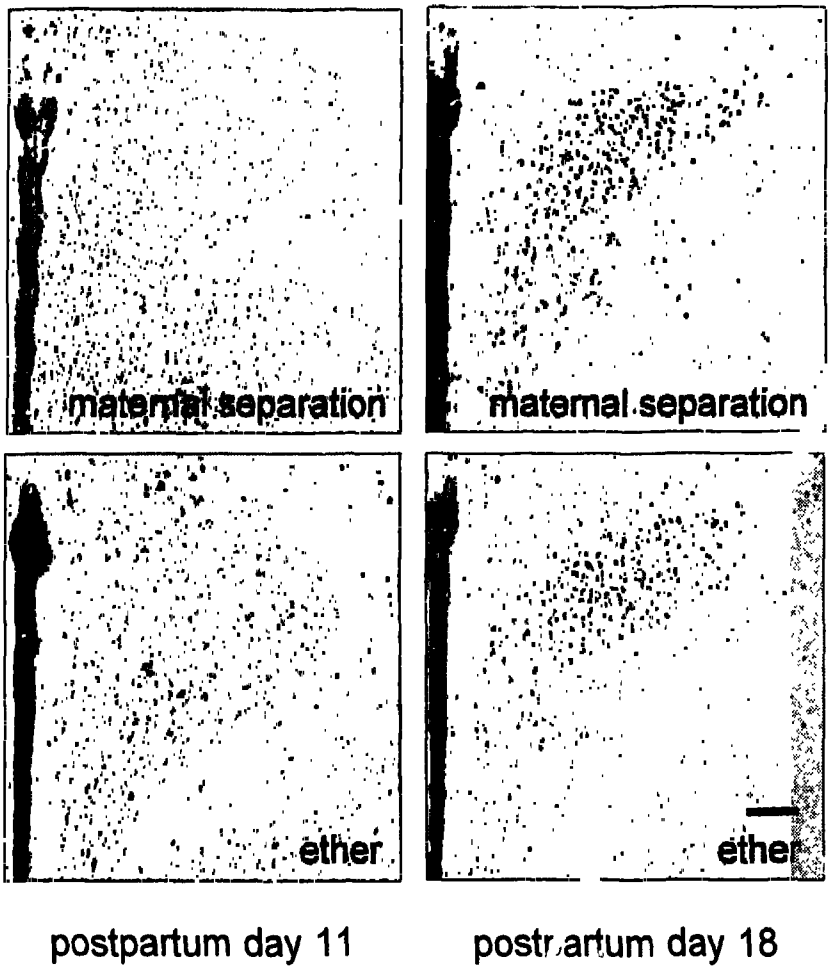


Figure 1.3.2 FLI in the PVN of 11 day old, and 18 day old male Sprague-Dawley rats killed 4 hours after separation from mother (maternal separation), or ether anaesthesia (10 min) and separation from mother. Scale bar = 100 μm.

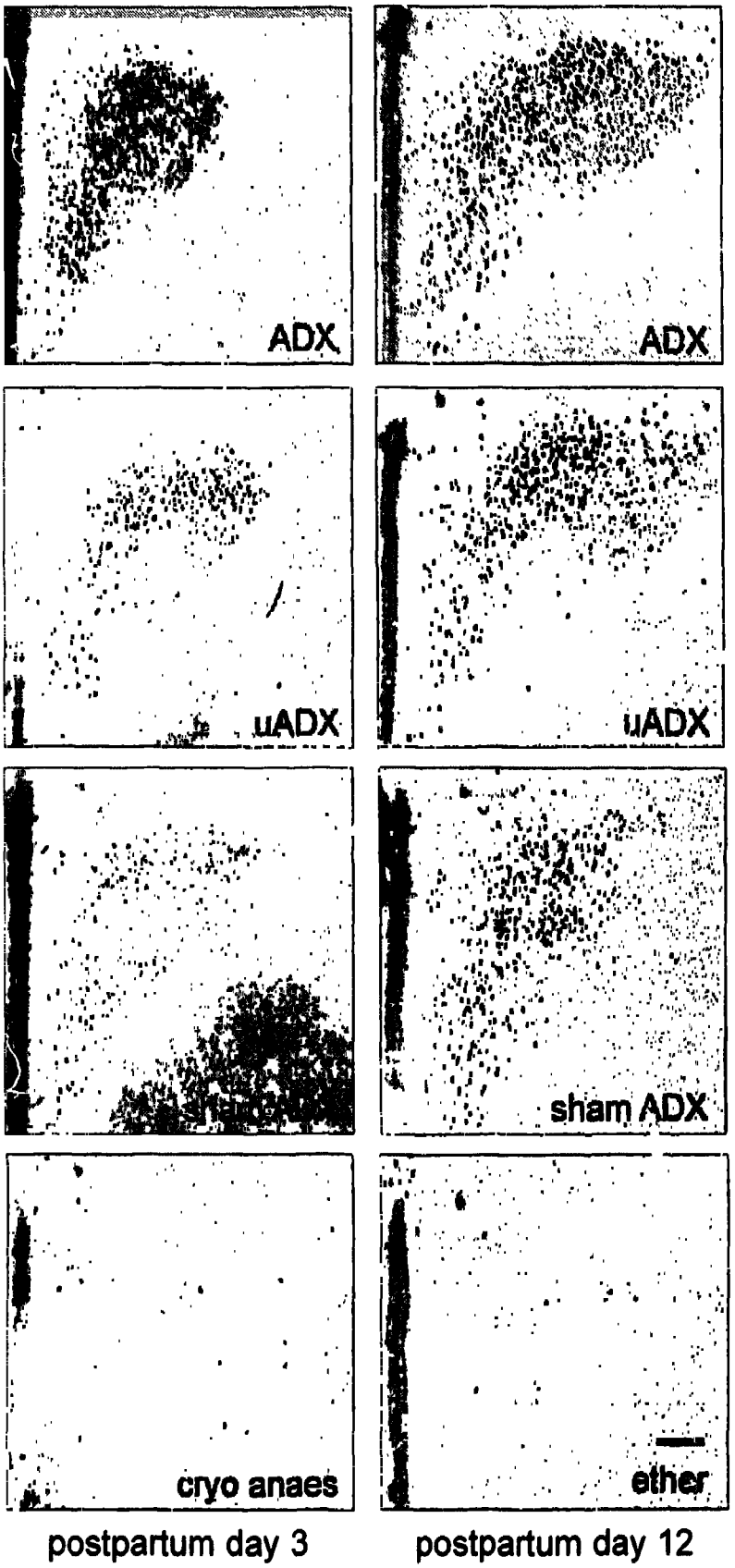


Figure 1.3.3 FLI in the PVN of 3 and 12 day old female Sprague-Dawley rats killed 4 hours after ADX, uADX, sham ADX, or anaesthesia [10 min.; day 3 by cooling (cryo anaes), day 12 by ether] without surgery. Scale bar = 100μm.

(Figures 1.3.1 and 1.3.2 vs. Figure 1.3.4). Levels of FLI in the PVN of 11 day old males killed 2 hours following ADX appeared to be somewhat higher than levels seen in males killed following uADX or sham ADX (ADX > uADX = sham ADX).

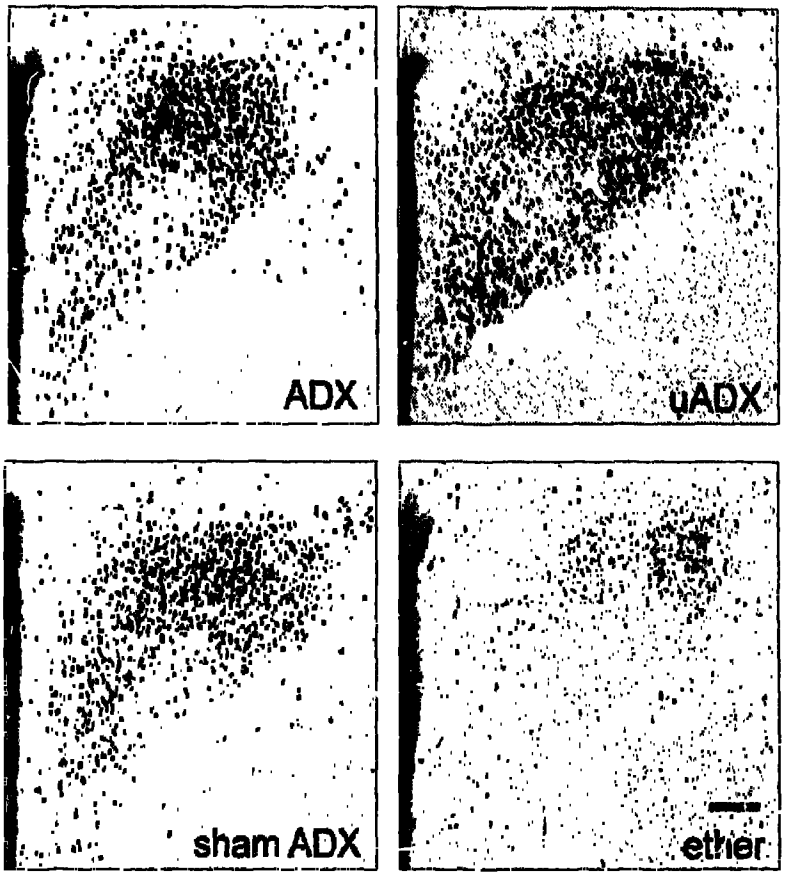


Figure 1.3.4 FLI in the PVN of 11 day old male Sprague-Dawley rats killed 2 hours after ADX, uADX, sham ADX, or ether anaesthesia (10 min.) without surgery. Scale bar = 100 μm.

Experiment 1.4. Effect of ADX on FLI in the PVN of Lewis Rats.

1.4 Methods

Male arthritis-susceptible Lewis rats (175-200 g.; n=2 per treatment) were anaesthetized and returned to their home cages, or anaesthetized and bilaterally, unilaterally, or sham adrenalectomized. Four hours following surgery or anaesthesia without surgery, animals, along with untouched control animals, were killed and perfused. Brains were removed, postfixed, and sectioned, and alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

1.4 Results

Digital images of brain sections from adult male Lewis rats are presented in Figures 1.4.1 and 1.4.2 alongside images from like-treated S-D males from experiment 1.1 (see also Figure 1.1.1 and 1.1.3). Levels of FLI in the PVN of untouched Lewis animals were low, and only marginal increases were detected in Lewis rats killed 4 hours after Somnotol anaesthesia without surgery. Both untouched and anaesthetized animals resembled like-treated S-D males (Figure 1.4.1). Levels of FLI in the PVN of Lewis rats killed 4 hours after ADX were comparable to those seen in ADX S-D males, but levels of FLI in the PVN of Lewis rats killed 4 hours after uADX or sham ADX were noticeably lower compared to like-treated S-D animals (Figure 1.4.2). In contrast to results seen in S-D rats, levels of FLI in the PVN of uADX Lewis rats were not lower than those seen in sham ADX Lewis animals.

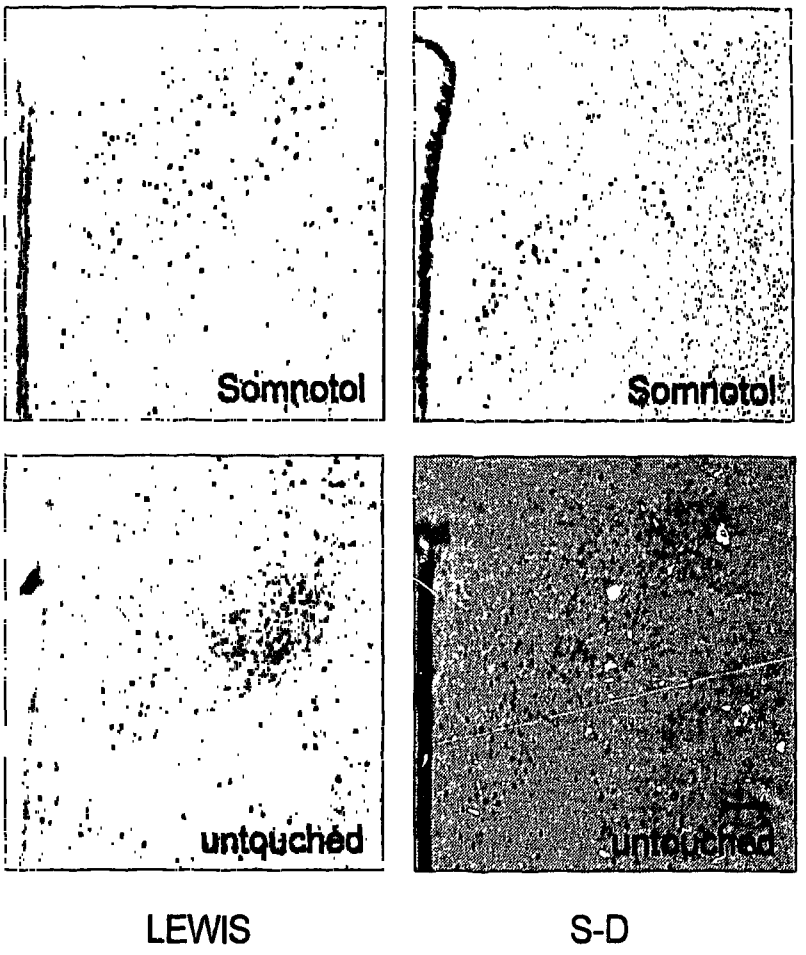


Figure 1.4.1 FLI in the PVN of untouched adult male Lewis and Sprague-Dawley (S-D) rats, and adult male Lewis and S-D rats killed 4 hours following Somnotol anaesthesia without surgery. Scale bar = 100 µm.

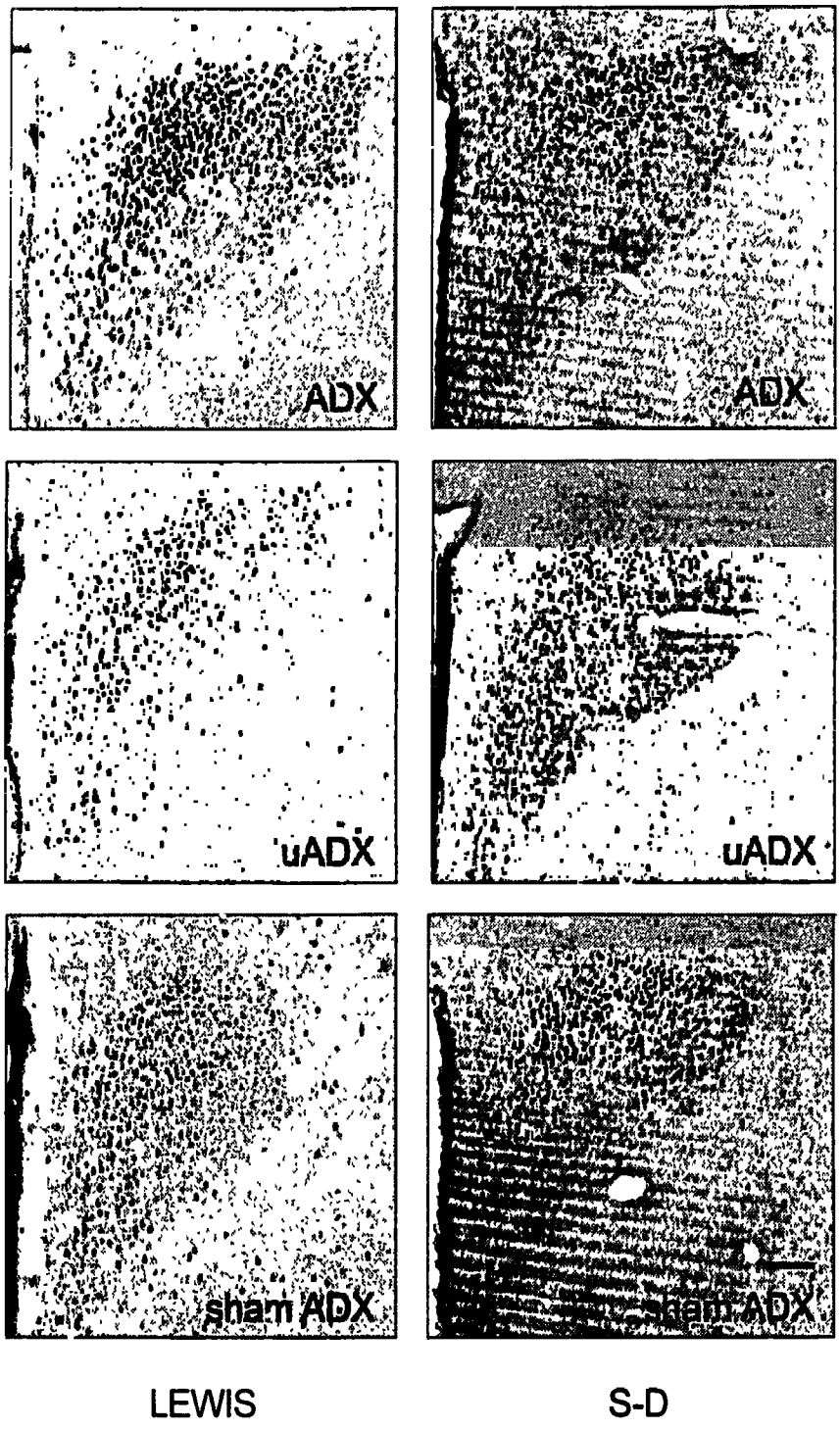


Figure 1.4.2 FLI in the PVN of adult male Lewis and Sprague-Dawley (S-D) rats killed 4 hours following ADX, uADX, or sham ADX. Scale bar = 100 μm.

Experiment 1.5 Effect of corticosterone priming on FLI in the PVN.

1.5 Methods

Adult male S-D rats (275-400 g.; 2 animals per treatment) received an injection of CORT (4-Pregnene-11ß,21-diol-3,20-dione; Sigma, St. Louis MO; 1 mg./kg. b.w. in 0.1 ml sesame oil, s.c.) at 2 hours, and at 5 minutes prior to being anaesthetized and bilaterally, unilaterally, or sham adrenalectomized. Following surgery, animals were returned to their home cages. In addition, 2 males received the same CORT dosages as the operated animals followed by anaesthetic without surgery. Two hours after surgery or anaesthesia, animals were anaesthetized and perfused. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

1.5 Results

Digital images of brain sections from CORT-primed ADX, uADX, and sham ADX male S-D rats are presented in Figure 1.5.1 alongside images from non-CORT-primed, like-treated S-D males from experiment 1.1 (see also Figure 1.1.2). Small increases in FLI were seen in the PVN of all CORT-primed animals killed 2 hours after adrenal surgery but all groups of CORT-primed rats displayed smaller increases in FLI compared to animals from experiment 1.1 which received similar surgeries without CORT priming (Figure 1.5.1). No FLI was seen in the PVN of CORT-primed rats which received anaesthetic without surgery (not shown).

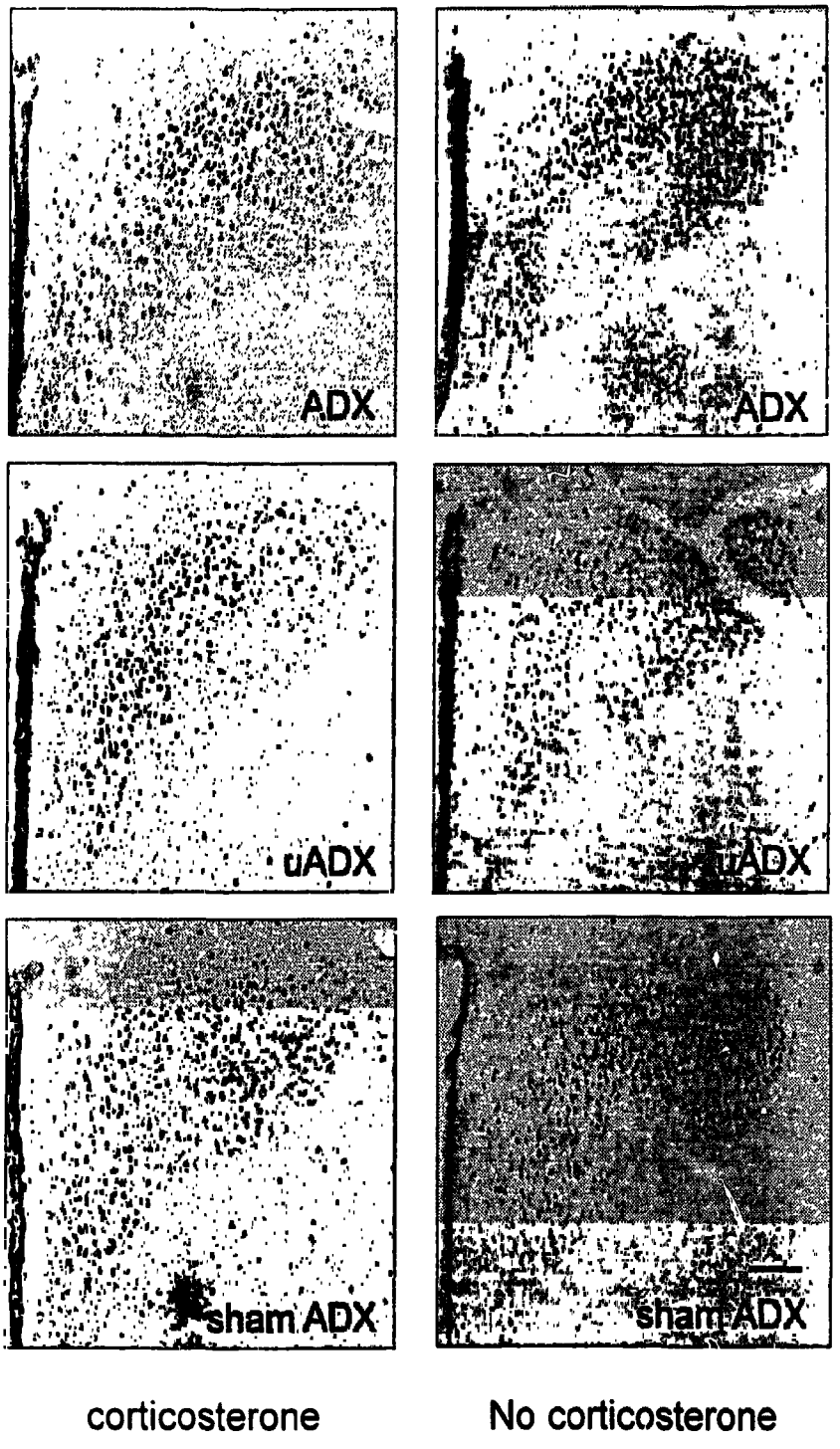


Figure 1.5.1 FLI in the PVN of adult male Sprague-Dawley rats killed 2 hours after ADX, uADX, or sham ADX, with or without corticosteroid priming (1.0 mg./kg. bw) at 2 hours and at 5 minutes prior to surgery. Scale bar = 100 μm.

Experiment 1.6 Effect of ovariectomy or laparotomy on FLI in the PVN of adrenalectomized adult S-D rats.

1.6 Methods

Adult male and female S-D rats (6 animals per treatment) were anaesthetized, bilaterally adrenalectomized, and returned to their home cages for recovery (3 animals per cage). Throughout the post-ADX period, animals had free access to food, water, and isotonic saline. Seven days later, animals were again anaesthetized, and males received either sham ADX (laparotomy) or no surgery, and females received either OVX or sham OVX (laparotomy). Four hours following surgery or anaesthesia without surgery, animals were killed and perfused. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

1.6 Results

Digital images of brain sections from rats used in this experiment are presented in Figures 1.6.1 and 1.6.2. Levels of FLI in the PVN of 7 day-ADX female rats killed 4 hours following OVX were dramatically elevated compared to levels seen in untouched animals (see Figures 1.1.1 and 1.6.1). Levels of FLI in the PVN of female 7 day-ADX rats killed 4 hours after laparotomy were also elevated but to a lesser degree than those seen in 7 day-ADX OVX females. Levels of FLI in the PVN of 7 day-ADX laparotomized males were comparable to those seen in 7 day-ADX OVX females. Results obtained from 7 day-ADX males killed 4 hours after the onset of anaesthesia without surgery were extremely variable. Of the three animals in this group, one displayed dramatically elevated levels of FLI in the PVN (comparable to those seen in animals killed 4 hours after

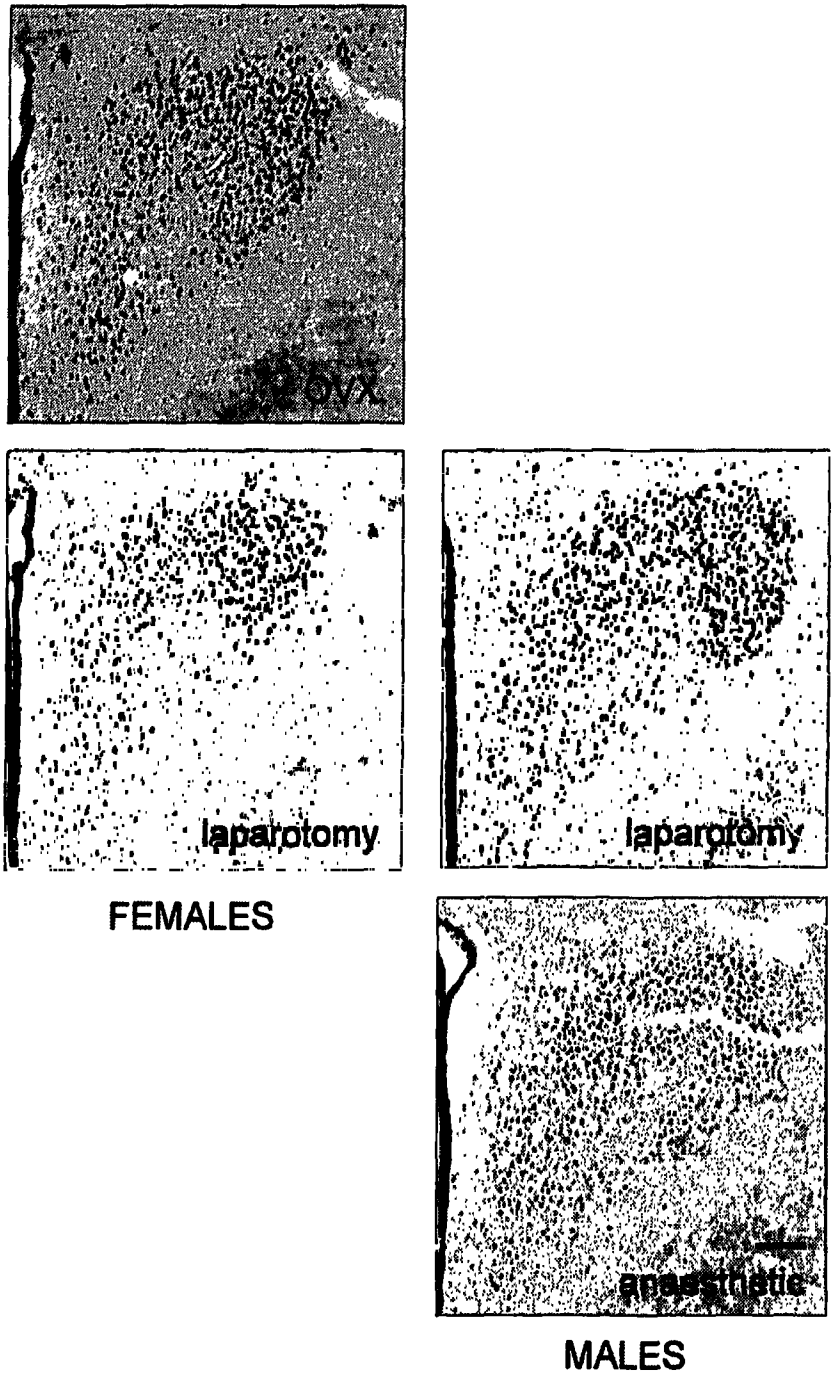


Figure 1.6.1 FLI in the PVN of adrenalectomized (7 days) adult female and male Sprague-Dawley rats killed 4 hours after OVX or laparotomy (females), or laparotomy or anaesthesia without surgery (males). Scale bar = 100 μm.

ADX), one animal displayed moderately elevated levels of FLI in the PVN, and one displayed no evidence of increased FLI (see Figure 1.6.2).

Figure 1.6.2 FLI in the PVN of 3 adrenalectomized (7 days) adult male Sprague-Dawley rats killed 4 hours after anaesthesia without surgery. Scale bar = 100 μm.

Discussion

As outlined in the introduction to this chapter, experiments were designed to survey various aspects which influence *c-fos* gene activation in the PVN with the intent of establishing a suitable model for further research. In the course of these experiments, several valuable lessons were learned, and some shortcomings of this approach were identified. The results are discussed below under a number of subheadings.

Time Course

The results of experiment 1.1 indicate that levels of FLI in the PVN of adult male rats are transiently increased following ADX, uADX, or sham ADX. The absence of FLI in the PVN of untouched rats was expected and has been reported by other investigators (Rivest and Rivier 1994; Takayama et al. 1994b). Nonetheless, it verifies that the ambient living conditions of the animals used in these experiments do not contribute to FLI in the PVN, and provides a baseline against which levels of FLI detected following experimental manipulation can be compared. Levels of FLI were elevated in the PVN of animals killed 30 minutes to 8 hours following adrenal surgery, but not in the PVN of animals killed 24 hours or longer after surgery (see Figures 1.1.1 and 1.1.2). The observation that elevated levels of FLI were not seen in animals killed 10 minutes after surgery confirms the contention of Hoffman et al. (1992) that the appearance of Fos protein is delayed 30-45 minutes after the onset of the stimulation which elicits it. A survey of published reports of *c-fos* gene activation (by various diverse stimuli), revealed that the time course for ADX-induced FLI established in experiment 1.1, coincides with the time course for Fos protein synthesis and degradation reported by most authors. For example, the time course reported for

both kainic acid-induced FLI (Le Gal La Salle 1988), and acute exercise-induced FLI (Rivest and Rivier 1995) resembles that seen following adrenal surgery.

The absence of FLI in the PVN of both untouched animals and animals killed 10 minutes after adrenal surgery (see Figure 1.1.1) is particularly significant. Given this result, it is reasonable to assume that the elevated levels of FLI observed in the PVN of animals killed following survival times ≥30 minutes, reflect the effect of adrenal surgery and, as suggested by Hoffman et al. (1992), are not influenced by stressors associated with sacrifice and perfusion.

The absence of FLI in the PVN of animals killed 1, 3, or 7 days after ADX is consistent with the results of preliminary experiments conducted using two other Fos antibodies (Medac and Oncogene Science), which also revealed no evidence of increased FLI in the PVN of rats killed 1 to 7 days after ADX (results not shown). These results, however, contradict the report of Jacobson et al. (1990), that levels of FLI in the PVN remained elevated for up to 7 days following ADX. The inconsistency between the results reported in this thesis, and those reported by Jacobson's group, are likely related to differences in the antibody specificity. As described earlier (see the Introduction to this thesis), Fos antibodies raised against amino acids 127-152 of the Fos protein are more likely to detect both Fos and Fra than antibodies raised against amino acid sequences taken from the N-terminal region of the Fos protein (Morgan and Curran 1991; Hoffman et al. 1993). The antibody used by Jacobson et al. (obtained commercially from Microbiological Associates, Bethesda, MD) was raised against amino acids 132-154 of the Fos protein. This sequence overlaps a substantial portion of the Fos amino acid sequence shared by several Fra. In contrast, the Fos antibody used in experiments in this thesis was raised against amino acids 2-17 of the Fos protein. This sequence is reported to be relatively specific to Fos [Santa Cruz Biotechnology Inc. (Santa Cruz CA) markets a similar Fos antibody (catalogue #

sc-52) raised against amino acids 3-16, which is non cross-reactive with Fos-B, Fra-1, or Fra-2]. Given the apparent lack of specificity of the Microbiological Associates Fos antibody, and current knowledge concerning the longevity of Fos and Fra, it is likely that the persistent (i.e., ≥ 24 hours) immunoreactivity seen by Jacobson's group reflects cross-reactivity of their antibody with a longer lived Fra.

Circadian Variation

The failure to detect circadian variation in basal FLI in the PVN (Figure 1.2), given the known diurnal variation in blood CORT levels, suggests that diurnal variations in CORT levels do not affect FLI in the PVN. This observation also confirms that differences in the time at which a surgical procedure is conducted is not critical in these experiments. This is significant as, in the experiments in this chapter, surgeries were frequently performed consecutively on a series of animals (occasionally over a number of hours).

The absence of a circadian effect on the magnitude of ADX-induced FLI in the PVN, might indicate that the effect of adrenal surgery on the HPA axis obliterates the influence of circadian variations on FLI activation in the PVN. A search of the literature failed to uncover any reference to circadian fluctuations in basal FLI in the PVN. Given that the daily CORT/ACTH rhythm is likely driven by CRH neurons, it will be interesting to determine whether parallel diurnal changes in FLI occur in the PVN. This will likely require application of more sensitive quantitative methods, such as those used in chapters 2 and 3 of this thesis, to reveal subtle differences which elude detection by visual inspection of a series of microscope slides or photographs.

Role of CORT Feedback

Two experiments in this chapter examine the role of CORT negative feedback in mediating stress-induced changes in FLI in the PVN. In experiment 1.5, the effect of feedback from exogenous CORT was examined, and in experiment 1.6, endogenous CORT feedback was eliminated by prior ADX.

The observation that exogenous CORT attenuates adrenal surgery-induced increases in FLI in the PVN (Figure 1.5.1), suggests that elevated levels of circulating CORT inhibit *c-fos* gene activation in CRH neurons in the PVN (at least in the short term). The observation that CORT inhibition of ADX-induced ACTH release is blocked by lesion of the PVN (Levin et al. 1988), provides further support for the contention that neurons of the PVN are a target for CORT feedback inhibition. The absence of FLI in the PVN of CORT primed male and female rats killed following anaesthesia without surgery (Figure 1.5.2) confirms that exogenous CORT, at the dosages used in this experiment, does not induce FLI in the PVN at the time sampled.

Regrettably, animals in this experiment were killed 2 hours following surgery whereas, apart from time course experiments, much of the data presented in this chapter were obtained from animals, killed 4 hours after surgery. It would be useful to repeat this experiment with the following modifications: 1) inclusion of a saline-primed control group, 2) inclusion of "anaesthetic without surgery", and "untouched" animals in each group, and 3) extension of the post-surgical survival time to 4 hours.

The results of experiment 1.5 indicate that CORT exerts a degree of negative feedback on *c-fos* gene activation in the PVN. In experiment 1.6, the observation of elevated levels of OVX- and laparotomy-induced FLI in the PVN of 7 day post-ADX animals (Figure 1.6.1) clearly demonstrates that *c-fos* gene activation occurs in these neurons in the absence of a CORT feedback signal. The higher levels of

laparotomy-induced FLI seen in the PVN of 7 day post-ADX male rats (Figure 1.6.1) compared to those seen in like-treated (sham ADX) intact males in experiment 1.1 (see Figure 1.1.3) suggests that the threshold for *c-fos* gene activation is lower in the PVN of ADX animals. In both ADX and intact animals, surgical trauma stimulates nociceptive and sympathetic neural pathways which impinge upon the PVN (see Figure 1 in the Introduction to this thesis). The relatively higher levels of laparotomy-induced FLI seen in the PVN of ADX animals likely reflects the absence of CORT feedback inhibition, which normally damps the influence of these pathways (see Figure 9 in the introduction to this thesis). The observation that laparotomy induces larger increases in plasma ACTH in 24 hour post-ADX rats compared to 24 hour post-sham ADX rats or 24 hour post-ADX animals given CORT replacement prior to laparotomy (Dallman et al 1972) provides further support for this contention.

The variable results observed in 7 day post-ADX male rats subjected to anaesthesia without surgery (see Figure 1.6.2) might also indicate that the threshold of activation of PVN neurons is lower in ADX animals. In the absence of the moderating effect of CORT on PVN neurons, slight differences in the treatment of animals during the administration of anaesthetic might translate into large differences in FLI in the PVN. Alternatively, in some animals, some adrenal tissue might have remained following ADX. Completeness of ADX could have been verified by analysis of blood collected at sacrifice, or by immunostaining the PVN using an antibody against CRH. CRH cannot be detected immunocytochemically in the PVN of intact rats, but is elevated in ADX or colchicine treated animals (Alonso et al. 1986; Sawchenko 1987). In this instance, statistical analysis of quantified data might prove useful to assess the impact that the variability observed in anaesthetic-treated animals might have on the overall results of this experiment.

It is tempting, given the similar distribution of laparotomy-induced FLI within the PVN of ADX and intact rats (see Figures 1.1.3 and 1.6.1) to conclude that the same population of neurons are labeled in both instances. Following ADX, many CRH neurons in the PVN coexpress VP (Alonso et al. 1986; Sawchenko 1987). Thus, the neurochemical consequences of activating a particular population of neurons might be profoundly altered in ADX animals. It will be interesting to determine the effect of laparotomy on hypothalamic and circulating CRH and VP levels in intact vs. ADX rats. Further experiments, using double labeling techniques, will also be useful to establish whether the same population of PVN neurons expres. FLI following laparotomy in intact and 7 day post-ADX animals, and to determine whether the result of activation of these neurons is the same in both cases.

Effect of uADX

The overall pattern of FLI observed in the PVN of adult animals following ADX, uADX, and sham ADX (i.e., ADX ≥ sham ADX > uADX; see Figures 1.1.1, 1.1.2, and 1.1.3) is somewhat surprising, given that uADX involves more extensive tissue trauma (and might on this basis be considered more stressful) than sham ADX. One explanation for this phenomenon might be that, uADX elicits a more robust CORT response than sham ADX, and the ensuing CORT feedback damps activation of CRH neurons in the PVN. This interpretation, however, is unlikely, as Dallman et al. (1977) reported that 2 hours after surgery, circulating CORT (and ACTH) levels in uADX rats do not differ f om those of sham ADX animals. In short, the regulation of uADX-induced FLI in the PVN likely involves neural mechanisms. The absence of this pattern in neonatal animals (see Figure 1.3.1) suggests that these mechanisms do not mature until after the end of the second postnatal week.

Evidence suggests that uADX activates a neural circuit or circuits which alter the level of activity in various brain regions, including the PVN. In addition to the transient increase in ACTH and CORT reported by Dallman et al. (1977), uADX is known to initiate non-ACTH-dependent compensatory hypertrophy in the remaining adrenal gland (Dallman et al. 1977; Holzwarth and Dallman 1979). It will be interesting to determine whether pharmacological blockade of CORT secretion, or manipulations known to block or attenuate compensatory hypertrophy of the remaining adrenal gland (i.e., spinal cord hemisection or local anaesthesia of the adrenal prior to its removal) might also alter the FLI response in the PVN following uADX.

Ontogenic Development

The results obtained from neonatal rats suggest that, in spite of the stress-hyporesponsiveness that characterizes this period of development, ADX induces a more robust FLI-response in the PVN of 3 and 11 day old rats compared to older animals (see Figure 1.3.1). Low circulating CORT levels, which characterize this period (Walker and Vrana 1993) might account for the potentiated FLI response seen in the PVN. It has been argued that during the second postnatal week, low circulating CBG and pituitary transcortin levels effectively amplify the CORT negative feedback signal at the level of the pituitary (see the introduction to this thesis; De Kloet et al. 1988; Rosenfeld et al. 1992). This hypothesis is supported by the observation that during this period, the CORT response to exogenous CRH is significantly depressed (Guillet et al. 1980; Walker et al. 1986a). Also, during this period the hypothalamic-hypophyseal portal system is not fully developed (see De Kloet et al. 1988). Taken together, these observations indicate that during the second week of life, the ability of CRH neurons in the PVN to stimulate ACTH secretion from the pituitary, and

thereby CORT secretion from the adrenals, is severely diminished. This being the case, the potentiated FLI response to ADX seen in the PVN of 3 and 11 day old rats is not inconsistent with the stress hyporesponsiveness reported at the level of the pituitary and adrenals. The smaller FLI response to ADX seen in the PVN of 18 day old animals provides further support for this interpretation as pituitary transcortin levels reach adult levels by postnatal day 10 (see Sapolsky and Meaney 1986), circulating CBG levels begin to rise on day 9 (D'Agostino and Henning 1981), and circulating CORT levels reach adult values by day 15 (Meaney et al. 1985; Vazquez and Akil 1992). During the first 2 postnatal days, rat pups are reported to exhibit an adult-like CORT response to many stressors (Arai and Widmaier 1991; Walker et al. 1991). It will be interesting to determine whether animals of this age also exhibit adult-like FLI responses to ADX. Preliminary data indicate that in adult animals, prior injections of CORT reduce the magnitude of ADX-induced increases in FLI in the PVN (see experiment 1.7). The possible effects of CORT on FLI levels in neonates also need to be determined.

An alternate explanation for the elevated FLI response to ADX seen in the PVN of day 3 and day 11 animals is that immature afferent neural circuitry might result in attenuated inhibitory inputs to the PVN. Low hippocampal GC receptor concentrations render the HC incapable of inhibiting the HPA axis in animals younger than 9 days (Meaney et al. 1985; Sapolsky and Meaney 1986). It is possible that the potentiated FLI response to ADX observed on day 3 and on day 11 might reflect this.

To date, the role of endogenous excitatory amino acids in regulation of the HPA axis is largely unknown (see Oliver et al. 1996). In contrast to the hyporesponsiveness to many stressors which characterizes the neonatal SHRP, animals of this age display a heightened sensitivity to excitatory amino acids

(Chautard et al 1993; McDonald et al 1988; McDonald and Johnston 1990). The potentiated ADX-induced FLI response seen in the PVN of 3 and 11 day old rats suggests that exploration of the role of glutamatergic neural pathways in the FLI response to ADX might yield interesting results. The role of glutamatergic neurons in activation of the HPA axis is considered in detail in Chapter 3 of this thesis.

The attenuated FLI response (compared to pd 18 males) seen in the PVN of 11 day old male rats killed 4 hours after ether anaesthesia (Figure 1.3.2) is consistent with the observation that CORT response to ether is also attenuated during the SHRP (Walker et al. 1986a, 1991; Eck and Kuhn 1992). The absence of FLI in the PVN of 11 day old male rats killed 4 hours after separation from their mother (Figure 1.3.2) is supported by findings by other investigators. For example, Levine et al. (1994) noted that 3 hours of maternal separation had no effect on the plasma ACTH levels of 5-12 day old rats. The appearance of FLI in the PVN of maternally-deprived 18 day old rats (Figure 1.3.2) is compatible with the observation that the ability of maternal contact to inhibit infant stress-response diminishes with time since parturition (Stanton and Levine 1990).

The absence of a sex difference in ADX-induced FLI in 3 and 11-12 day old rats (Figure 1.3.1 vs. 1.3.3) is not surprising as no report of sex differences in the HPA-response of prepubertal animals could be found in the literature. The relatively higher levels of FLI seen in the PVN of 11 day old male rats killed 2 hours after adrenal surgery or anaesthesia without surgery, compared to like-treated animals killed at 4 hours post-treatment, suggests that the time course of FLI induction or FLI degradation in the PVN might be somewhat accelerated in young rats compared to adults. In experiment 1.1, the levels of FLI in the PVN of adult male rats subjected to adrenal surgery peaked between 2 and 8 hours after surgery. Results from like-treated 11 day old male rats suggests that in animals

of this age, levels of FLI are declining by 4 hours after surgery (Figure 1.3.1 vs. 1.3.4). This time course resembles that seen for NMDA-induced FLI in the arcuate nucleus of 30 day old rats reported by MacDonald et al. (1993).

Initial results obtained from 3-21 day old animals suggested that during the neonatal SHRP, the magnitude of ADX-induced FLI response in the PVN follows a U-shaped path (i.e., lower levels were displayed in the PVN of 11 day old animals compared to 3 day old or 18-21 day old animals). These results were obtained using the CRB Fos antibody (lot #823). With the completion of further studies using the successor of the above antibody (CRB, lot #824), slightly different results were obtained. If suppression of the FLI response to ADX does occur in the PVN during the neonatal SHRP, it is not visually apparent in tissue sections processed for Fos immunocytochemistry using the newer CRB antibody (#824). In this instance, the potential of quantitative image analysis is apparent. Except when within treatment variability is low and between groups differences are (relatively) high, visual inspection of tissue sections cannot be considered an infallible technique for drawing conclusions from data of this type. However, subtle age differences in ADX-induced FLI in the PVN might be revealed by statistical analyses applied to quantified results.

The sections from 3 day old animals presented in Figure 1.3.1 were cut at 100 µm. (vs. 50 µm. for older animals). This discrepancy could account for at least some of the apparently higher concentration of FLI-positive nuclear profiles seen in the PVN of 3 day old animals. Again, by applying quantitative image analysis techniques, it might be possible to correct for differences in section thickness. If the section thickness and the number of FLI-positive nuclear profiles are known, the number of profiles which would be present in thicker or thinner sections can be estimated (see Coggeshall 1992). Note, however, that although quantitative

methods allow accurate comparisons between sections of varying thickness, it is still preferable to apply quantitative methods to sections of uniform thickness.

Strain Differences

The experiment designed to determine whether *c-fos* expression might be different in a known hyporesponsive strain (Lewis rats) gave interesting results. These results should be interpreted cautiously, however, as they are based on a small number of observations (2 Lewis rats per treatment), and utilize S-D animals from earlier experiments for comparison purposes. The observation that basal levels of FLI in the PVN of adult male Lewis rats were extremely low and did not differ from those seen in adult male S-D rats (Figure 1.4.1), confirms the observation of Rivest and Rivier (1994) who reported no detectable FLI in the PVN of untouched Lewis rats and S-D rats. The observed strain differences in FLI in the PVN of Lewis vs. S-D rats following uADX or sham ADX, but not following ADX (see Figure 1.4.1), is somewhat surprising given the observation that no strain differences in FLI were seen in the PVN of Lewis and S-D rats following footshock or interleukin-1ß injection (Rivest and Rivier 1994). A number of authors have reported that, compared to S-D animals, Lewis rats exhibit a blunted CORT response to a variety of stressors (Sternberg et al. 1989a,b; Aksentijevich et al. 1992; Glowa et al. 1992; Rivest and Rivier 1994). A significant, sustained rise in blood CORT level is precipitated by uADX or sham ADX, but no rise in blood CORT occurs following ADX. Thus, a strain difference in CORT negative feedback sensitivity could explain both the strain differences in FLI response seen following uADX or sham ADX, and the absence of a strain differences following ADX.

The hyporesponsiveness in the HPA axis of Lewis rats has been linked to a defect in hypothalamic CRH secreting neurons (Calogero et al. 1992). It has been

suggested that CRH gene regulation, possibly related to coordinate regulation of the CRH and enkephalin genes, might be responsible for this defect (Sternberg et al. 1989b). Given that differences in the function of the HPA axis of Lewis vs. S-D rats are only apparent under stressful circumstances (i.e., when circulating CORT levels are elevated), the defect in Lewis rat CRH neuron gene expression might be dependent upon elevated levels of CORT. Thus, following ADX, which precludes further increases in blood CORT, this defect might not be revealed. Alternatively, ADX might simply represent a stimulus which overwhelms the stress-hyporesponsiveness of the Lewis rat HPA system. Further research is necessary to determine whether either of these explanations is correct. Also, to verify the validity of the reported results, this experiment should be repeated with a larger number of Lewis rats and an equal number of S-D animals.

Contribution of Sex Steroids

A further regulatory component of PVN response may be sex steroid dependent (this is discussed in detail in the introduction of Chapter 2 of this thesis). The observation that laparotomy induces a greater increase in FLI in the PVN of male rats compared to like-treated females suggests that, in the absence of normal circulating CORT levels, ovarian hormones might exert an attenuating effect on CRH neurons in the PVN. The similarity between the FLI-response seen in the PVN of ADX males killed 4 hours following laparotomy, and that seen in ADX females killed 4 hours after OVX provides further support for this explanation. To confirm whether this is indeed the case, it will be necessary to compare the effect of laparotomy on ADX males and ADX/OVX females.

Summary

Basal levels of FLI are extremely low in the PVN of adult S-D rats. Following adrenal surgery, FLI appears in the PVN within 30 minutes, accumulates progressively until between 2 and 8 hours following surgery, and returns to basal levels within 24 hours following surgery (this time course might be accelerated in younger animals). The magnitude of FLI response observed in the PVN following adrenal surgery, appears to be influenced by the nature of the surgery, and the age and strain of the animal. In many cases, the magnitude of FLI induced by adrenal surgery adhered to a uniform general pattern (i.e., ADX ≥ sham ADX > uADX) although this was not evident in neonates. Overall levels of adrenal surgery-induced FLI in the PVN appeared higher in 3-11 day old rats relative to older animals. In adult male Lewis rats, the response to uADX and sham ADX was depressed compared to S-D animals, but the response to ADX was not. FLI response to adrenal surgery was also depressed in CORT-primed animals. Differences in plasma CORT feedback intensity appears to play a central role in mediation of the FLI response in the PVN.

As anticipated, *c-fos* immunocytochemistry provides an approach to examining activation of the HPA axis exclusive of stressful events which occur within 30 minutes of death (also see Hoffman et al. 1992). While this eliminates the influence of one source of stress (i.e., handling, etc., associated with sacrifice), it does not isolate the specific effects of ADX from surgical stress which impinges upon PVN neurons via nociceptive and sympathetic pathways. The specific effects of ADX on neurons of the PVN are difficult to unravel as they are masked by the effects of activation of these pathways. Given the anatomical and functional complexity of the stress system, it is difficult to eliminate the influence of general stressors upon specific stress pathways, although the Lewis rat appears to be a useful model. An alternate approach, which will be discussed in

detail in chapter 2, utilizes a different stress-hyporesponsive rat model (lactating rats) in an attempt to minimize non-specific stress-pathway activation (background noise in the system).

An important issue repeatedly raised by this series of experiments is the necessity to quantify FLI. In several experiments, within treatment variability made selection of a single representative brain section difficult or impossible (for example, see Figure 1.6.2). Also, many of these studies (for example, experiment 1.3) yielded complex panels of images (see Figure 1.3.1) which further complicated the already subjective assessment of effects. In dealing within the following chapters, results presented in the form of digital images of tissue sections are supplemented by quantitative image analysis and statistical evaluation of quantified data.

CHAPTER 2: THE EFFECT OF LACTATION ON THE INDUCTION OF FLI IN THE PVN OF THE RAT.

Introduction

Chapter 1 examined the effect of ADX, uADX, and sham ADX on the induction of FLI in the PVN of adult S-D rats, and 2 stress-hyporesponsive rat models (i.e., neonates and Lewis rats). In this chapter, a third hyporesponsive rat model (lactating females) is investigated.

In this chapter also, the development of a technique for quantifying immunocytochemical data using computer assisted densitometry is described. While this technique does not completely eliminate the subjective component of quantification, it significantly reduces the opportunity for subjective error. It also affords the opportunity to include summary statistics to scrutinize quantitative results. In concert with representative digital images, these convey more information concerning within- and between-group variation than can be transmitted in a collage of photographs or images alone. It is also anticipated that this technique will enable the detection of subtle differences in FLI in the PVN which are difficult to detect by sequential examination of microscope slides or photographs.

Lactation-induced alterations in HPA function are summarized in the general Introduction to this thesis. Evidence suggests that many of these changes are suckling dependent. Briefly, compared to non-lactating females, lactating animals exhibit elevated basal levels of circulating CORT and ACTH, depressed levels of CBG (see Tucker 1994), and an attenuated CORT and ACTH response to a variety of stressors (Thoman et al. 1970; Stern et al. 1973; Stern and Voogt 1973/74; Schlein et al. 1974; Myers et al. 1975; Walker et al. 1992; Lightman and Young 1989; Walker et al. 1992). Basal levels of several other hormones including

epinephrine, NE, GH, prolactin, oxytocin, and TSH are also elevated in lactating rats. Basal levels of FLI in the PVN and other brain areas are extremely low in lactating rats (Smith and Lee 1990; Fenelon et al. 1993, 1994), and during lactation, the FLI response to stress is attenuated in several brain regions (Smith and Lee 1990; Abbud et al. 1992, 1993, 1994; Fenelon et al. 1994).

The results discussed in chapter 1 established that the magnitude of induced FLI in the PVN of infant rats subjected to adrenal surgery follows a U-shaped distribution over the first 3 postnatal weeks. Experiment 2.1 (this chapter) is designed to test the hypothesis that the magnitude of adrenal surgery-induced FLI in the PVN of lactating mothers follows a similar U-shaped path, and to establish whether the pattern of FLI response to ADX and sham ADX identified in chapter 1 (i.e., ADX ≥ sham ADX) is preserved throughout lactation.

Suckling and other maternal behaviours are essential to sustaining lactation, and are reported to contribute significantly to maintaining stress-hyporesponsiveness in lactating rats. Within 24 hours of pup removal, circulating CORT (Stern et al 1973; Walker et al 1992), and prolactin (Stern and Voogt 1973/74) return to non-lactating levels; and the magnitude of NMA-induced FLI in the cortex and HC, which is attenuated in lactating rats suckling pups, reaches that seen in non-lactating females (Abbud et al. 1992). Experiment 2.2 is designed to test the hypothesis that the number of ADX-induced FLI-positive nuclear profiles counted in the PVN of mother rats is increased by litter removal during, but not after, the LSHRP.

In chapter 1, was suggested that the time course of adrenal surgery-induced FLI in the PVN of suckling pups might differ from that seen in adult rats. Experiment 2.3, tests the hypothesis that a change in the time course of adrenal surgery-induced FLI in the PVN might also be seen in lactating animals.

Abbud et al. (1992, 1993, 1994) and others have reported attenuated FLI response in selected brain areas of lactating rats following treatment with the glutamate agonist, NMA. Lactating animals are also reported to be refractory to the behavioural effects of NMA (Smith et al. 1990). In experiment 2.4 NMA is used as a probe to test the hypothesis that the blunted FLI response to ADX seen in the PVN of lactating rats, is mediated through glutamatergic pathways (i.e., that the FLI response to NMA is attenuated in lactating females).

The convulsant Metrazole is reported to induce FLI in the PVN (Shehab et al. 1992). Experiment 2.5, examines the hypothesis that the magnitude of Metrazole-induced FLI is attenuated in the PVN of lactating female rats compared to non-lactating animals.

Quantitative Image Analysis

From each animal, 8 sections, which included all rostro-caudal levels of the PVN, were processed and the four sections displaying the highest levels of FLI were selected for quantitative image analysis. Because no unilateral effects were detected, analysis was performed on one hemisphere of the PVN (final magnification = 63x). Composite colour images of brain sections were transmitted to a Macintosh IIci as described earlier in this thesis in the section on digital imaging for Figure preparation. Using Adobe Photoshop with NuVista "capture" image acquisition software, images were captured using high levels of light intensity, converted to greyscale, and resized to fill the screen. Grey levels were adjusted using the Adobe Photoshop "Levels auto-adjust" command, the black level was then set to the lower end of the intensity histogram for that image, and the image was stored, as a TIFF file (resolution = 72 pixels per inch), to a 88 Mb SyQuest removable hard disk cartridge.

The 88 Mb SyQuest cartridge containing the digital images of brain sections was transferred to a second SyQuest drive connected to the Power Macintosh 6100/60AV computer described above, and the stored TIFF file was accessed using NIH Image software (version 1.55b57, National Institutes of Health, USA). For each image, three randomly chosen rectangular areas containing no FLI-labeled profiles were selected (outlined) and the NIH "measure" command was used to calculate the mean grey value of the aggregate area. This value, which varied somewhat from section to section, was used as a baseline to correct for between-image differences in intensity of non-specific (background) staining. The area containing the PVN was then selected (outlined), and the image was converted to a density slice which included all grey values from saturated black to a value 150 greyscale units above the calculated background grey value for that image. The resulting image was converted to black and white by applying a

look-up-table (LUT) included in the NIH-Image program. The number of FLI positive nuclear profiles within the PVN were then counted by converting to threshold mode and using the NIH-Image "analyze particles" function. For each animal, the total number of FLI-positive profiles summed over four images was used in all subsequent analyses.

Experiment 2.1. Effect of time since parturition on ADX-induced FLI in the PVN of lactating and post-lactating female S-D rats.

Lactating adult female S-D rats which had given birth 3, 11, or 21 days earlier, and post-lactating females which had delivered 28 days earlier were used in this experiment. Lactating animals were housed individually with their litters, and post-lactating animals, which had been separated from their pups on pd 21, were housed 2 per cage. Animals were removed from their home cages, anaesthetized, bilaterally adrenalectomized or sham adrenalectomized, and returned to their home cages. Four hours following the completion of surgery, animals were anaesthetized and perfused (n=3 animals/group except where otherwise noted), and brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

2.1 Results

Digital images of brain sections from animals used in this experiment are presented in Figure 2.1.1. Dramatic increases in FLI were seen in the PVN of animals killed 4 hours after ADX on pd 3, and 28. Much smaller increases in FLI were seen in the PVN of lactating animals killed 4 hours after ADX on pd 11 or 21. Slight to moderate increases in FLI were seen in the PVN of lactating rats killed 4 hours after sham ADX on pd 3. No evidence of elevated FLI was seen in the PVN of animals killed 4 hours after sham ADX on pd 11 or 21, but levels were again elevated on pd 28. Quantified data from these animals are summarized in Table 2.1.1 and Figure 2.1.2. A two-way ANOVA performed on these data revealed a significant main effect of time since parturition on the number of FLI-positive nuclear profiles counted in the PVN of lactating and post-lactating females killed 4 hours after ADX or sham ADX ($F_{3, 22}$=5.146, p=.0076).

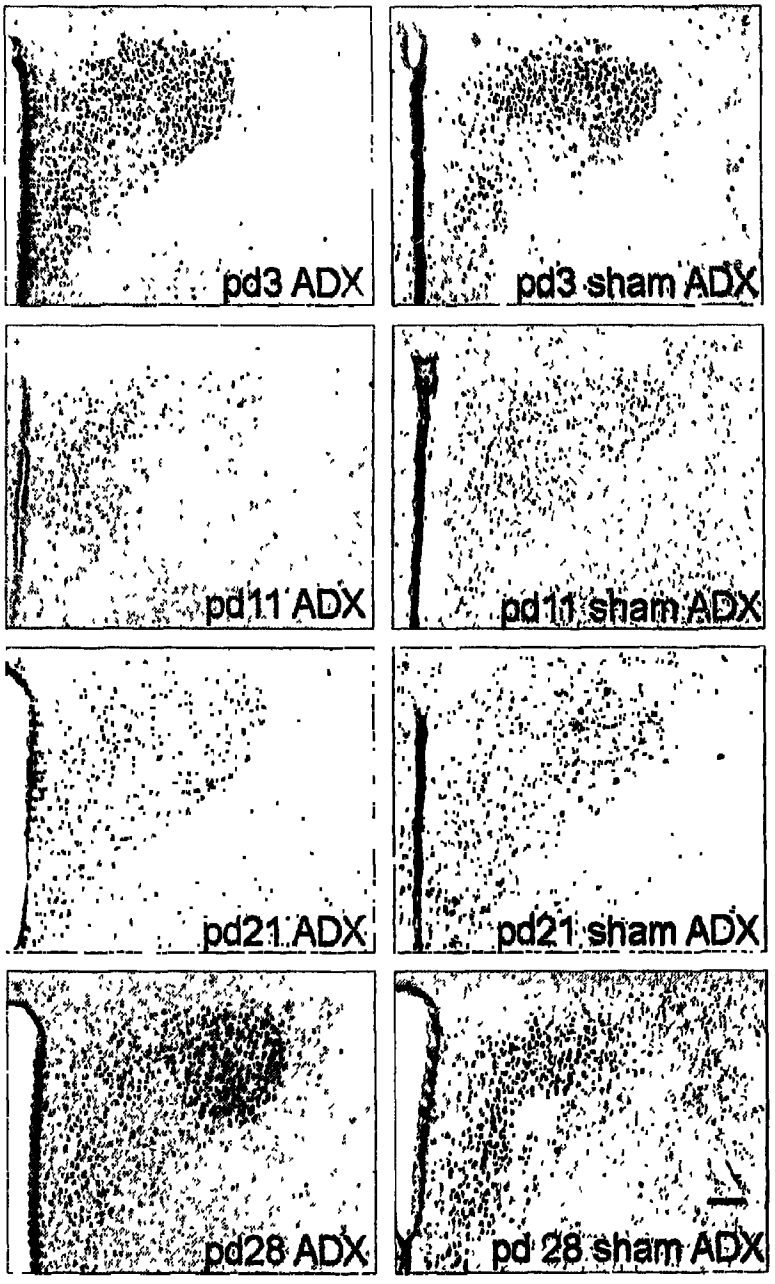


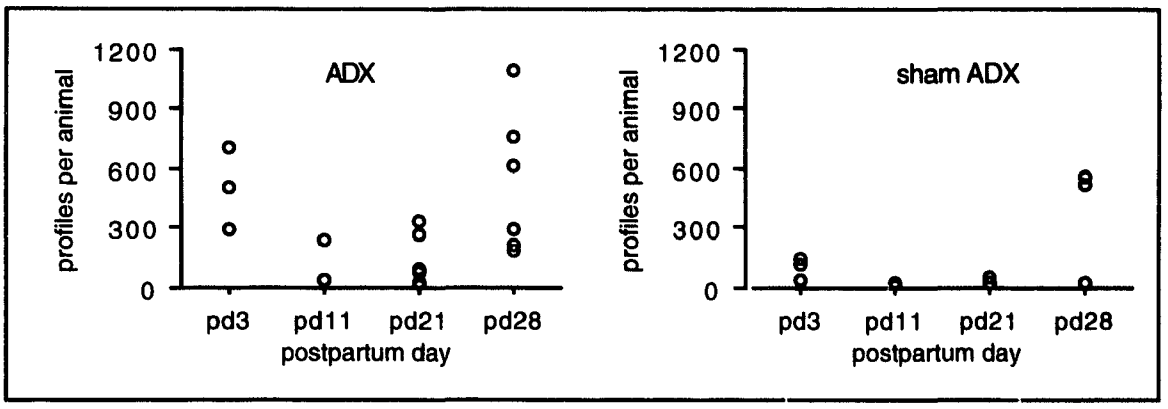
Figure 2.1.1 FLI in the PVN of lactating and post-lactating female Sprague-Dawley rats killed 4 hours after ADX or sham ADX (pd = postpartum day). Scale bar = 100 μm.

A significant main effect of surgery (i.e., ADX vs.. sham ADX) was also detected in these animals ($F_{1,22}$=4.967, p=.0364). The time since parturition/surgery interaction effect was not significant ($F_{3,22}$=0.707, p=.5583). To establish whether the FLI response to adrenal surgery was significantly diminished during the LSHRP, a one-tailed t-test was performed on combined data from postpartum days 11 and 21 (LSHRP) vs. combined data from postpartum days 3 and 28 (non-LSHRP). This analysis revealed a significant effect of LSHRP on the number of FLI-positive nuclear profiles counted in the PVN (p=.0067).

Table 2.1.1 *Number of FLI-positive profiles in the PVN of lactating females killed 4 hours after ADX or sham ADX on pd 3, 11, 21, or 28.*

	ADX			sham-ADX		
postpartum day	n	profiles*	SEM	n	profiles*	SEM
3	3	502.7	118.7	3	101.0	31.2
11	3	105.3	69.3	3	24.0	5.9
21	6	136.8	53.9	3	34.7	9.7
28	6	526.3	148.9	3	367.3	173.0

*(*mean number of FLI-positive nuclear profiles per animal)*



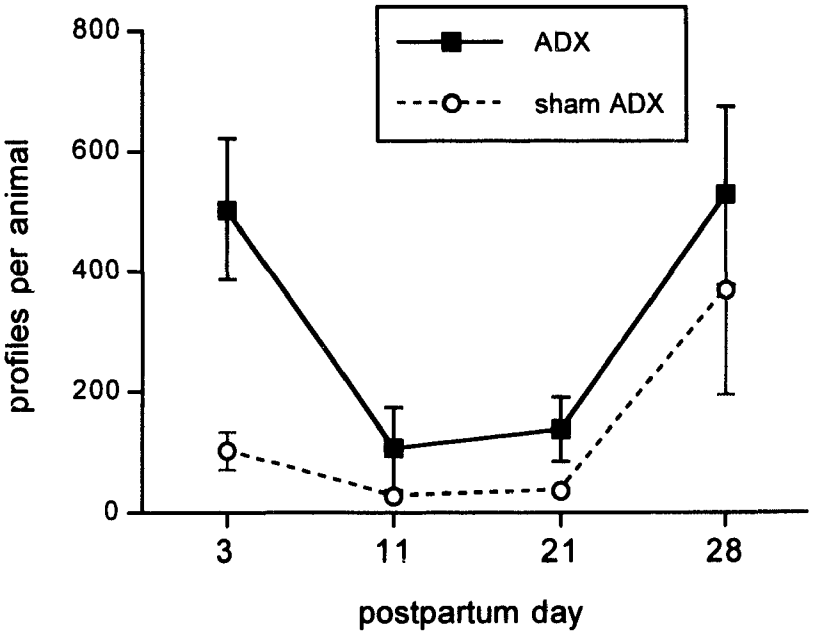


Figure 2.1.2 FLI in the PVN of lactating and post-lactating female S-D rats killed 4 hours after ADX or sham-ADX. Scatterplots show number of FLI-positive nuclear profiles per animal; line plot shows mean number of FLI-positive nuclear profiles per animal ±SEM.

Experiment 2.2. Effect of suckling on ADX-induced FLI in the PVN of pd21 female S-D rats.

Lactating female S-D rats were divided into four groups and the litters of one group were removed on pd 14. Seven days later (pd 21) animals in the pups-removed group ("no pups" group), and one other group of mothers ("pups" group) were anaesthetized and bilaterally adrenalectomized. The litters of one remaining group were removed on pd 21, and 7 days later (pd 28), these animals, along with those in the final group (who remained with their litters until pd 28) were bilaterally adrenalectomized.

All animals were killed and perfused 4 hours after surgery. Brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

2.2 Results

Digital images of brain sections from animals used in this experiment are presented in Figure 2.2.1. Levels of ADX-induced FLI in the PVN of pd 21 females nursing litters were attenuated compared to levels seen in pd 21 animals deprived of litters for 7 days prior to surgery. In contrast, levels of ADX-induced FLI in the PVN of pd 28 females housed with their pups did not differ noticeably from those seen in pd 28 females separated from their pups 7 days previously. Quantified data from this experiment are summarized in Table 2.2.1 and Figure 2.2.2. A two-way ANOVA performed on these data revealed a significant main effect of litter presence ($F_{1,14}$=5.698, p=.0316), but not of postpartum day ($F_{1,14}$=0.451, p=.4741) on the number of FLI-positive nuclear profiles counted in the PVN. The litter/pd interaction effect was not significant ($F_{1,14}$=1.787, p=.2026). Given the significant main effect of litter presence, one-tailed t-tests were performed on data split by postpartum day. These revealed a significant

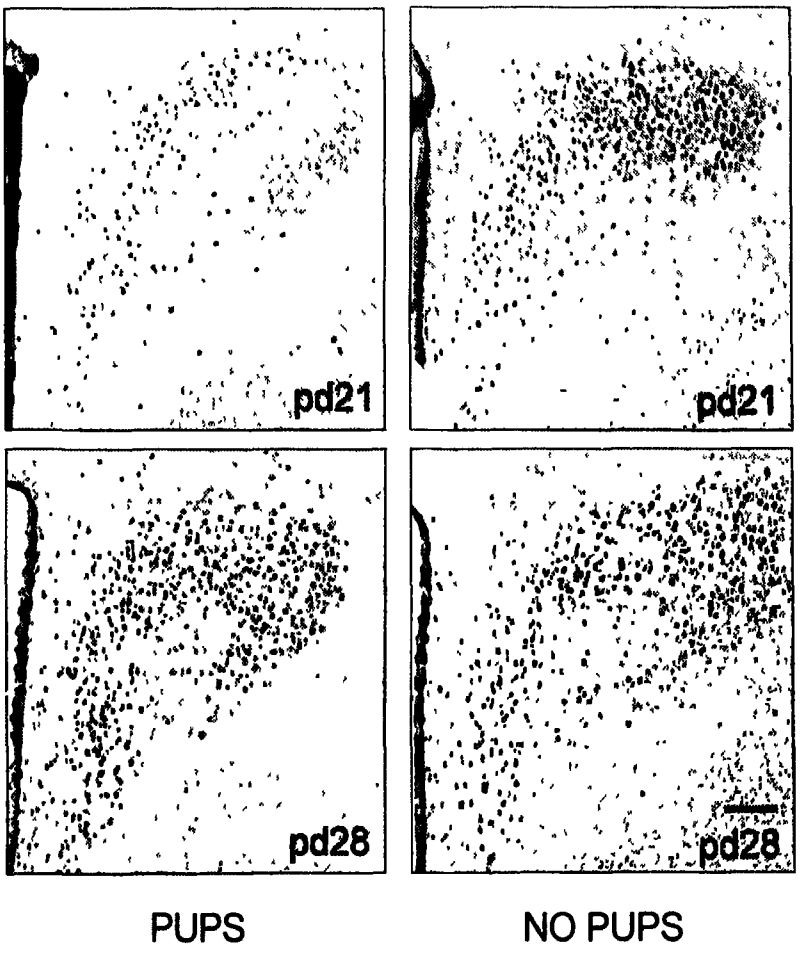


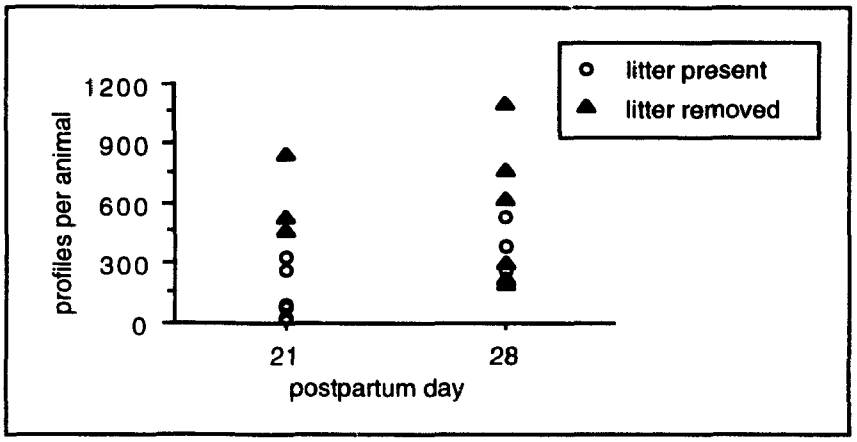
Figure 2.2.1 FLI in the PVN of female Sprague-Dawley rats killed 4 hours following ADX on postpartum day 21 or 28 (pups = housed with litters until time of surgery; no pups = litters removed 7 days prior to surgery). Scale bar = 100 μm,

effect of litter removal on animals killed on pd 21 (p=.0021) but not on animals killed on postpartum day 28 (p=.2880).

Table 2.2.1 *Number of FLI-positive profiles in the PVN of pd 21 and pd 28 female rats with and without litters, killed 4 hours after ADX or sham ADX.*

	litter present			litter removed		
postpartum day	n	profiles*	SEM	n	profiles*	SEM
21	6	136.8	53.9	3	601.3	119.3
28	3	395.3	75.5	6	526.3	148.9

*(*mean number of FLI-positive nuclear profiles per animal)*



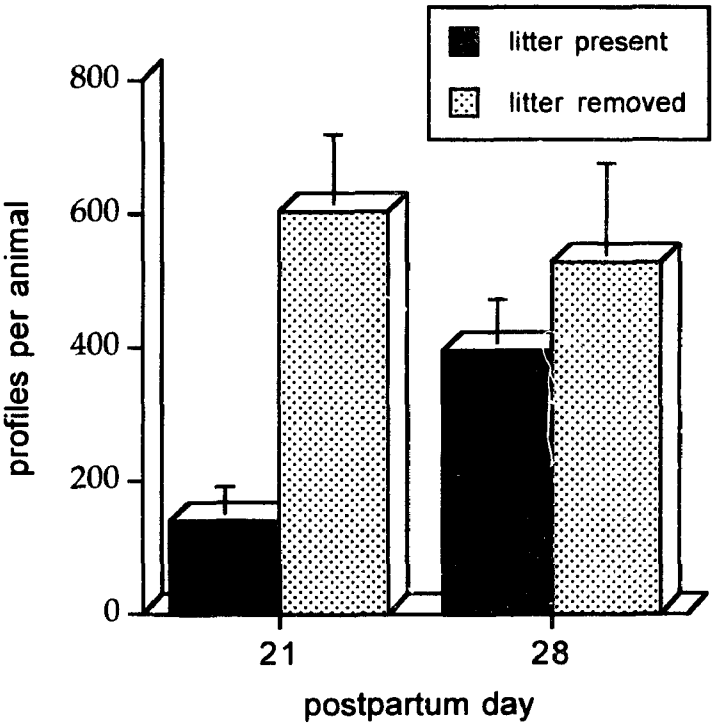


Figure 2.2.2 FLI in the PVN of female S-D rats killed 4 hours following ADX on pd21 or 28, with or without litter removed 7 days earlier. Scatterplot shows number of FLI-positive nuclear profiles per animal; bar plot shows mean number of FLI-positive nuclear profiles per animal ±SEM.

Experiment 2.3 Time course of ADX-induced FLI in the PVN of pd 11 lactating female S-D rats.

Lactating female S-D rats (postpartum day 11) were anaesthetized, bilaterally- or sham-adrenalectomized as described, and returned to their home cages. Four hours, 8 hours, 24 hours, or 3 days following surgery, animals were killed and perfused, and brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

2.3 Results

Digital images of brain sections from animals used in this experiment are presented in Figure 2.3.1. Elevated levels of FLI were seen in the PVN of animals killed 4 or 8 hours following ADX, and somewhat smaller increases were seen in rats killed 4 or 8 hours following sham-ADX. Marginal increases were seen in rats killed 24 hours after ADX or sham ADX, and no increases were seen in animals killed 3 days after ADX or sham ADX. Quantified data from these animals are summarized in Table 2.3.1 and Figure 2.3.2. The results of a two-way ANOVA performed on these data showed no statistically significant effects of treatment or survival time on FLI in the PVN (surgery main effect, $F_{1,11}$=2.717, p=.1276; survival time main effect, $F_{2,11}$=0.855, p=.4515; surgery/survival time interaction, $F_{2,11}$=0.878, p=.4430).

Table 2.3.1 *Number of FLI-positive profiles in the PVN of lactating females killed 4, 8, or 24 hours after ADX or sham ADX 11.*

	ADX			sham-ADX		
survival time (hours)	n	profiles*	SEM	n	profiles*	SEM
4	3	105.3	69.3	3	24.0	5.9
8	3	102.3	49.4	2	23.5	18.5
24	3	18.7	2.8	3	24.3	5.9

*(*mean number of FLI-positive nuclear profiles per animal)*

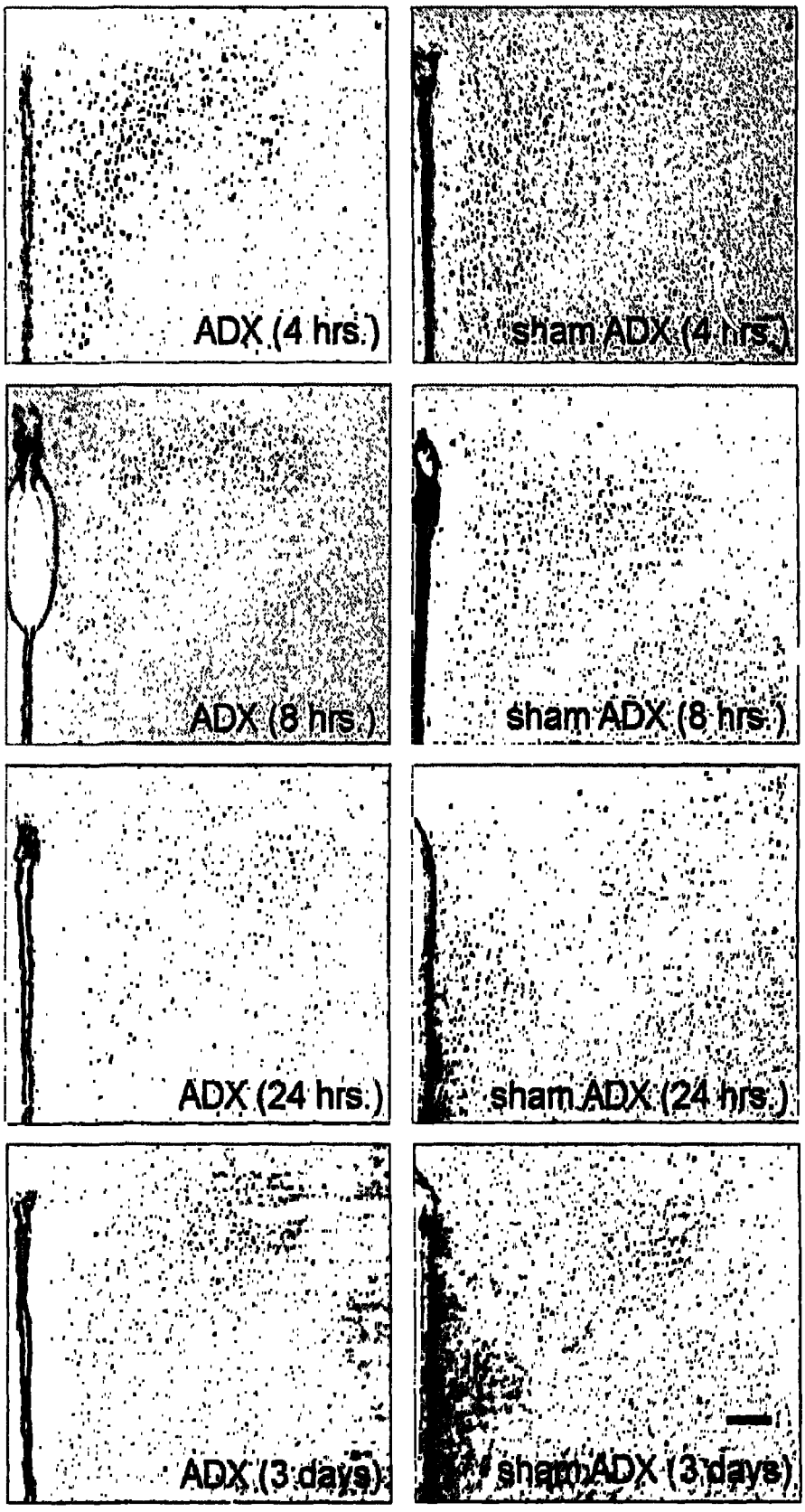
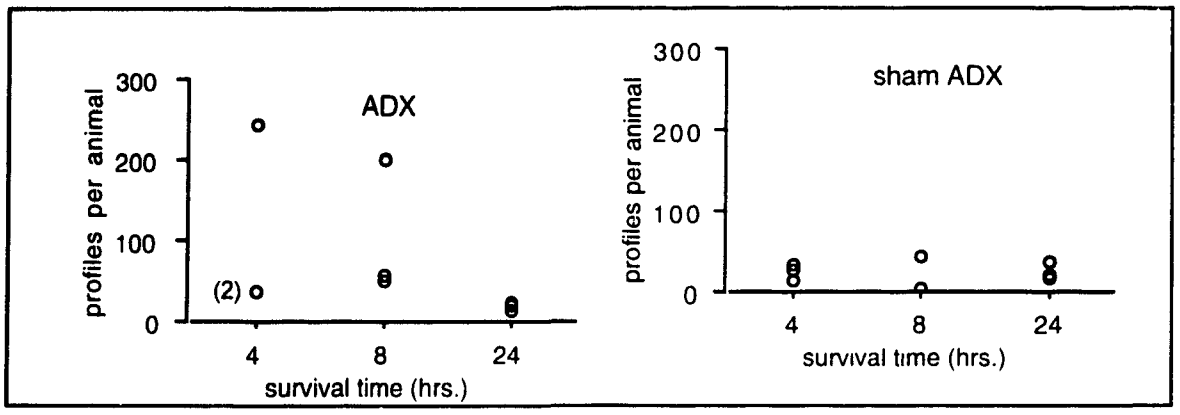


Figure 2.3.1 FLI in the PVN of pd 11 lactating female S-D rats killed 4 hours, 8 hours, 24 hours, or 3 days after ADX or sham ADX. Scale bar = 100 μm.



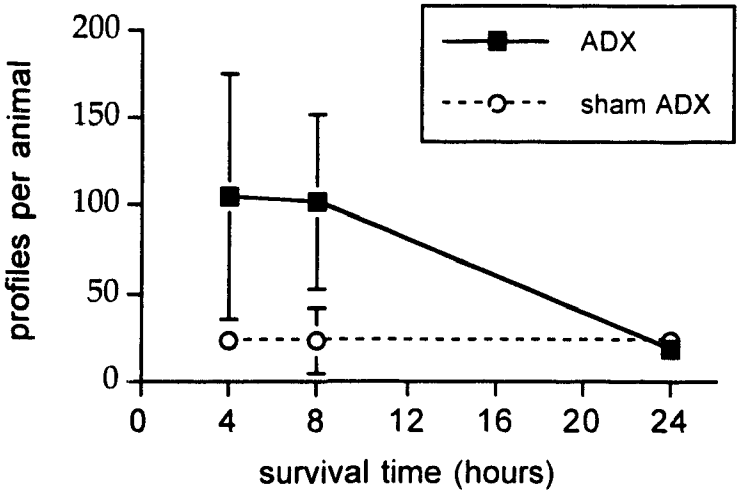


Figure 2.3.2 FLI in the PVN of lactating female Sprague-Dawley rats killed 4, 8, or 24 hours following ADX or sham-ADX on pd11. Scatterplots show number of FLI-positive nuclear profiles per animal; line plot shows mean number of FLI-positive nuclear profiles per animal ±SEM.

Experiment 2.4. NMA-induced FLI in the PVN of pd7 lactating female S-D rats.

On postpartum day 7, lactating female S-D rats and non-lactating adult females (200-325 g.; 3 animals per group) received a single s.c. injection of NMA (N-methyl-$_{D-L}$-aspartic acid, Sigma, St. Louis, MO; 50 mg./kg. b.w., in saline), or saline (0.1 ml.). Two hours later, animals were killed and perfused; brains were removed, postfixed, and sectioned and alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry. Selected coronal sections through the arcuate nucleus (a known target of NMA stimulation; MacDonald et al. 1990) were also included.

2.4 Results

Digital images of brain sections from animals used in this experiment are presented in Figure 2.4.1. FLI was elevated in the PVN of both lactating and non-lactating female rats killed 2 hours after NMA injection, but the level of FLI induced in the PVN of lactating animals was lower than that observed in like-treated non-lactating females. Quantified data from the PVN of these animals are presented in Table 2.4.1 and Figure 2.4.2. A two-way ANOVA performed on these data revealed a significant main effect of treatment (ie., NMA v.s. saline; $F_{1,7}$=24.650, p=.0016), and of lactation status ($F_{1,7}$=7.001, p=.0331) on the number of FLI-positive nuclear profiles counted in the PVN. The treatment/lactation status interaction effect was not significant ($F_{1,7}$=5.066, p=.0591). Subsequent one tailed t-tests performed on these data split by treatment confirmed that the FLI response to NMA is significantly suppressed in lactating females compared to non-lactating females (p=.0248). One tailed t-tests performed on data split by lactation status established that compared to saline, NMA induced significantly higher levels of FLI in the PVN of non-lactating female rats (p=.0044).

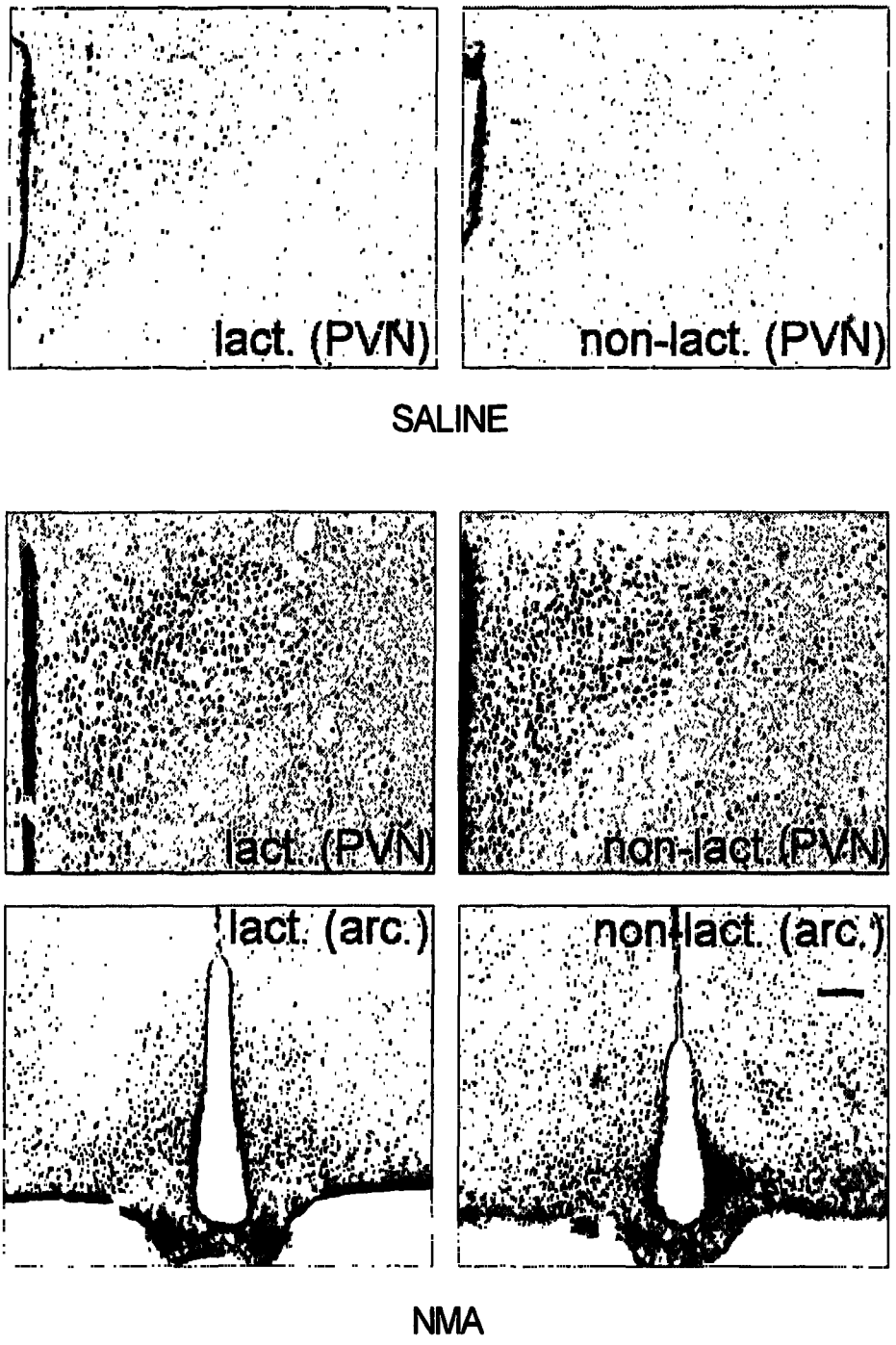


Figure 2.4.1 FLI in the PVN and arcuate nucleus (arc.) of lactating (lact.) and non-lactating (non-lact.) female Sprague-Dawley rats killed 2 hours following injection of saline (0.1 ml.) or NMA (50 mg./kg.). Scale bar = 100 μm.

Differences detected in NMA- vs. saline-treated lactating rats were not significant (p=.0521).

Table 2.4.1 *Number of FLI-positive profiles in the PVN of lactating (pd 7) and non-lactating female S-D rats killed 2 hours following injection of NMA (50 mg./kg.).*

	lactating females			non-lactating females		
treatment	n	profiles*	SEM	n	profiles*	SEM
NMA	3	212.0	69.1	3	583.7	114.3
saline	4	27.5	10.5	2	6.0	3.0

*(*mean number of FLI-positive nuclear profiles per animal)*

Selected sections through the arcuate nucleus were processed for *c-fos* immunocytochemistry to confirm the effectiveness of NMA injection. Elevated levels of FLI were evident in arcuate sections from lactating and non-lactating animals (see Figure 2.4.1). Visual inspection of these sections indicates there may be a lactation effect in NMA-induced FLI levels in the arcuate nucleus. The number of available sections were, however, insufficient to allow quantification. A more thorough investigation is warranted.

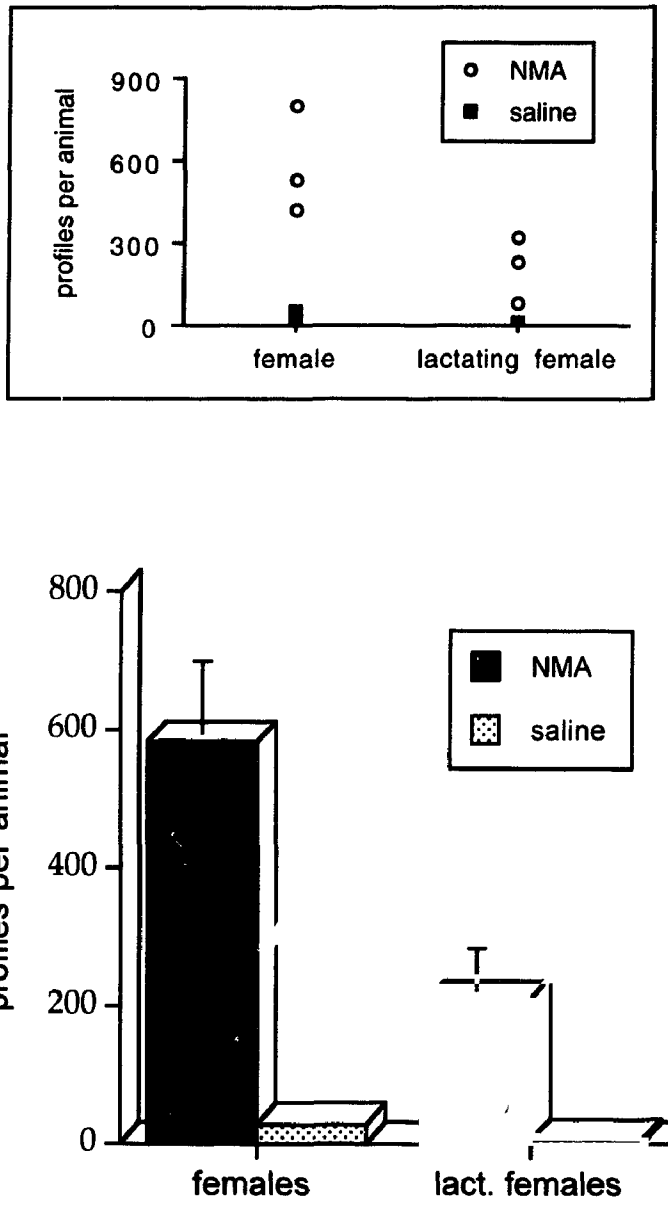


Figure 2.4.2 FLI in the PVN of non-lactating (female) and lactating (lact.) female Sprague-Dawley rats killed 2 hours following s.c. injection of NMA (50 mg./kg. b.w., in saline). Scatterplot shows number of FLI-positive nuclear profiles per animal; line plot shows mean number of FLI-positive nuclear profiles per animal ±SEM.

Experiment 2.5. Seizure-induced FLI in the PVN of pd 12 lactating female S-D rats.

Lactating (pd 12) and non-lactating female S-D rats received a single i.p. injection of pentylenetetrazole (Metrazole, RBI, Natick, MA; 5 mg./kg. b.w., in saline) and were killed and perfused 2 hours later (saline-injected animals from experiment 2.4 served as controls, as this experiment and experiment 2.4 were conducted concurrently). Brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

2.5 Results

One Metrazole-injected non-lactating animal was dropped from this study as it failed to exhibit behavioural effects consistent with the dose of drug administered (no initial motor seizure). The remaining two Metrazole-injected non-lactating females salivated profusely and experienced level 4 motor seizures which began within 2 minutes of Metrazole injection and recurred throughout the following 2 hours. Behavioural responses in Metrazole-injected lactating animals were far less profound consisting of an initial motor seizure, which began 2 to 4 minutes after Metrazole injection, followed by an extended period of ataxia. No behavioural perturbations were seen in saline-injected animals.

Digital images of brain sections from animals used in this experiment are presented in Figure 2.5.1. Levels of FLI in the PVN, piriform cortex, and HC of Metrazole-injected animals were noticably elevated compared to saline-injected rats (see Figure 2.4.1). The magnitude of FLI in the PVN of Metrazole-injected lactating females was markedly attenuated compared to like-treated non-lactating females. Levels of FLI in the piriform cortex also appeared to be somewhat lower in Metrazole-treated lactating females, but levels of FLI in the

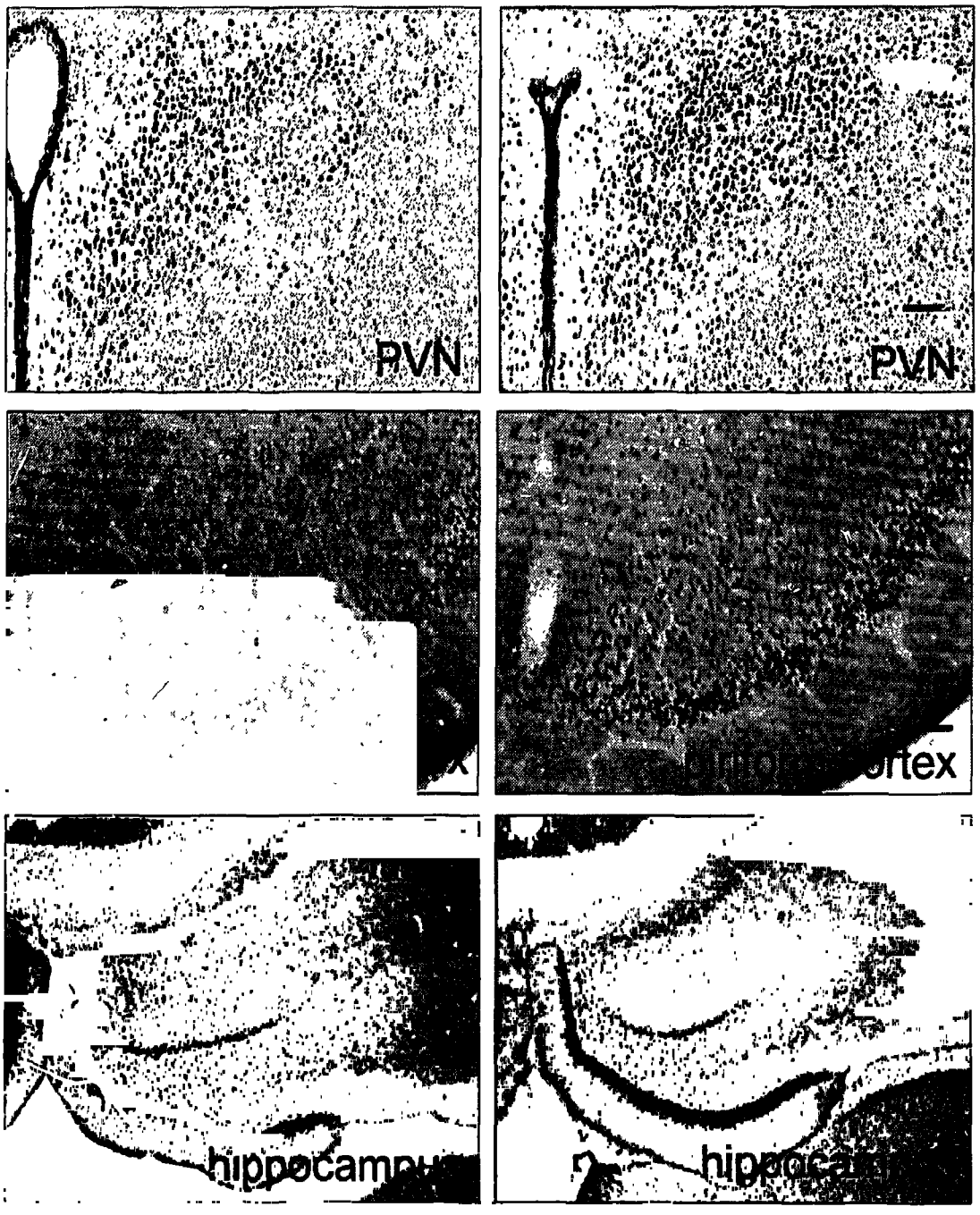


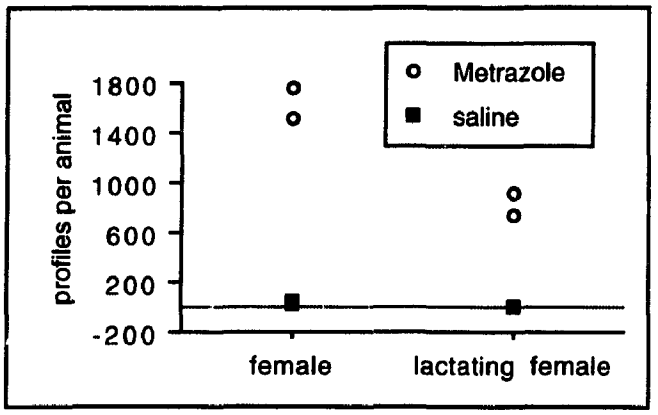
Figure 2.5.1 FLI in the PVN, piriform cortex, and hippocampus of lactating and non-lactating female Sprague-Dawley rats killed 2 hours following injection of Metrazole. Scale bars = 100 μm.

HC of Metrazole-treated lactating animals were comparable to those seen in non-lactating females. Quantified data from the PVN of these animals are presented in Table 2.5.1 and Figure 2.5.2. A two-way ANOVA performed on these data revealed a significant main effect of treatment (ie., Metrazole v.s. saline; $F_{1,5}$=325.857, p=<.0001), and lactation status ($F_{1,5}$=38.862, p=.0016) on the number of FLI-positive nuclear profiles counted in the PVN. A significant interaction effect was also noted ($F_{1,5}$=33.471, p=.0022). One tailed t-tests performed on these data split by treatment revealed significantly lower FLI-positive nuclear profile counts in the PVN of Metrazole-injected lactating female rats compared to like-treated non-lactating females (p=.0177). One tailed t-tests performed on these data split by lactation status revealed a significant treatment effect in the PVN of both non-lactating (p=.0003) and lactating (p=.0056) females. Data from areas outside the PVN were not quantified.

Table 2.5.1 *Number of FLI-positive profiles in the PVN of lactating female rats killed 2 hours after injection of Metrazole (5 mg./kg.) or saline.*

	lactating females			non-lactating females		
treatment	n	profiles*	SEM	n	profiles*	SEM
Metrazole	2	826.5	87.5	2	1630.5	128.5
saline**	2	6.0	3.0	3	36.0	8.7

*(*mean number of FLI-positive nuclear profiles per animal; **data from experiment 2.4)*



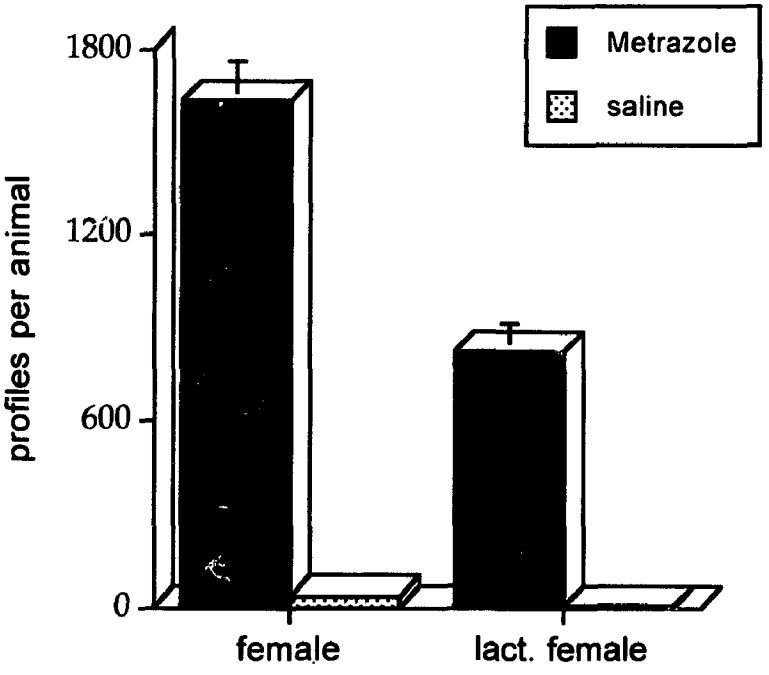


Figure 2.5.2 FLI in the PVN of non-lactating (female) and lactating (lact.) female Sprague-Dawley rats killed 2 hours following i.p. Metrazole (5 mg./kg. in saline), or saline (0.1 ml.). Scatterplot shows number of FLI-positive nuclear profiles per animal; line plot shows mean number of FLI-positive nuclear profiles per animal ±SEM.

Discussion

The results of experiment 2.1 indicate that following parturition, the magnitude of ADX- and sham ADX-induced increases in FLI in the PVN of lactating female rats follows a U-shaped distribution similar to that detected in young suckling rats (Figures 2.1.1 and 2.1.2). The onset of the period of blunted FLI induction in the PVN of lactating rats coincides with that detected for neonates, but the duration seems longer in lactating animals (pd4-15 for neonates vs. pd 4-21 for lactating females). The recovery of FLI response seen in the PVN of post-lactating females killed 4 hours following ADX or sham ADX on pd 28 (7 days after the removal of litters) suggests that either: 1) the magnitude of FLI response to adrenal surgery observed in postpartum lactating females is internally regulated, and is dependent exclusively upon time since parturition, or 2) external factors such as interaction with pups, mediate this phenomenon. However, the results of experiment 2.2 indicate that, removal of litters 7 days prior to ADX on pd 21 significantly elevates the FLI response of postpartum female rats, whereas removal of litters 7 days prior to ADX on pd 28 has little or no effect on induction of FLI in the PVN (see Figures 2.2.1 and 2.2.2). The observation that the FLI response in the PVN of pd 21 females without litters resembles that seen in pd 28 females with or without litters suggests that the efficacy of mother/pup interaction in suppressing ADX-induced FLI in the PVN diminishes following the end of the third postpartum week i.e., the FLI response to ADX recovers even in the presence of pups. This raises the question of whether the age of the litter, and/or the postpartum age of the mother are critical to maintaining an attenuated FLI response to ADX in postpartum females.

It is possible to extend the period of lactation to as long as 70 days by repeatedly replacing litters with younger animals which, in contrast to older pups which suckle progressively less frequently, continue to suckle vigorously

(Nicoll and Meites 1959; Tucker et al. 1963; Flint et al. 1984). It will be interesting to determine whether extending the period of lactation (by substituting younger litters) also extends the period during which the FLI response to ADX is attenuated (and thus, the period during which pup removal alters maternal FLI response to ADX). While the results of experiment 2.2 indicate that postpartum day 28 females housed with 28 day old pups do not display an attenuated FLI response to ADX, the extent to which 28 day old rats continue to suckle, or attempt to suckle was not monitored. It will be interesting to determine whether, for example, postpartum day 28 lactating females suckling surrogate litters of 7 day old pups display an attenuated FLI response to ADX.

Circulating CORT levels, which are low through most of pregnancy, increase dramatically over the last few days before parturition. The effect of this increase on the availability of biologically active CORT is potentiated by a decrease in plasma CBG which occurs in the immediate prepartum period (see Tucker 1994). Thus, the attenuated FLI response to ADX and sham ADX on pd 11 and pd 21 might reflect the effect of increased level of CORT feedback inhibition on CRH neurons in the PVN. The failure of this mechanism to suppress FLI response to ADX on pd 3 (in spite of elevated CORT and depressed CBG levels) indicates that an undetermined mechanism overwhelms CORT feedback inhibition during the first few prenatal days. The suckling stimulus is necessary to sustain the elevated levels of CORT which characterize the lactation period (Stern et al. 1973; Walker et al. 1992). It is possible that the suckling stimulus also initiates a sequence of events which culminate in the delayed onset of the SHRP associated with lactation. Alternatively, the onset of the SHRP during lactation (LSHRP) might be dependent upon the age of the suckling pups. Pups younger than 3 days old may be unable to exert the stimulus necessary to initiate the LSHRP. This hypothesis could be tested by examining the effect of ADX on FLI in the

PVN of lactating mothers who beginning on pd 2, had their litters replaced every 2 days with litters of newborn pups. If this procedure successfully blocks the onset of the LSHRP, one could conclude that the stimulus controlling the onset of the LSHRP is an external one originating in the pups. Further experiments will then be necessary to determine the nature of the stimulus. These experiments might incorporate the olfactory desensitization and ventral analgesia protocols used by Fleming and Walsh (1994) to determine whether olfactory or tactile cues play a significant role in initiating the LSHRP.

The results of experiment 2.3 suggest that, although the magnitude of ADX- and sham ADX-induced FLI in the PVN of lactating females is attenuated compared to like-treated non-lactating animals, the time course, as far as was examined, is similar (Figures 2.3.1 and 2.3.2). Maximal accumulations of FLI were detected at 4 hours and 8 hours, and by 24 hours, basal levels were restored. As was the case in experiment 1.3, insufficient evidence is available to determine whether the lower levels of ADX-induced FLI observed in the PVN of lactating rats reflect a blunted FLI response or an altered time course for FLI-induction. Fos is a short-lived protein which accumulates progressively over time, peaks, and then degrades. The relatively equal accumulations of FLI detected at 4 hours and 8 hours following ADX suggest that the peak lies somewhere between these two time points. This interpretation however, is based upon inference, and in order to establish whether it is accurate, it will be necessary to extend experiment 2.3 to include additional, perhaps shorter, survival times. A survey of relevant literature reveals close agreement on the time course for induction of Fos protein. The results of experiment 1.3, however, suggest the possibility that in some instances, this time course might vary. Levels of FLI in the PVN of 11 day old rats killed 2 hours after ADX were noticeably higher than those observed in age-matched animals killed 4 hours

after ADX (see Figures 1.3a and 1.3d). Prior to pursuing further experiments to determine whether induction of FLI in lactating animals follows a modified time course, it would seem prudent to ascertain whether this is the case in neonates. In retrospect, given the results of experiments 1.1 and 1.3, it would be useful to have data from both neonates and lactating animals killed after shorter survival times (i.e., 10 minutes to 2 hours) following ADX. Further evidence in support of the contention that the time course for Fos protein accumulation might vary in some instances is offered in a recent abstract by Bhatnagar and Dallman (1995). They report that following restraint stress, FLI in the PVN peaked at 30 minutes. It should be noted, however, that this observation is based upon preliminary data.

A number of authors have reported NMA-, or NMDA-induced FLI in various brain regions, but only one reference was located which specifically discusses NMDA-induced FLI in the PVN, and that study employed a mouse model (Saitoh et al. 1991). In this thesis, the elevated levels of NMA-induced FLI seen in the hypothalamic arcuate nucleus of both lactating and non-lactating adult female rats (Figure 2.4.1) is consistent with the effect seen in NMDA-treated immature female rats (MacDonald et al. 1990, 1993).

In adult rats, NMDA receptor agonists, including NMA, induce FLI in the HC and other brain areas (Kaczmarek et al 1988; Abbud et al. 1992, 1994). Given that the HC is a major source of inhibitory afferents to the PVN (Herman et al. 1989; Feldman and Weidenfeld 1995), the relatively lower level of NMA-induced FLI observed in the PVN of lactating females compared to non-lactating cohorts (see Figure 2.4.1) might be accounted for in terms of increased inhibition of PVN neurons by hippocampal neurons. Abbud et al (1992, 1994) also reported lower levels of NMA-induced FLI in the HC of lactating female rats compared to cycling females. Note however, that, in contrast to the single s.c. injection of

NMA (50 mg./kg. b.w.) given in experiment 2.4 (this thesis), animals in the studies of Abbud et al. received multiple intravenous infusions of NMA. It would be useful to repeat experiment 2.4 using varying doses of NMA, and to examine both the HC and the PVN to determine whether the lactating/non-lactating differences in hippocampal FLI reported by Abbud et al. (1992, 1994) prevail in animals given a single dose of NMA.

The results obtained from Metrazole injected animals are particularly interesting. Metrazole elicited far less behavioural disruption, and significantly smaller increases in FLI in the PVN of lactating female rats compared to non-lactating females, but no differences were seen in hippocampal FLI. This indicates that during lactation, the susceptibility of the PVN to Metrazole, a general CNS stimulant, is selectively attenuated. Further investigation will be required to determine whether this is a direct consequence of elevated CORT inhibition of CRH neurons in the PVN. One approach will be to examine the effect of surgical or pharmacological ADX on Metrazole-induced FLI in the PVN of lactating rats. Alternatively, it will be instructive to determine whether CORT-priming (see experiment 1.7) reduces the magnitude of Metrazole-induced FLI in the PVN of non-lactating female rats.

An additional mechanism might involve glutamate receptors, consistent with the NMA results described above. Zawia and Bondy (1990) report that electrically-induced convulsions induce FLI via an NMDA-mediated pathway whereas Cole et al. (1990) report the opposite i.e., blockade with MK801 does not prevent induction of *c-fos*. Prior application of MK801 (a non-competitive NMDA receptor antagonist) effectively blocks the onset of Metrazole-induced tonic extensor seizures in mice (Nevins and Arnolde 1989). This suggests that the convulsant effects of Metrazole are mediated, at least in part, by glutamatergic pathways. An examination of the effect of MK801 on Metrazole-induced FLI in

the PVN will reveal whether the mechanisms of Metrazole activation of these neurons also involves glutamatergic pathways.

In summary, a pronounced decrease in ADX-induced FLI is seen in lactating rats from about postpartum day 4 until the end of the lactating period. This decrease can be terminated prematurely by litter removal, but it is not known whether its natural termination is determined by the age of the offspring or the time elapsed since parturition. The time course for ADX-induced FLI in the PVN of lactating females appears to be similar to that observed in adult males, but further research is needed to confirm this. The effect of lactation on NMA-induced FLI in the PVN implicates glutamatergic neuronal circuits in suppression of FLI in the PVN of lactating animals, and the specific effect of lactation upon Metrazole-induced FLI in the PVN suggests that the refractoriness of the FLI response (to Metrazole) of neurons in the PVN of lactating rats does not extend to all brain regions influenced by Metrazole.

Experiments in this thesis identified a quantitative difference in FLI in the PVN of lactating rats, but the neurochemical identity of the neurons involved was not determined. Further experiments will be necessary to establish whether the observed differences reflect changes in the activity of CRH neurons in the PVN, or whether other populations of neurons are involved.

Further experiments with a variety of chemical probes will elucidate precise neurotransmitter involvement in lactational hyporesponsiveness of PVN neurons. For example, bombesin and CCK (Bonaz et al. 1993), α-adrenergic receptors (Tsujino et al. 1992), muscarinic receptors (Hughes and Dragunow 1993), and serotonin (Torres and Rivier 1994) have all been implicated in the regulation of *c-fos* expression in the PVN. Chapter 3 of this thesis describes experiments with one such probe, MK801, which is a specific antagonist for NMDA-type glutamate receptors.

CHAPTER 3: NMDA RECEPTOR BLOCKADE INDUCES FLI IN THE PVN OF THE RAT: EFFECT OF SEX STEROIDS.

Introduction

In earlier work (see Chapter 2, experiment 4) an attempt to block NMA-induced FLI with the non-competitive NMDA receptor antagonist dizocilpine maleate (MK801) revealed an unexpected stimulation of *c-fos* expression in the PVN. This led to a more detailed investigation of this phenomenon. In this chapter, the effect of injection of MK801 on FLI in the PVN is examined. The possible role of gonadal steroids as mediators of the FLI-response to MK801 is also investigated. As in chapter 2, computer assisted quantitative image analysis is utilized to evaluate immunocytochemical results.

MK801 acts at a site within the NMDA receptor-coupled calcium channel, by blocking agonist-induced cellular calcium uptake (Kemp et al. 1987). It is reported to possess potent anticonvulsant, sympathomimetic, and anxiolytic properties (Nevins and Arnolde 1989; Blanchard et al. 1992; Young and Dragunow 1994). In some models, it is also reported to protect neurons from NMDA-related excitotoxicity (Rogawski 1993) and damage associated with transient cerebral hypoxia-ischemia (Kato et al. 1990; Priestley et al. 1990). Stereotypical, dose dependent behavioural effects including hyperlocomotion, head weaving, circling, ataxia, hindlimb abduction, and general sensorimotor deficits have been described in MK801-injected rats (Koek et al. 1988; Löscher and Hönack 1991, 1992, 1993; Hargreaves and Cain 1992).

An investigation of the literature revealed that systemic administration of MK801 is known to activate the HPA axis in rats as evidenced by increased secretion of ACTH and CORT (Pechnick et al. 1989), and to elevate levels of FLI in CRH neurons in the PVN (Lee et al. 1994). The results of preliminary

experiments designed to investigate the influence of MK801 on FLI in the PVN of lactating rats, suggested that sex steroids may influence the responsiveness of NMDA receptors in the PVN. The effect of gonadal steroids on the HPA axis is well documented and the existence of sex differences in HPA function has been known for some time (Critchlow et al. 1963).

Several lines of evidence suggest that the action of MK801 on CNS neurons may be modified by gonadal steroids, particularly estrogen. At low doses, MK801 induces more pronounced behavioural perturbations in female rats than in male rats (Blanchard et al. 1992; Löscher and Hönack 1993; Hönack and Löscher 1993) and, on the basis of behavioural evidence, appears to exert a greater anxiolytic effect in males than in females (Blanchard et al. 1992). Further, MK801 injection decreases median eminence dopamine synthesis and metabolism in intact female rats and in ovariectomized females with estrogen replacement, but not in intact or castrated males, or ovariectomized females without estrogen replacement (Wagner et al. 1993). In mice, sex differences have been reported in the effect of MK801 on morphine and stress-induced analgesia (Lipa and Kavaliers 1990), and in forebrain MK801 receptor binding following acute swim stress (Akinci and Johnston 1993). Finally, an intriguing influence of estrogen/progesterone on the paradoxical "neurotoxic" effects of MK801 has been reported (Farber and Olney 1994). Olney's group noted that MK801 treatment caused vacuolization and necrosis in rat retrosplenial cortex, an effect which was blocked during pregnancy

The general objective of chapter 3 is to determine whether the reported sex differences in susceptibility to the effects of MK801 might, in part, be explained by steroid-dependent differences in activation of specific neuronal populations in the PVN. In experiment 3.1, the effect of MK801 on FLI in the PVN of intact and gonadectomized adult male and female rats, and lactating (pd 7) female rats is

examined to: 1) confirm that MK801 (1 mg./kg.) induces FLI in the PVN of adult S-D rats; 2) establish whether a sex difference in FLI response to MK801 is seen in the PVN of intact rats; 3) determine whether the FLI response of PVN neurons is reduced in lactating rats compared to non-lactating animals; and 4) to examine the effect of removal of circulating gonadal steroids on MK801-induced FLI in the PVN.

In experiment 3.2, the effect of MK801 on FLI in the PVN of prepubertal rats within (pd 14) and beyond (pd 25) the neonatal SHRP is examined to determine: 1) whether the magnitude of MK801-induced increases in FLI in the PVN is attenuated during the neonatal SHRP; and 2) to determine whether a sex difference in FLI-response to MK801 exists at the ages sampled.

Experiment 3.3 examines whether the FLI-response of PVN neurons of adult rats to MK801 is dose-dependent, and whether a sex difference is evident following administration of low doses of MK801.

Exposure of 2 day old female rats to elevated levels of circulating testosterone results in androgenization which persists into adulthood (Barraclough 1961). In experiment 3.4, the effect of MK801 on FLI in the PVN of female rats androgenized by testosterone injection on postnatal day 2 is compared to its effect on age-matched intact and gonadectomized male and female animals. The results of this experiment should establish the relative contributions of sex differences in brain morphology, and neuro-endocrine function to sex-differences in the FLI response to MK801 in the PVN of adult rats.

Experiment 3.1. MK801-induced FLI in the PVN of male, cycling and lactating female, and gonadectomized adult S-D rats.

Intact and gonadectomized adult male and female S-D rats (150-300 g.) and lactating female S-D rats (200-325 g.) with litters were ordered for use in this experiment. Following a minimum of 5 days in our animal care facility, intact male and female rats received a single s.c. injection of either MK801 (1 mg./kg. b.w., in 0.1 ml. saline), or 0.1 ml. saline. Lactating female rats arrived 2 days after delivering litters and 5 days later (pd 7), lactating animals received a single s.c. injection of either MK801 (1 mg./kg. b.w., in 0.1 ml. saline), or 0.1 ml. saline. Gonadectomized animals arrived in our animal care facility 2 days following surgery. Ten days following arrival, castrated males received a single s.c. injection of either testosterone [1 mg. testosterone propionate (TP) in 0.1 ml sesame oil] or sesame oil [(oil); 0.1 ml), and OVX females received a single s.c. injection of either estradiol benzoate [(EB); 10 µg 17-ß-estradiol-3-benzoate; Sigma, St. Louis, MO in 0.1 ml sesame oil] or sesame oil (0.1 ml). Twenty four hours following steroid or oil injection, all gonadectomized animals, along with groups of intact male and cycling female rats, received a single s.c. injection of MK801 (1.0 mg./kg. b.w., in 0.1 ml. saline), or saline (0.1 ml.). Two hours after MK801 or saline injection, animals were killed and perfused; brains were removed, postfixed, and sectioned; and alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

3.1 Results

Behavioural Effects

At a dose of 1.0 mg./kg. (s.c.), MK801 induced profound behavioural changes. Within 5 minutes of injection, head bobbing and weaving was seen in all animals. Over the next 10 minutes, circling movements, hyperactivity, and

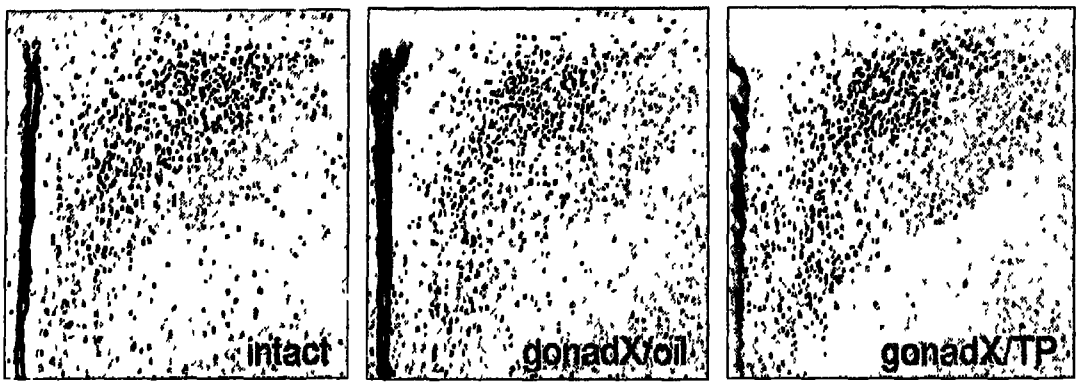
loss of balance and motor co-ordination developed, and within 15 minutes, all animals were prostrate and ataxic. This condition persisted throughout the first hour following injection.

At one hour following MK801 injection, lactating and cycling females lay completely immobile, whereas males, though still prostrate and ataxic, were capable of moving their heads and appeared somewhat more alert.

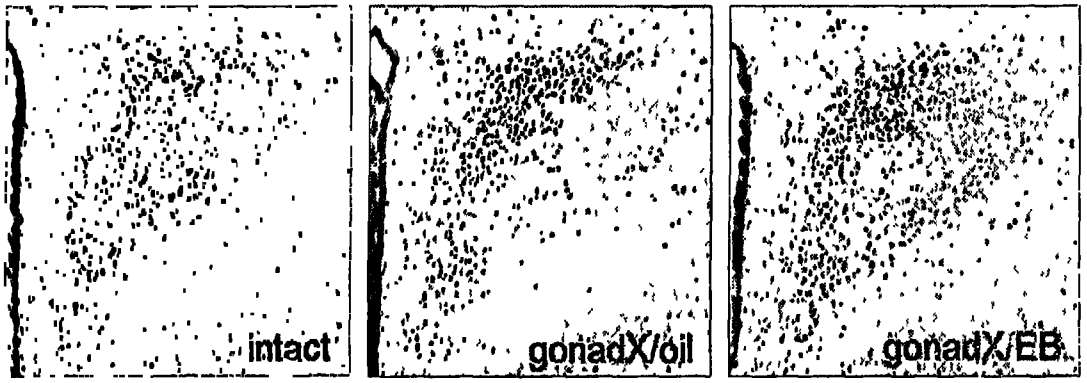
Two hours after MK801 injection, cycling females were still prostrate and almost completely ataxic, and lactating females, though exhibiting slightly more recovery of function than cycling females, continued to exhibit severely limited sensorimotor function. In marked contrast, males displayed significant recovery of function. All males had regained at least some control of their forelimbs and were moving around in their cages. Some had also regained hindlimb function and moved around their cages with an unsteady gait, while still others exhibited hindlimb abduction. Unlike cycling and lactating females which still appeared to be too motor-impaired to resist, all males struggled and attempted to escape when picked up and injected with anaesthetic prior to perfusion. No attempts were made to quantify behavioural effects of MK801, but they remained consistent through several experiments.

Effects on FLI

Digital images of brain sections from animals used in this experiment are presented in Figure 3.1.1. Little or no FLI was seen in the PVN of saline-injected animals, but dramatic increases were observed in animals injected with MK801. Levels of FLI in the PVN of MK801-injected male rats appeared somewhat higher than those seen in like treated cycling or lactating female rats. No discernible differences were noticed between levels of FLI in the PVN of MK801-treated gonadectomized rats pre-injected with sesame oil or steroid (TP or EB) compared



MALES (MK801)



FEMALES (MK801)

LACTATING FEMALES

Figure 3.1.1 FLI in the PVN of intact and gonadectomized male and female Sprague-Dawley rats killed 2 hours after s.c. injection of MK801 (1.0 mg./kg. b.w. in 0.1 ml saline), and lactating females killed 2 hours after injection with MK801 or saline. Twenty four hours prior to MK801 injection, gonadectomized (gonadX) males were injected with testosterone propionate (TP) or vehicle (oil), and gonadX females were injected with estradiol benzoate (EB) or oil. Scale bar = 100 μm.

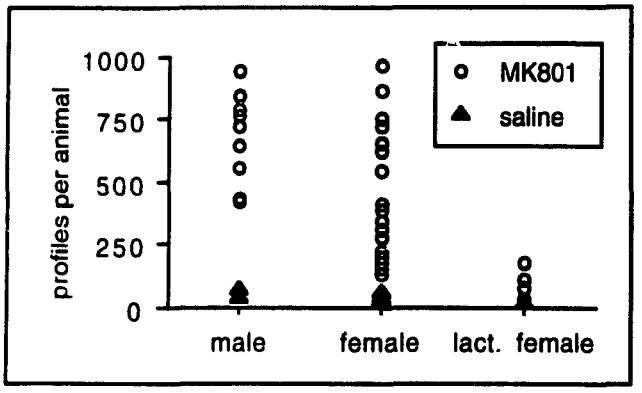
to like-treated, same-sex intact animals (although levels of FLI in the PVN of 2 of 3 gonadectomized males pre-injected with sesame oil did appear to be lower than levels seen in like-treated intact males).

Table 3.1.1 *Number of FLI-positive nuclear profiles in the PVN of intact male and cycling and lactating female rats killed 2 hours after injection of MK801 (1.0 mg./kg. b.w.) or saline.*

	MK801 (1.0 mg./kg.)			saline		
sex	n	profiles*	SEM	n	profiles*	SEM
male	9	680.7	61.0	2	51.0	19.0
cycling female	18	433.9	64.7	4	27.5	10.5
lactating female	4	100.0	30.2	2	6.0	3.0

*(*mean number of FLI-positive nuclear profiles per animal)*

The results of quantitative image analyses (summarized in Tables 3.1.1 and 3.1.2, and Figures 3.1.2 and 3.13) are consistent with these impressions. A two-way ANOVA performed on quantified data revealed a significant main effect of MK801 injection (compared to saline injection) on the number of FLI-positive nuclear profiles counted in the PVN of intact animals ($F_{1,33}$=16.192, p=.0003). The main effect of sex/lactation status ($F_{2,33}$=3.041, p=.0614), and the (sex/lactation status)/treatment interaction effect ($F_{2,33}$=2.232, p=.1232) were not significant. Subsequent one tailed t-tests performed on these data split on the basis of sex/lactation status, revealed a significant treatment effect (i.e., MK801 vs. saline) in male rats (p=.0006) and cycling female rats (p=.0044). Differences in the magnitude of MK801- vs. saline-induced FLI in the PVN of lactating female rats were not significant (p=.0535). Two tailed t-tests performed on data split by treatment revealed a significant difference in the number of FLI-positive nuclear profiles counted in the PVN of MK801-treated males compared to lactating females (p<.0001). Differences between MK801-treated males vs. females (p=.0227), and females vs. lactating females (p=.0275) were not significant given



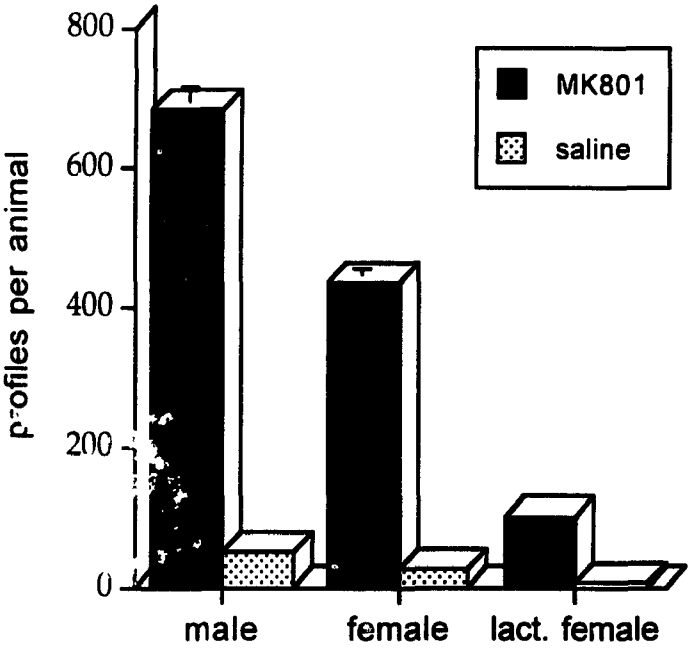
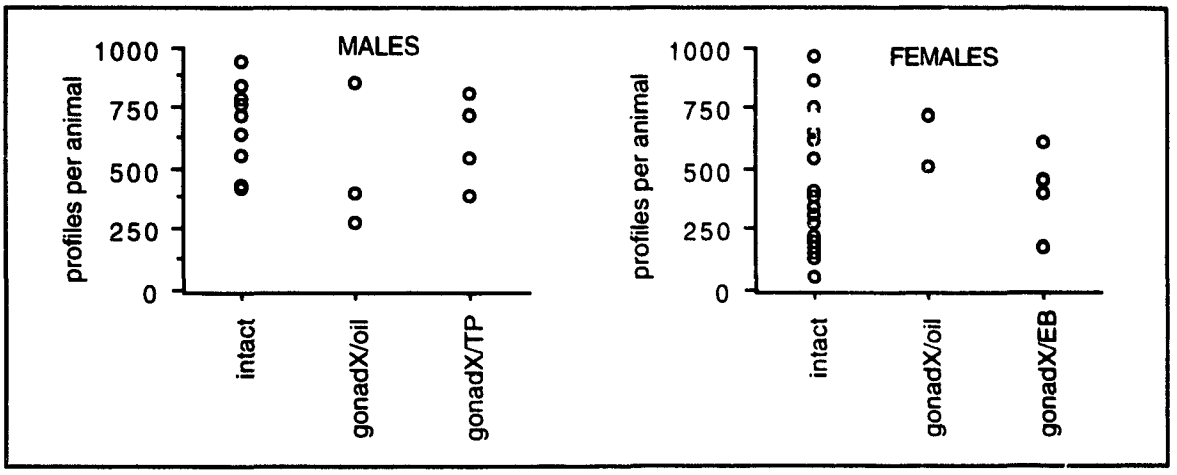


Figure 3.1.2 FLI in the PVN of adult male and non-lactating and lactating (lact.) female S-D rats killed 2 hours after s.c. injection of MK801 (1.0 mg./kg. b.w. in 0.1 ml saline), or 0.1 ml of saline. Scatterplot shows number of FLI-positive nuclear profiles per animal; bar plot shows number of FLI-positive nuclear profiles per animal ±SEM.



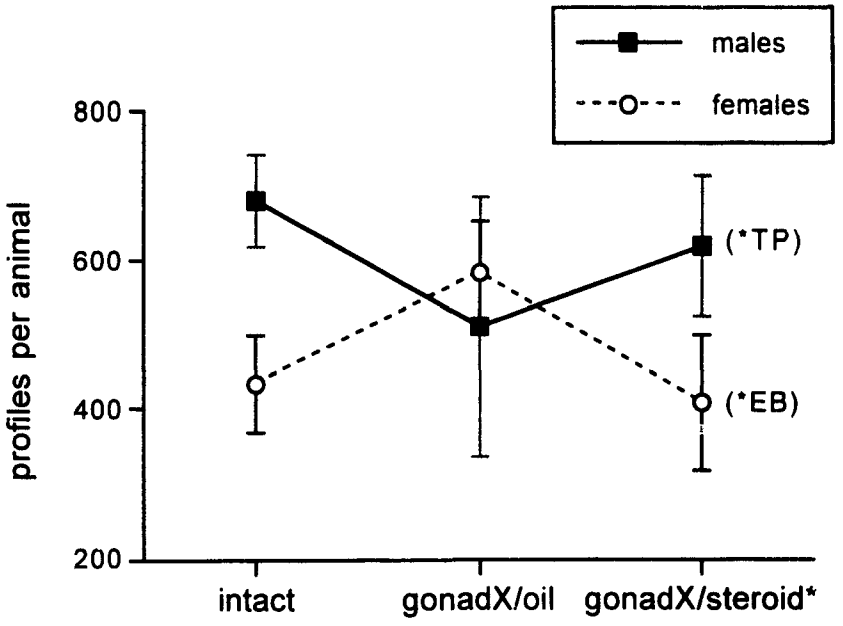


Figure 3.1.3 FLI in the PVN of intact and gonadectomized (gonadX), male and female Sprague-Dawley rats killed 2 hours after s.c. injection of MK801 (1.0 mg./kg. b.w. in 0.1 ml saline). Gonadectomized animals were injected with steroid (TP or EB) or vehicle (oil) 24 hours prior to MK801 injection (TP = testosterone propionate; EB = estradiol benzoate; oil = sesame oil). Scatterplots show number of FLI-positive nuclear profiles per animal; line plot shows number of FLI-positive nuclear profiles per animal ±SEM.

the Bonferroni inequality. Sex differences in saline-induced FLI in the PVN were also not significant as measured by two tailed t-tests (male/female, p=.2965; male/lactating female, p=.1442; female/lactating female, p=.2469).

A two-way ANOVA comparing MK801-treated intact male and female animals with gonadectomized animals pretreated with sesame oil or steroid revealed no significant main effect of sex ($F_{1,35}$=1.984, p=.1678) or pretreatment ($F_{2,35}$=0.102, p=.9034), and no significant sex/pretreatment interaction effect ($F_{2,35}$=1.088, p=.3480) on the number of FLI-positive nuclear profiles counted in the PVN.

Table 3.1.2 *Number of FLI-positive nuclear profiles in the PVN of intact and gonadectomized male and female rats with and without steroid replacement, killed 2 hours after MK801 injection (1.0 mg./kg. b.w.).*

	male			female		
surgery/pre-injection	n	profiles*	SEM	n	profiles*	SEM
intact/none	9	680.7	61.0	18	433.9	64.7
gonadX/oil	3	510.0	175.6	3	581.0	71.5
gonadX/steroid**	4	618.5	93.3	4	408.8	90.2

*(*mean number of FLI-positive nuclear profiles per animal; **male=TP. female=EB)*

Experiment 3.2. Sex differences in MK801-induced FLI in the PVN of prepubertal S-D rats

Preweanling (aged 14 days) and weanling (aged 25 days) male and female S-D rat pups (n=3 animals/group) received a single s.c. injection of MK801 (1 mg./kg. b.w. in saline). Two hours after MK801 injection, animals were killed and perfused; brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

3.2 Results

Digital images of brain sections from animals used in this experiment are presented in Figure 3.2.1. Elevated levels of FLI were seen in the PVN of all 14 day old and 25 day old rats killed 2 hours after MK801 injection, but levels observed in 25 day old rats were higher than those seen in 14 day old animals. Quantitative data from this experiment are summarized in Table 3.2.1 and Figure 3.2.2. Results of a two-way ANOVA performed on these data revealed a significant main effect of age on the number of FLI-positive nuclear profiles counted in the PVN of MK801-injected rats ($F_{1,8}$=31.881, p=.0005). The main effect of sex ($F_{1,8}$=2.843, p=.1303), and sex/age interaction effect ($F_{1,8}$=2.843, p=.1303) were not significant. Subsequent two tailed t-tests performed on data split on the basis of sex, revealed a significant age effect in females (p=.0001), but not in males (p=.1109).

Table 3.2.1 *Number of FLI-positive nuclear profiles in the PVN of prepubertal male and female rats killed 2 hours after MK801 injection (1.0 mg./kg. b.w.) on pd 14 or pd 25.*

	male			female		
age	n	profiles*	SEM	n	profiles*	SEM
14 days	3	272.3	54.6	3	272.3	7.3
25 days	3	532.7	115.3	3	754.3	30.9

*(*mean number of FLI-positive nuclear profiles per animal)*

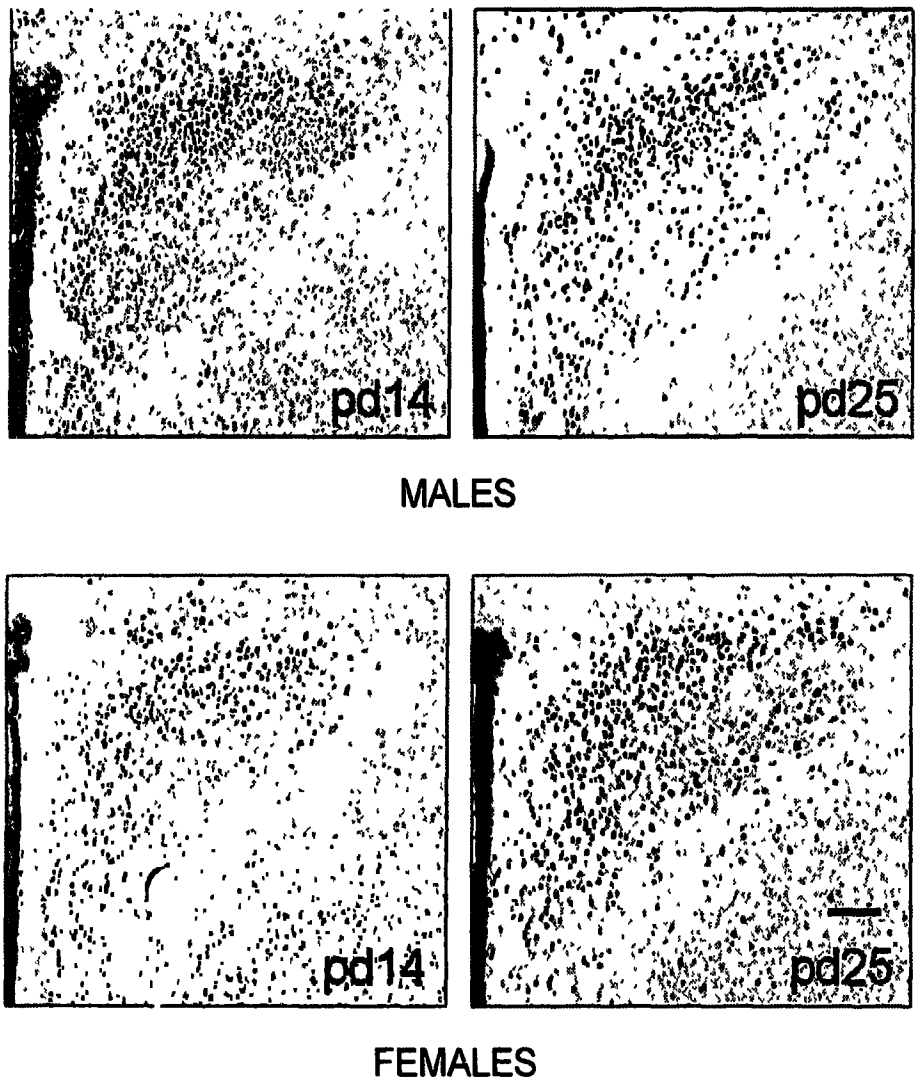
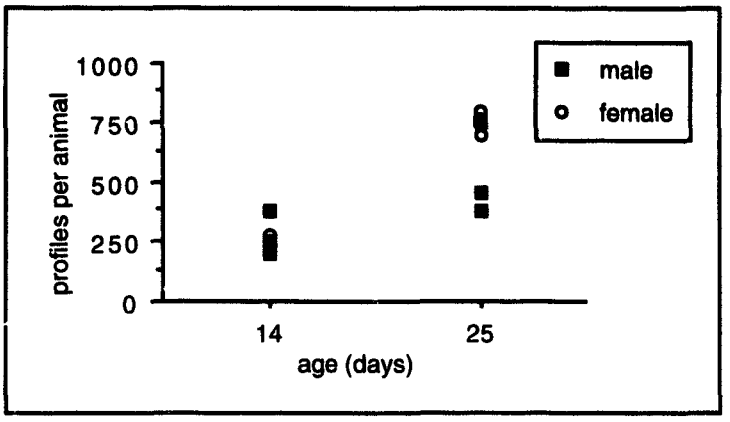


Figure 3.2.1 FLI in the PVN of prepubertal male and female Sprague-Dawley rats killed 2 hours after s.c. injection of MK801 (1.0 mg./kg. b.w. in 0.1 ml saline) on postpartum day 14 (pd14) or postpartum day 25 (pd25). Scale bar = 100 µm.



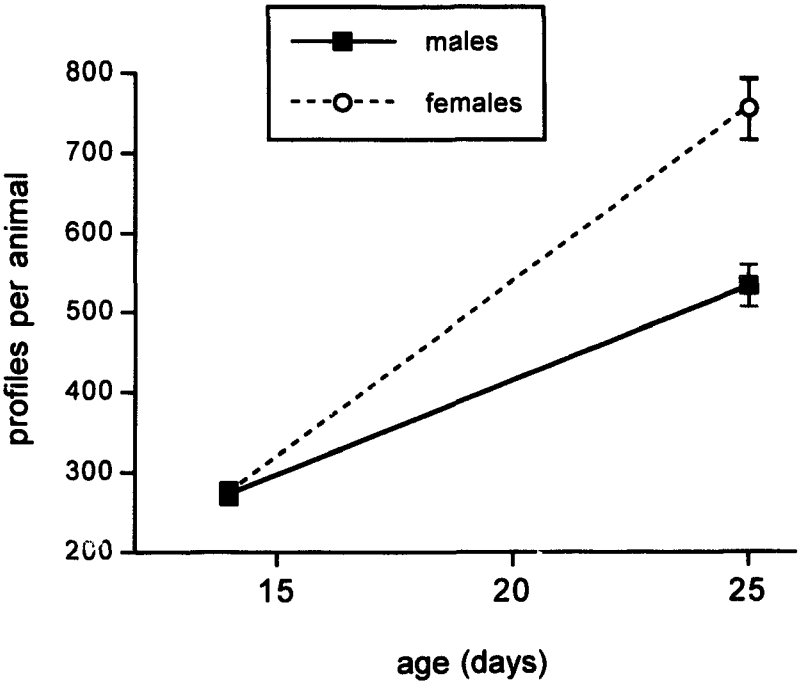


Figure 3.2.2 FLI in the PVN of prepubertal male and female S-D rats killed 2 hours after s.c. injection of MK801 (1.0 mg./kg. b.w. in 0.1 ml saline). Scatterplot shows number of FLI-positive nuclear profiles per animal; line plot shows number of FLI-positive nuclear profiles per animal ±SEM.

Experiment 3.3. Effect of low-dose MK801 on FLI in the PVN of adult male and female S-D rats.

Adult male (375-450 g.) and (non-lactating) female (250-325 g.) S-D rats received a single s.c. injection of MK801 (0.1 mg./kg. b.w. in 0.1 ml. saline), MK801 (0.3 mg./kg. b.w. in 0.1 ml. saline) or saline (0.1 ml.). Lactating female rats (300-350 g.) received either MK801 (0.1 mg./kg. b.w. in 0.1 ml. saline) or saline (0.1 ml.). Two hours after MK801 or saline injection, animals were killed and perfused, and brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

3.3 Results

Behavioural Effects

At both dose levels, MK801 induced behavioural changes in all females injected. Within 10 minutes of MK801 injection, non-lactating and lactating females treated with MK801 (0.1 mg./kg.) displayed grooming, head bobbing and weaving, and moved erratically about their cages. At 60 minutes, females were noticeably prostrated and exhibited ataxia and impaired motor control. By 2 hours, prostration and ataxia was greatly diminished and unstable circling movements were noted. In general, non-lactating females exhibited less behavioural disruption than like-treated lactating females.

Females receiving 0.3 mg./kg. of MK801 resembled lower dose females at 10 minutes following injection but, by 60 minutes they were completely prostrated and exhibited almost total ataxia. Most animals exhibited pronounced hindlimb abduction and extension, and appeared unable to balance on their feet, resting instead upon their elbows and knees. At 2 hours, some females lay completely flat and immobile while others demonstrated limited recovery of hindlimb

function and were able to move around the cage in an erratic, swimming-like manner. At both dose levels, males exhibited less pronounced behavioural perturbations than did females. Males receiving 0.1 mg./kg. of MK801 exhibited no behavioural changes at 10 minutes following administration. Slight ataxia and aberrant movement was noted at 60 minutes following MK801 injection, but by 2 hours following injection, their behaviour had returned to normal. At 10 minutes following injection, males which received 0.3 mg./kg. of MK801 appeared agitated and somewhat prostrated and exhibited head bobbing and weaving. By 60 minutes, they were prostrate and immobile but to a lesser degree than similarly treated females. Pronounced hindlimb abduction/flexion was seen in only one animal. By 2 hours post-injection, co-ordination was largely restored in most males. Although some ataxia and agitation persisted, no evidence of prostration was noted. In contrast to females, most males were capable of coordinated struggling and escape attempts when picked up for anaesthetic injection prior to perfusion. No behavioural changes were observed in animals which received saline alone.

Effects on FLI

Digital images of brain sections from animals used in this experiment are presented in Figure 3.3.1. Immunocytochemical results were consistent with behavioural observations. In both males and females, slightly more FLI was seen in the PVN of animals which received 0.3 mg./kg. compared to those receiving 0.1 mg./kg., and at both dosages, more FLI was seen in the PVN of females compared to males. Quantitative data from this experiment are presented in Table 3.3.1 and Figure 3.3.2, alongside saline and high dose (1.0 mg./kg.) MK801 data from experiment 3.1. A two-way ANOVA performed on these data revealed a significant main effect of MK801 dosage on the number of

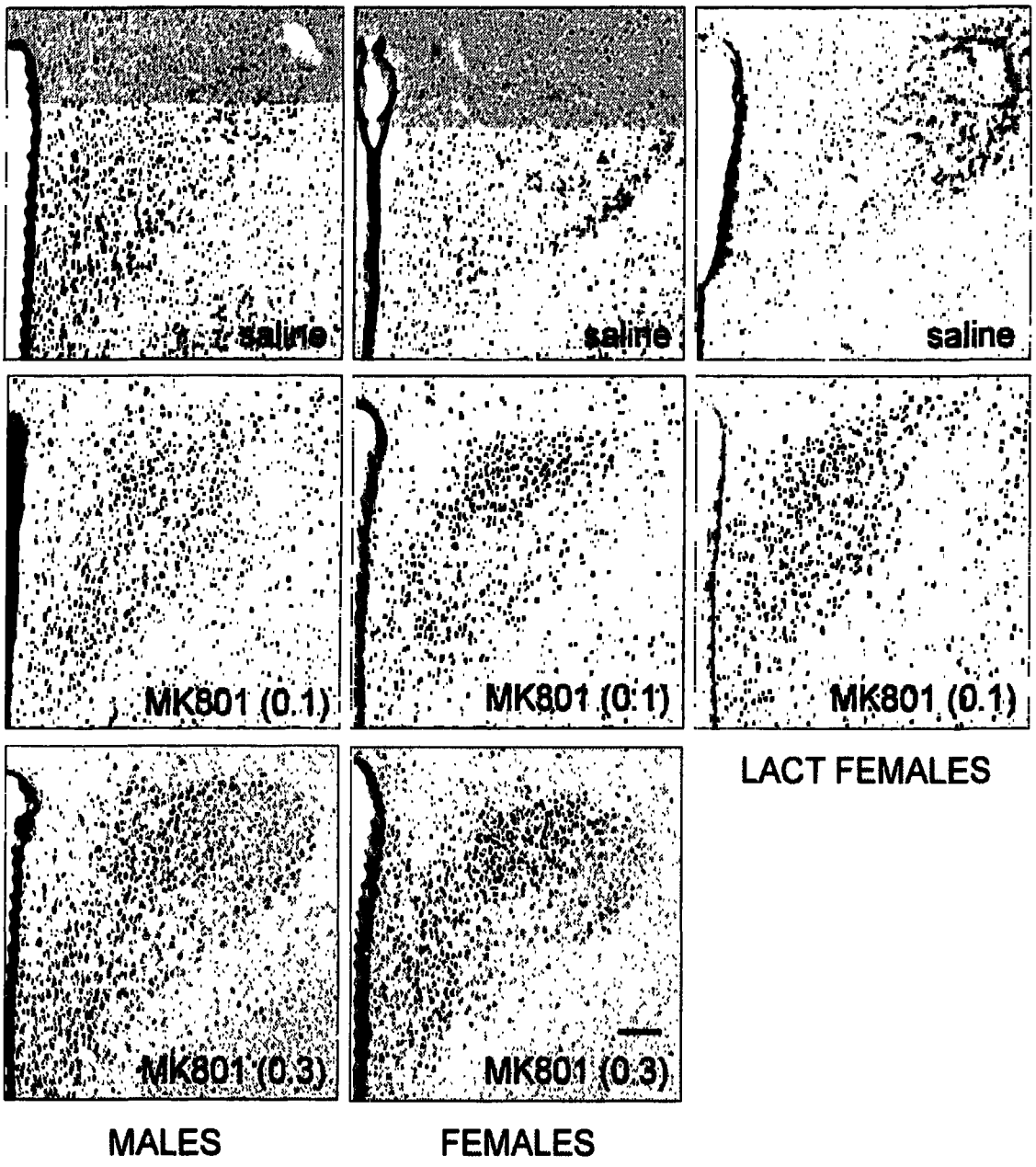


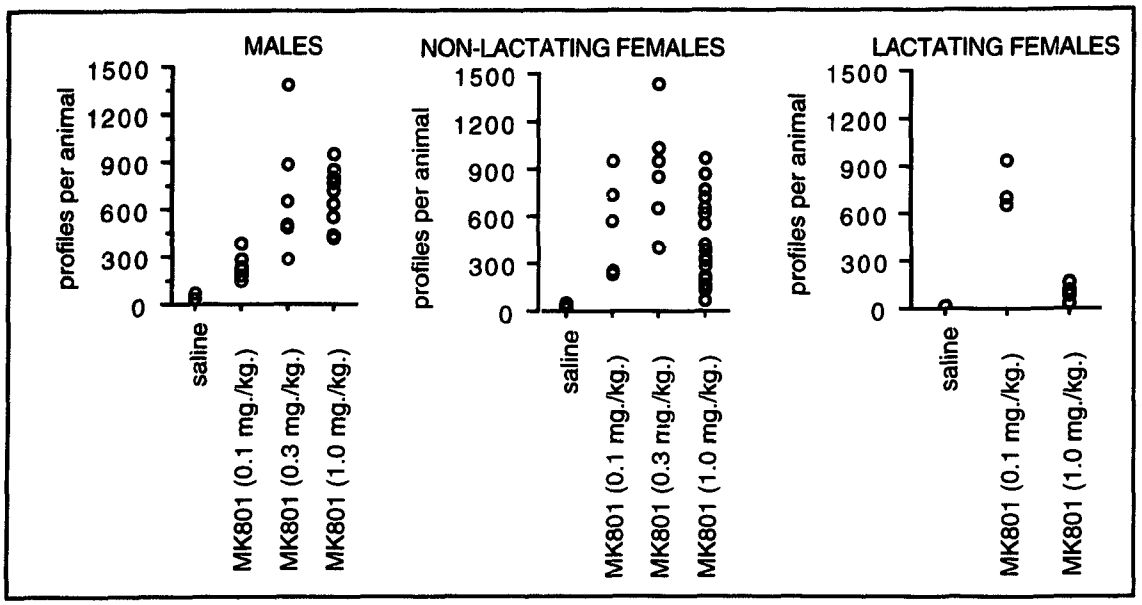
Figure 3.3.1 FLI in the PVN of male, non-lactating female (female) and lactating (lact) female Sprague-Dawley rats killed 2 hours after s.c. injection of MK801 (0.1, or 0.3 mg./kg. b.w. in 0.1 ml saline) or 0.1 ml. saline. Scale bar = 100 μm.

FLI-positive profiles counted in the PVN ($F_{2,55}$=8.75, p=.0005). The main effect of sex/lactation status was not significant ($F_{1,55}$=0.280, p=.6007), but a significant (sex/lactation status)/dose interaction effect was detected ($F_{5,55}$=5.040, p=.0007). Subsequent two tailed t-tests performed on data split by dosage revealed a significant difference between MK801-treated males and lactating females (0.1 mg./kg. dose, p=.0002; 1.0 mg./kg. dose, p<.0001). All other pairwise comparisons yielded insignificant results.

***Table** 3.3.1 Number of FLI-positive nuclear profiles in the PVN of adult male, and non-lactating and lactating female rats killed 2 hours after injection with MK801 (0.1, 0.3, or 1.0 mg./kg. b.w.) or 0.1 ml. saline.*

	male			non-lactating female			lactating female		
MK801 dosage	n	profiles	SEM	n	profiles*	SEM	n	profiles	SEM
0.1 mg./kg.	6	247.2	34.6	6	496.7	124.1	3	762.3	82.9
0.3 mg./kg.	6	696.3	157.8	6	883.7	143.5	-	-	-
1.0 mg./kg.**	9	680.7	61.0	18	433.9	64.7	4	100.0	30.2
saline**	2	51.0	19.0	4	27.5	10.5	2	6.0	3.0

*(*mean number of FLI-positive nuclear profiles per animal; **data from experiment 3.1)*



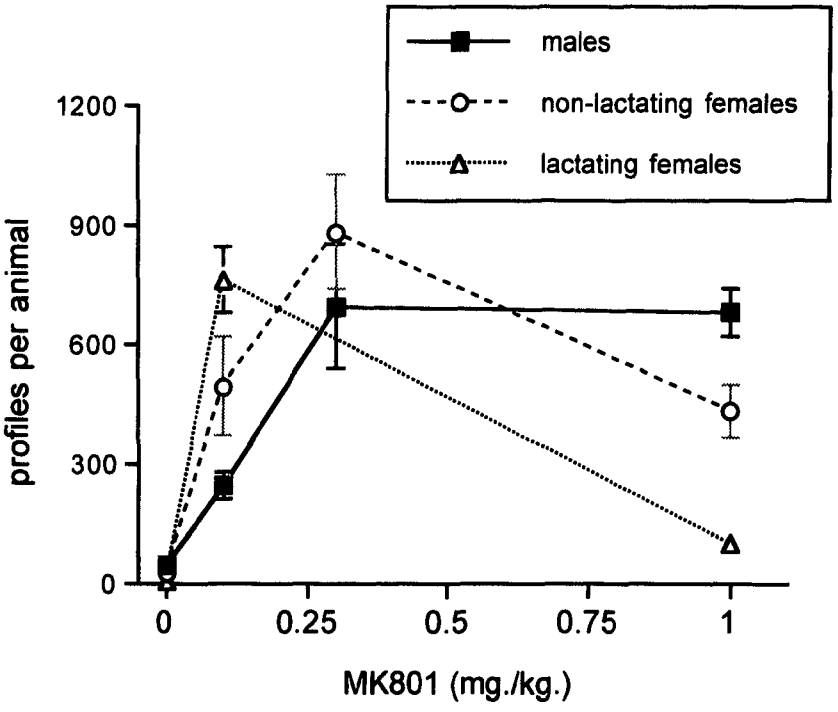


Figure 3.3.2 FLI in the PVN of adult male, and non-lactating and lactating female Sprague-Dawley rats killed 2 hours after s.c. injection of MK801 (0.1, 0.3, or 1.0 mg./kg. b.w. in 0.1 ml saline). The initial value (0 mg./kg. MK801) represents saline injection. Scatterplots show number of FLI-positive nuclear profiles per animal; line plot shows number of FLI-positive nuclear profiles per animal ±SEM.

Experiment 3.4. MK801-induced FLI in the PVN of normal and androgenized female S-D rats.

Litters of androgenized (ANDR) and non-androgenized (NANDR) female rats (aged 60-72 days) were used in this experiment along with groups of age-matched intact and gonadectomized (12 days post-surgery) males and females.

Androgenization

Litters of 2 day old female S-D rats (8-10 animals per litter) were given a s.c. injection of either testosterone propionate (TP; 1.0 mg. in 0.1 ml sesame oil) or sesame oil (oil; 0.1 ml) and returned to their home cages. On postnatal day 21, mothers were removed but litters of weanlings remained together. Animals were checked periodically for vaginal opening and body weights were recorded at 40, 50, 60, and 72 days of age. Following perfusion (after MK801 treatment on day 60 or 72), ovaries were removed and weighed to confirm whether androgenization had occurred in TP-injected animals.

MK801 Injections

On postnatal day 60 or 72, ANDR and NANDR females (6 animals per group), along with groups of age-matched intact (also used as a comparison group in experiment 3.3) and gonadectomized (12 days) male and female rats received a single injection of MK801 (0.1 mg./kg., s.c.). Some gonadectomized males received a s.c. injection of either TP (1.0 mg. in 0.1 ml sesame oil) or sesame oil (0.1 ml.) 24 hours prior to MK801 injection. One group of intact females also received a pre-injection of TP (1 mg. in 0.1 ml. sesame oil) 24 hours before MK801 administration. Two hours after MK801 injection, animals were killed and perfused, and brains were removed, postfixed, and sectioned. Alternate coronal sections through the PVN were processed for *c-fos* immunocytochemistry.

Behavioural effects of MK801 were recorded throughout the post-injection period.

3.4 Results

At 50 days of age, ANDR females displayed marked pudendal hypertrophy, and were significantly heavier than NANDR littermates as measured by a one tailed t-test (p=.0081; see Table 3.4.1 and Figure 3.4.1). Throughout the experimental period, ANDR continued to grow at a faster rate than NANDR animals. With the exception of the youngest age at which body weights were recorded (day 40), between group weight differences were statistically significant as measured by one tailed t-tests (day 40, p=.3961; day 50, P=.0054; day 60, p=.0003; day 72, p=.0029).

Table 3.4.1 *Mean body weight of non-androgenized (NANDR), and androgenized (ANDR) female rats on postpartum days 40, 50, 60, and 72.*

	NANDR			ANDR		
age (days)	n	b.w. (g.)	SEM	n	b.w. (g)	SEM
40	16	140.8	3.9	15	142.3	4.2
50	16	177.9	2.9	15	190.1	3.9
60	16	211.3	3.2	15	234.2	4.9
72	10	247.8	4.7	9	275.8	7.7

Ovarian Weights

Ovaries from NANDR 60 day old females were of normal size, weight, and appearance. Follicles and corpora lutea were visible, confirming that these animals were cycling. In contrast, ovaries removed from 60 day old ANDR females were small, pale and unstimulated. Between group differences in

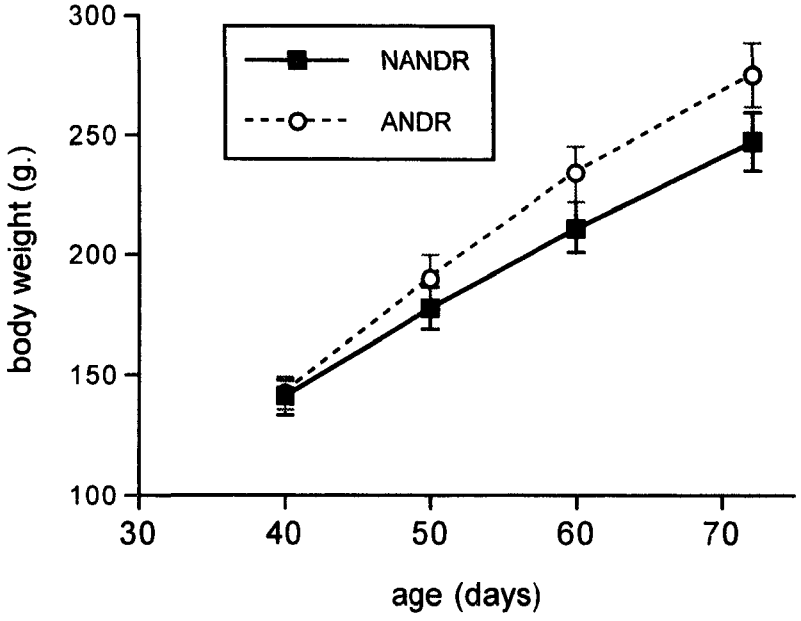


Figure 3.4.1 Body weights of Sprague-Dawley rats injected with sesame oil (NANDR) or testosterone propionate (ANDR) on postpartum day 2. Values shown are mean weight per animal ± SEM.

ovarian weights were significantly different as determined by a one tailed t-test (p=.0014; see Table 3.4.2 and Figure 3.4.2).

Table 3.4.2 *Mean ovarian weight of non-androgenized (NANDR) and androgenized (ANDR) female rats killed on pd 60.*

group	n	weight (mg.)*	SEM
NANDR	7	77.00	4.6
ANDR	7	33.25	1.2

*(*total ovarian weight per animal)*

Behavioural Results

Within 10 minutes of injection, behavioural anomalies were observed in all animals receiving low dose MK801 (0.1 mg./kg.). Hyperlocomotion and unsteady gait was observed in ANDR and NANDR females, and to a lesser degree, in gonadectomized females and intact females pre-injected with TP. In intact males, behavioural disruption at 10 minutes following MK801 (0.1 mg./kg.) was limited to grooming and head bobbing. Some gonadectomized males displayed hyperactivity, grooming, and head bobbing, others displayed only grooming and head bobbing. Slight head bobbing was also noted in some gonadectomized males which had received TP replacement 24 hours earlier.

At 60 minutes following MK801 injection, ANDR females, though markedly prostrated, were still able to control all 4 limbs. Though still displaying hyperlocomotion, no rearing or hindlimb abduction was seen in these animals. NANDR females displayed similar behavioural disruptions to ANDR females but appeared to be slightly more impaired. Repeated rearing with loss of balance was frequently seen. At 60 minutes following MK801 injection, gonadectomized females exhibited unsteady gait and some loss of balance but to a lesser extent than that seen in NADR or NANDR intact females. Apart from slight flattening

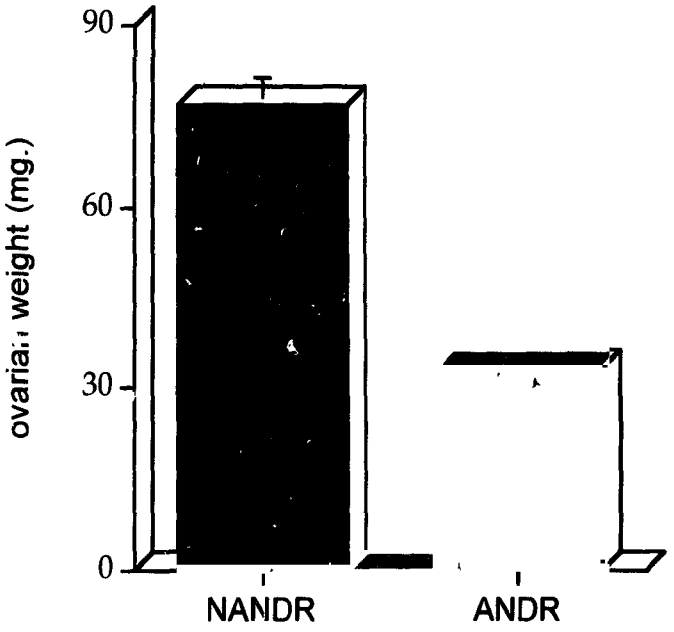


Figure 3.4.2 Ovarian weights of 60 day old Sprague-Dawley rats injected with sesame oil (NANDR) or testosterone propionate (ANDR) on postpartum day 2. Values shown are group means of ovarian pair weight per animal ±SEM.

and unsteadiness of gait seen in some animals, intact and gonadectomized males were moving normally and showed very little evidence of motor impairment.

At 2 hours following MK801 (0.1 mg./kg.), ANDR females were still flattened and hyperactive. NANDR females were flattened and unsteady with poor hind-limb control. They moved around their cages with a swimming-like motion, dragging themselves primarily with their forelimbs. By 2 hours following MK801 injection, gonadectomized females were still somewhat flattened but otherwise, were moving normally. Gonadectomized males showed less residual motor impairment than females. Some showed slight hyperactivity and were still unable to maintain their balance on their hind limbs, while others appeared to be fully recovered. In contrast to all other groups, intact males were completely recovered and were resting or sleeping together as is normal at that time of day.

Effects on FLI

Digital images of brain sections from animals used in this experiment are presented in Figure 3.4.3 and 3.4.3b. Elevated levels of FLI were present in the PVN of all MK801-injected animals. Levels induced in the PVN of intact males appeared lower than those seen in gonadectomized males, and levels of FLI in the PVN of gonadectomized males which received TP 24 hours before MK801 injection did not differ appreciably from those seen in other gonadectomized males. In general, levels of FLI seen in the PVN of MK801-injected females showed more within group variability than did males. No discernible between group differences in level of FLI in the PVN were apparent among females.

Quantified data from this experiment, along with comparison data from experiment 3.3, are summarized in Table 3.4.3 and Figure 3.4.5. In experiment 3.3 of this thesis, a significant sex difference was reported in intact animals killed

2 hours after injection of 0.1 mg./kg. of MK801. Results of a one-way ANOVA performed on quantified data from male animals used in this experiment revealed a significant main effect of gonadectomy on the number of FLI-positive profiles counted in the PVN ($F_{1,13}$=15.351, p=.0012). In contrast, a one-way ANOVA performed on quantified data from females revealed no main effect of gonadectomy ($F_{1,20}$=.0920, p=.7653). Further analyses using two-tailed t-tests revealed significant differences between intact males and gonadectomized males pre-injected with oil (p=.0008) or TP (p=.0033). Differences in MK801-induced FLI in the PVN of ANDR vs. NANDR females were not statistically significant (p=.3896).

Table 3.4.3 *Number of FLI-positive nuclear profiles in the PVN of androgenized (ANDR) and non-androgenized (NANDR) female rats, intact and gonadectomized (gonadX) male and female rats, intact females pre-injected with testosterone propionate (TP). and gonadX males pre-injected with either sesame oil (oil) or TP. All animals were killed 2 hours after injection of MK801 (0.1 mg./kg., b.w.).*

	FEMALES			MALES		
	n	profiles*	SEM	n	profiles*	SEM
NANDR females	6	452.8	140.0	-	-	-
ANDR females	6	621.3	124.5	-	-	-
intact**	6	496.3	124.1	6	247.2	34.5
gonadX	2	575.0	132.0	3	616.0	200.6
intact (24h TP)	2	386.5	79.5	-	-	-
gonadX (24h TP)	-	-	-	3	511.7	51.4
gonadX (24h oil)	-	-	-	3	607.7	59.3

*(*mean number of FLI-positive nuclear profiles per animal; **data from experiment 3.3)*

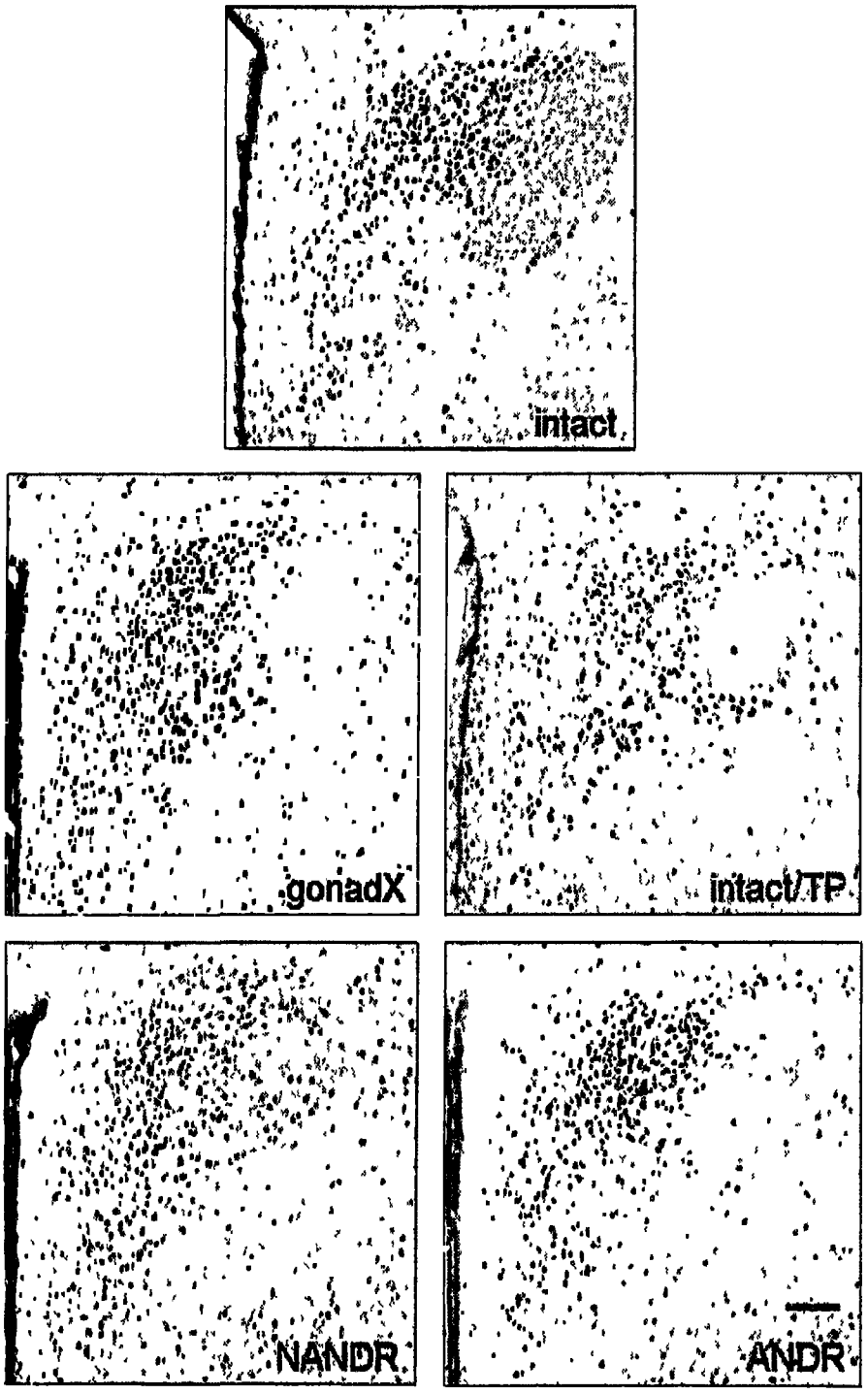


Figure 3.4.3 FLI in the PVN of intact and gonadectomized (gonadX) female Sprague-Dawley rats killed 2 hours after s.c. injection of 0.1 mg./kg. b.w. MK801 (intact/TP = injected with testosterone propionate 24 hours prior to MK801 injection; NANDR = injected with sesame oil at age 2 days; ANDR = injected with testosterone propionate at age 2 days. Scale bar = 100 µm.

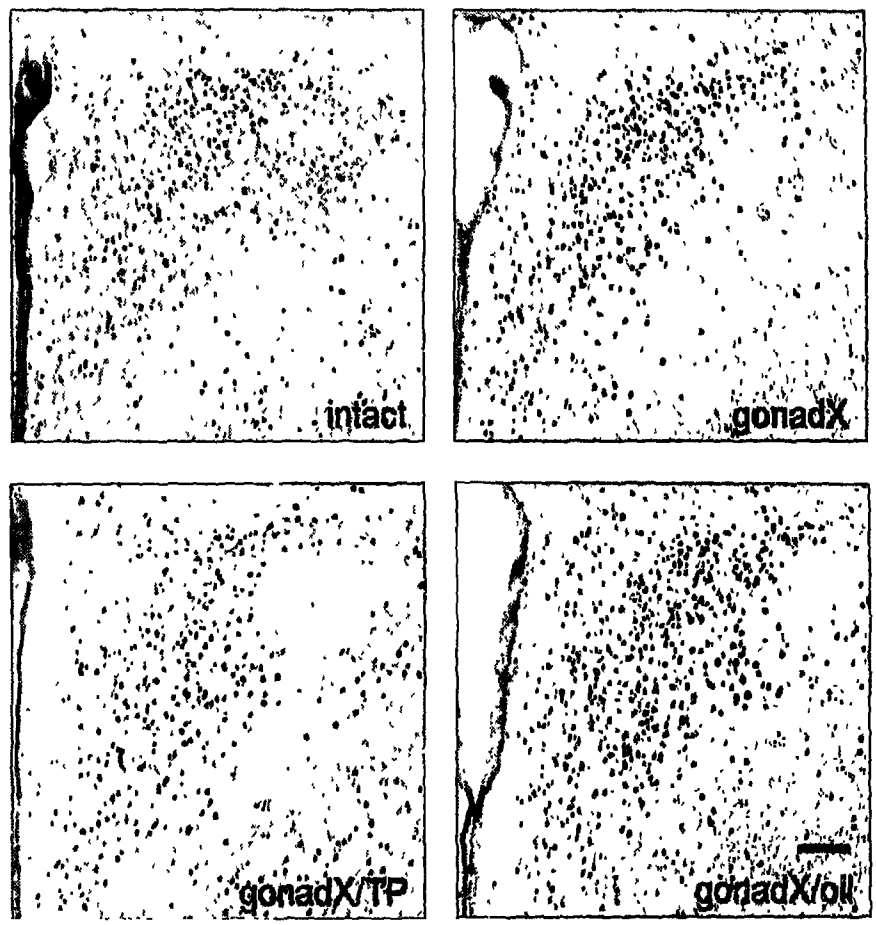
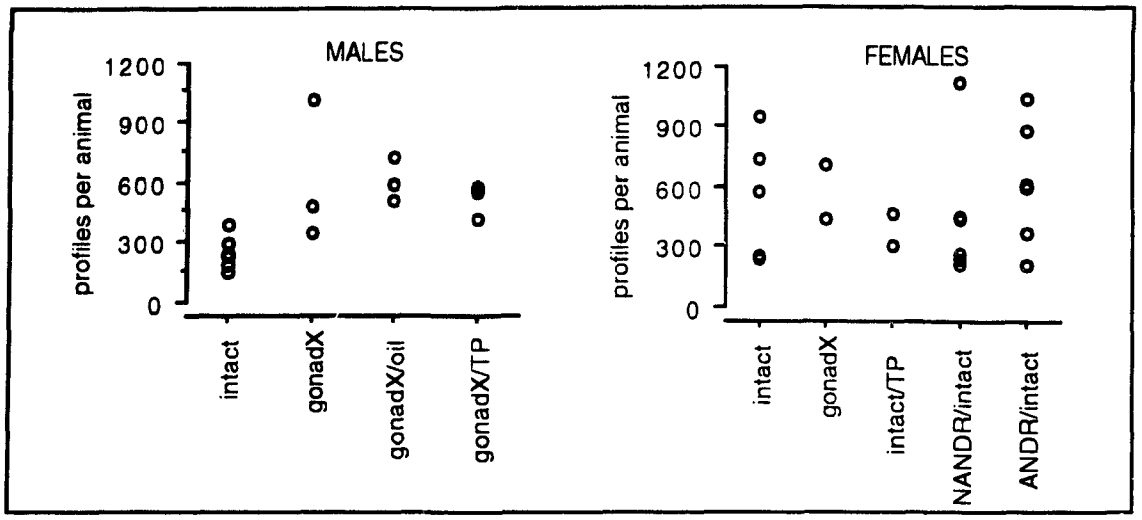


Figure 3.4.4 FLI in the PVN of intact and gonadectomized (gonadX) adult male rats, and gonadX rats injected with sesame oil (oil) or testosterone propionate (TP) 24 hours earlier, killed 2 hours after s.c. injection of 0.1 mg./kg. b.w. MK801. Scale bar = 100 μm.



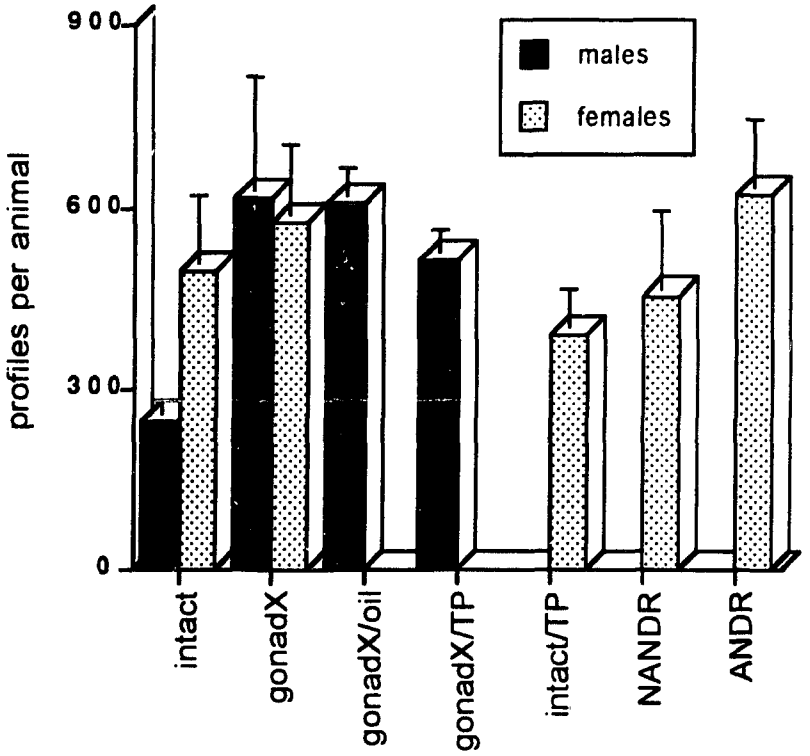


Figure 3.4.5 Number of FLI-positive nuclear profiles in the PVN of androgenized (ANDR) and non-androgenized (NANDR) female rats, intact and gonadectomized (gonadX) male and female rats, intact females pre-injected with testosterone propionate (TP), and gonadX males pre-injected with either sesame oil or TP. All animals were killed 2 hours after injection of MK801 (0.1 mg./kg., b.w.). Scatterplots show number of FLI-positive nuclear profiles per animal; bar plot shows group mean number of FLI-positive nuclear profiles per animal ±SEM.

Discussion

In general, the MK801-induced behavioural perturbations observed in this study resemble those reported by other investigators (Koek et al. 1988; Löscher and Hönack 1991, 1992, 1993; Hargreaves and Cain 1992). The observation that MK801 induces FLI in the PVN of adult rats is consistent with the findings of Lee et al. (1994) who reported FLI confined exclusively to parvocellular regions of the PVN of adult male rats killed 60 minutes following i.p. injection of 1.0 mg./kg. MK801. In the current study, the pattern of FLI in the PVN of MK801-treated adult male rats resembled that reported by Lee et al. (i.e., levels of FLI were elevated in parvocellular neurons but were seldom detected in magnocellular neurons), but the density, however, was noticeably higher. A number of differences in experimental protocol might account for this difference. Lee's group used a different Fos antibody at a different concentration (Oncogene Science @ 1:1,000 vs. CRB @ 1:10,000-1:20,000), a different route of administration (i.p. vs. s.c.), and a shorter survival time (60 minutes vs. 2 hours). In pilot studies related to this thesis, the Oncogene Science Fos antibody (1:1,000-1:2,000) consistently yielded less intense ADX-induced FLI compared to CRB (1:10,000-1:20,000; results not shown). In other preliminary work related to this chapter, i.p. injections of MK801 induced noticeably lower levels of FLI in the PVN of adult rats compared to identical doses injected s.c. (results not shown). This difference might be related to different rates of uptake of i.p.- vs. s.c.-injected substances to the blood. Alternatively, it might reflect the proportion of i.p.- vs. s.c.-injected substances which pass through the liver prior to reaching the cerebral circulation. Given the reversal of dose-response seen in females in experiment 3.1 of this thesis, it will be interesting to determine whether s.c. and i.p. injections of MK801 yield parallel dose-response curves.

Lee et al. (1994) selected a survival time of 60 minutes on the basis of reports by other investigators that this survival time yields maximal accumulations of CRH- (Parkes et al. 1993) and cocaine-induced (Young et al. 1991) FLI in the PVN. Although a time course study of MK801-induced FLI has not been done, a time course study of adrenal surgery-induced FLI reported in experiment 1.1 of this thesis revealed noticeably denser accumulations of FLI in the PVN of adult male rats killed 2 hours after surgery, compared to like-treated animals killed 1 hour after surgery (Figure 1.1.1). Evidence suggests that the time course for *c-fos* gene activation might vary in some instances. For example, in Chapter 1 of this thesis, adrenal surgery-induced FLI appeared to peak earlier in infant rats (2-4 hours) compared to adults (4-8 hours). Also, in a recent abstract, Bhatnagar and Dallman (1995) reported that FLI accumulations in the PVN peak 30 minutes following the onset of restraint stress. Given these observations, it will be interesting to determine whether the time course of MK801-induced FLI coincides with that established for ADX-induced FLI (see Experiment 1.1).

The results of experiment 3.1 are somewhat paradoxical. *High dose* MK801 (1.0 mg./kg. b.w.), induced more profound, longer lasting behavioural effects in cycling and lactating female rats compared to intact males (lactating females ≥ non-lactating females > males), but induced more FLI in the PVN of intact males compared to cycling or lactating females (males > non-lactating females > lactating females; Figures 3.1.1, 3.1.2). The observation that in both males and females, the magnitude of high-dose MK801-induced FLI in the PVN of gonadectomized animals (with or without steroid replacement) did not differ significantly from levels detected in same-sex intact animals (Figure 3.1.3), suggests that sensitivity to MK801 is not dependent upon the presence of gonadal steroids. However, the finding of a significant sex difference in MK801-

treated intact animals which does not persist in gonadectomized animals indicates that gonadal steroids do contribute to maintaining this sex-difference.

The data presented in Figure 3.1.3, suggest that gonadectomy does affect MK801-induced FLI in the PVN. In 2 of 3 gonadectomized/oil-treated males, the number of MK801-induced FLI-positive nuclear profiles counted in the PVN approached the mean number counted in intact females. In contrast, the range of FLI-positive nuclear profile counts obtained from gonadectomized males given TP 24 hours earlier resembled that seen in like-treated intact males. The failure to detect a statistically significant difference in FLI in the PVN of intact vs. gonadectomized animals, is likely due to the small number of gonadectomized animals included in this study, and the broad range of results obtained from MK801-treated intact females (this variation might have been reduced by using gonadectomized females implanted with Silastic capsules containing estradiol in place of cycling intact animals; see Weiland 1992). Clearly, this interpretation leans heavily upon inference and conjecture. To determine whether the trends described above represent real effects, it will be necessary to repeat a portion of experiment 3.1 using larger numbers of animals.

Perhaps the most surprising result obtained in these experiments is the dose-related reversal in FLI response to MK801 seen only in female animals. High dose (1.0 mg./kg.) MK801 induced greater numbers of FLI-positive nuclear profiles in the PVN of male rats compared to non-lactating or lactating females (males >non-lactating females > lactating females; Figure 3.1.2) whereas in animals given a lower dose of MK801 (0.1 mg./kg.), this relationship was reversed (lactating females >non-lactating females > males; Figure 3.3.2). Male rats display a dose-related increase in FLI induction in the PVN following injection of low doses of MK801 (0.1 or 0.3 mg./kg.), but no further increase in FLI is seen when MK801 dose is increased to 1.0 mg./kg. (whether doses of

MK801 between 0.1 and 1.0 mg./kg. result in a greater FLI response in the PVN has not yet been determined). In contrast, the female MK801 dose-response curve, which lies above the curve for males at dose levels of 0.1 and 0.3 mg./kg., declines sharply when MK801 dose is increased from 0.3 mg./kg. to 1.0 mg./kg.. Although no lactating females were included in the 0.3 mg./kg. MK801 dosage group, data from lactating animals given low dose vs. high dose MK801 indicates that the pattern observed for non-lactating females persists during lactation. It will be interesting to compare the effects of other doses of MK801 on FLI in the PVN of male and female rats to determine the dose-response curve for this drug.

Results from experiment 3.4 suggest that sex differences in MK801 sensitivity are more likely linked to male rather than female gonadal steroids. Gonadectomy significantly altered the FLI-response in the PVN of low-dose MK801-treated male rats. Levels of FLI detected in the PVN of gonadectomized males approached those observed in like-treated females (Figure 3.4.5). In contrast, neither gonadectomy nor androgenization significantly altered the number of FLI-positive nuclear profiles counted in the PVN of low-dose MK801-treated females (Figure 3.4.5). The apparent failure of TP replacement to reverse the effect of gonadectomy on FLI response to MK801 in gonadectomized males possibly reflects inadequate or poorly timed hormone replacement. Repeating this experiment using gonadectomized males implanted with appropriate dose time-release TP pellets will determine whether this is the case. The failure of androgenization (i.e., neonatal TP) to masculinize the FLI response to MK801 given in adult female rats again implicates testosterone in the hyporesponsiveness seen in intact males (Figure 3.4.5). As noted above, experiments using longer term replacement of testosterone in gonadectomized males and androgenized females will be necessary to prove this hypothesis.

The observation that at all doses tested, MK801-treated females displayed more extensive behavioural disruption compared to like-treated males indicates that females are more sensitive to the effects of this drug than are males. This is consistent with reports that estradiol potentiates neuronal excitability by modulating excitatory amino acid (including glutamatergic) receptor systems (see Smith 1994). The observed reversal of MK801 dose-response in females might reflect a direct or indirect action of MK801 on neurons in the PVN. The higher sensitivity of females (compared to males) to MK801 doses of 0.1 or 0.3 mg./kg., suggests that the excitatory threshold of PVN neurons to MK801 might be lower in females than in males. If this is the case, these neurons might become refractory when overstimulated by high dose MK801. Alternatively, injection of high dose MK801 might suppress PVN neural activity by activating (or disinhibiting) a population of neurons projecting inhibitory afferents to the PVN.

Both androgen receptors (AR) and estrogen receptors (ER) are widely distributed in the rat brain but, apart from the central part of the medial preoptic area (which is 4 times larger in males and contains more AR and ER), and the anteroventral periventricular nucleus of the preoptic area (which is 2 times larger in females), no gross sexual dimorphisms have been detected (Simerly et al. 1990). All other brain regions of male and female rats contain comparable densities of AR and ER. In the PVN, high concentrations of AR are found in the posterior magnocellular region (which does not display elevated levels of FLI following MK801 injection), and lower, but detectable, concentrations are found in the dorsal and lateral parvocellular regions, and ventral portion of the medial parvocellular region. The dorsal medial parvocellular region of the PVN, which contains a large concentration of CRH neurons (Swanson et al 1987) and exhibits elevated levels of FLI in MK801-treated animals, contains no AR (Simerly et al

(1990). Thus, if androgen does mediate the observed sex difference in MK801 sensitivity, it is unlikely to do so by acting directly upon these neurons.

AR have been identified in many brain areas which project to the PVN. These include regions of the brainstem (NTS, parabrachial nucleus, and LC), limbic system (SFO, BNST, HC, and amygdala), and hypothalamus (SCN, arcuate nucleus, and POA) (Swanson 1991; Simerly et al. 1990). While the influence of androgens on MK801 sensitivity might originate in any (or all) of these areas, previous reports that gonadal steroids influence MK801 binding in the HC suggest it has a role in mediating sex differences to this drug. Weiland (1992) reported that significantly higher levels of MK801 binding were detected in the dentate gyrus of gonadectomized female rats compared to estradiol-treated cohorts. Handa et al. (1994) reported significantly higher levels of MK801 binding in regions of the CA1 cell group of the HC in gonadectomized male rats compared to intact males. This increase was blocked by testosterone replacement. Taken together, the above reports suggest that MK801 receptor concentrations in regions of the HC are significantly influenced by both androgens and estrogens. Given that the HC exerts a major inhibitory influence upon PVN neurons (see Jacobson and Sapolsky 1991), it appears likely that the sex differences in MK801-induced FLI detected in the PVN in experiments in this chapter are related to gonadal steroid dependent differences in these hippocampal MK801 receptor concentrations. It will be useful to compare MK801 binding in the HC of gonadectomized/steroid replaced male vs. female rats, and to determine whether the effects of other NMDA antagonists (i.e., competitive and non-competitive) on FLI in the PVN resemble those induced by MK801. Note, however, that both Weiland (1992) and Handa's group (1994) have drawn conclusions about sex steroid-induced changes in NMDA receptor concentrations based on changes in MK801 receptor binding to the ion channel.

If these hormones act directly upon the MK801 receptor rather than the NMDA receptor protein, this might not be the case.

It will be interesting to determine whether sex differences in NMDA receptors (as measured by differences in MK801 binding) extend to other types of glutamate receptors. The observation that estradiol replacement does not effect the density of hippocampal kainate or AMPA receptors in gonadectomized female rats (Weiland 1992) suggests that the sex differences seen in NMDA receptors are not generalizable to all glutamate receptors.

The failure to detect a sex difference in MK801 sensitivity in prepubertal animals (Figure 3.2.2) provides further support for the contention that the sex differences observed in adult animals are mediated by gonadal hormones (which do not reach adult levels until after puberty). The lower numbers of FLI-positive nuclear profiles counted in the PVN of 14 day old rats compared to 25 day old rats might reflect the general stress-hyporesponsiveness which characterizes the second postnatal week. It will be interesting to study the ontogeny of high dose MK801-induced FLI to determine whether the effect seen on day 14 is characteristic of the neonatal SHRP.

The observation that lactating female rats exhibit a potentiated FLI response to low-dose MK801, and a depressed FLI response to high-dose MK801 injection (compared to non-lactating animals) suggests that sensitivity to MK801 is elevated during lactation. Given that lactating female rats exhibit depressed levels of circulating gonadal steroids (estrogen and progesterone), these results are consistent with the hypothesis that gonadal hormones are important for maintaining the observed sex differences in MK801 sensitivity. As noted in chapter 2 of this thesis, lactating female rats demonstrate marked hyporesponsiveness to a variety of stressors. The apparent heightened sensitivity to MK801 observed in lactating rats might result in activation of

inhibitory inputs to the PVN at a lower level in lactating rats compared to non-lactating females. If this proves to be the case, a similar mechanism might also account for depressed levels of FLI observed in the PVN of lactating rats following other stimuli.

In summary, the extent of behavioural disruption, and the density of FLI in the PVN, are influenced by the sex and hormonal state of the animal and the relative dose of MK801 injected. MK801 in doses as low as 0.1 mg./kg. induces behavioural perturbations and elevated levels of FLI in the PVN of adult rats. Dose response data suggest that females are more sensitive to the effects of MK801 than males, and that this difference is mediated by gonadal hormones (particularly androgens). The lack of a positive relationship between extent and duration of behavioural perturbation, and magnitude of FLI induced in the PVN suggests that sex differences in sensitivity to the effects of MK801 might also be found in brain regions other than the PVN. The observed reversal of MK801-induced FLI in the PVN of female rats suggest that brain areas with inhibitory input to the PVN might be implicated in mediating sex differences in sensitivity to MK801. Data from other authors suggest that the HC is a likely candidate.

SUMMARY

The goal of this thesis was to test the hypothesis that expression of the immediate early gene *c-fos* in the PVN is modulated by adrenal and gonadal steroids. A further objective was to determine whether *c-fos* immunocytochemistry could be used to isolate the influence of experimental variables from the effects of general stressors associated with experimental manipulations. The results of the time course study conducted in chapter 1 (experiment 1.1) established that the *c-fos* immunocytochemistry technique used in this thesis is sensitive to manipulations performed 30 minutes to 8 hours prior to sacrifice. On this basis, it is reasonable to assume that results are not coloured by stresses associated with sacrifice and perfusion. Nonetheless, stresses related to maintenance and handling of animals must still be considered as potential causes of variable or spurious results. For example, although animals used in these experiments were housed under controlled conditions of light, temperature, feed etc., on one occasion an experiment was interrupted by a loud fire alarm bell which continued to sound for approximately 30 minutes. This experimental session was abandoned but it illustrates the need for vigilance and stringent controls when studying stress-system activation.

The results of chapter 1 confirm that basal levels of FLI in the PVN of S-D rats are extremely low, and establish that they are transiently elevated following adrenal surgeries. The observation that the magnitude of the FLI response in the PVN is attenuated in animals with elevated circulating CORT levels (lactating animals and those pretreated with exogenous CORT), and potentiated in animals with depressed levels of circulating CORT (previously ADX animals and 3-11 day old pups) suggests that activation of these neurons is modulated by CORT

negative feedback inhibition. The observed strain differences between Lewis and S-D rats offers further support for this hypothesis. A possible explanation for the attenuated increase in FLI response observed in uADX rats also implicates elevated blood CORT (secreted from the remaining adrenal). Given that CORT negative feedback appears to exert a damping effect upon *c-fos* gene expression in the PVN, the failure to detect a circadian variation in FLI is somewhat puzzling. If circadian differences do exist the approach used in Chapter 1 lacks the sensitivity needed to detect them.

Although neonatal rats are reported to be stress-hyporesponsive, this definition is largely based upon the magnitude of the CORT and ACTH response to stress [although Walker et al. (1993) reported adult-like ACTH responses to ADX in 10 day old rats]. Data from experiments in this thesis suggest that this hyporesponsiveness does not extend to neurons of the PVN. In fact, these results indicate that in neonates, PVN neurons might be hyperresponsive to adrenal surgery. This conclusion is supported by the observation that the neonatal HPA axis is known to mount an adult-like response to some stressors (for example, histamine; Walker et al 1991), and a potentiated response to others (for example, excitatory amino acids; MacDonald et al. 1993). Data from Iny et al. (1987) suggest that ß-endorphin levels in these animals also respond to stress.

The variable results obtained from experiments conducted on animals with depressed circulating CORT levels i.e., neonatal animals and ADX (7 day) adults, make subjective evaluation of results extremely difficult. The results of these experiments demonstrate the inadequacies of non-quantified data.

In general, the results obtained from lactating female rats were relatively stable. Neurons in the PVN of lactating animals displayed a consistent blunted FLI response to adrenal surgery, and to chemical stimulation by Metrazole and NMA. Although lactating animals appear to represent an excellent stress-

hyporesponsive model, the high cost of these animals (approximately fifty dollars per animal) is likely to discourage all but the most generously funded researchers.

The observation that MK801 activates FLI in the PVN implicates glutamatergic pathways in the modulation of these neurons. Sex differences in behavioural responses and dose responses to MK801 suggest that sex steroids act upon these pathways, or directly upon the PVN neurons themselves. Although experiments with gonadectomized and androgenized animals failed to establish clear effects of gonadal steroids on FLI in the PVN, the trends detected to date suggest that repeating these experiments with a larger number of animals might prove fruitful. In these experiments, quantitative image analysis has proven an invaluable tool for unraveling the complicated interactions involved. A comparison of the samples of brain sections shown in Figures 3.4.3a and 3.4.3b to the summary data presented in Figure 3.4.5 provides an excellent illustration of this point.

An obvious question which is raised by the results presented in this thesis concerns the phenotype of the FLI-positive neurons detected in the PVN in various experiments. It is tempting to assume that these are CRH neurons, and given their distribution within the PVN, the possibility seems reasonable. In pilot studies, the distribution of CRH-like immunoreactivity detected in the PVN of ADX rats (results not shown) resembled the distribution of FLI seen in experiments presented in this thesis. Nonetheless, it will be useful to determine the identity of FLI-positive neurons by double labeling techniques. It will also be essential, given the inconsistencies encountered with different antibodies, to confirm the results of these experiments using in situ hybridization techniques. It is encouraging that, working in the laboratory in which experiments reported in this thesis were conducted, and using the same antibody, MacDonald et al.

(1993), reported good agreement between the distribution of NMDA-induced FLI detected by immunocytochemistry, and *c-fos* mRNA detected by in situ hybridization. More importantly, close agreement was also seen between the results of manual counts of FLI-positive nuclear profiles and optical density measurements of corresponding autoradiographs. Application of the quantitative techniques used in this thesis to in situ hybridization studies will further increase the value of this approach.

In a number of experiments, higher variation was observed in adult female animals compared to males (for example, see Figure 3.1.2). Given that males and females received identical treatment, it seems likely that some of this variability is related to variation in the hormonal state of cycling females. No attempt was made to identify the stage of the estrous cycle for these rats. In retrospect, it would have been useful to collect this information (although the procedures involved in collecting vaginal smears clearly stress the animal). Alternatively, it might have been preferable to use ovariectomized females implanted with estrogen/progesterone time-release pellets.

In summary, the results presented in this thesis demonstrate that *c-fos* immunocytochemistry, combined with computer assisted quantitative analysis represents a useful approach to examine the influence of adrenal and gonadal steroids on PVN function. Although the lactating rat appears to be a useful model for further research, the expense of conducting experiments using these animals might prove prohibitive. In light of this, the Lewis rat, or alternatively, the lactating mouse might prove more practical. Taken together, the data suggest that chemical probes such as those used in the last two chapters of this thesis might prove more useful than surgical interventions for examining the role of steroids in the modulation of PVN function.

TECHNICAL NOTE

The data presented in chapters 2 and 3 were obtained from quantitative image analysis of microscope slides. Similar techniques have been described previously (Brennan et al. 1992; Pepperkok et al. 1993; Mize 1994; Petrov et al. 1994). In the absence of quantified data, it is easy to subjectively select for inclusion in publications, sections which best illustrate the point at hand, rather than sections which are most representative of the group in question. In this thesis, representative sections were selected from animals with profile counts as close as possible to the mean of their treatment group, as calculated by quantitative analysis. This approach allows composition of figures which faithfully reflect both the nature, and the magnitude of the differences in question. Also, the use of computerized counting methods, has enabled me to make full use of all of the data collected to determine the statistical significance of subtle differences in FLI-induction.

It should be noted that computerized counting procedures such as the one introduced in this thesis, do not eliminate subjective judgment from the measurement process. At some point, whether FLI-positive nuclear profiles are counted manually or by computer, it is necessary to establish a threshold below which profiles are considered unlabelled. Although it is desirable to establish a threshold which yields totals which are in close agreement with the results of manual counts, reliable results are primarily dependent upon maintaining a consistent threshold throughout all analyses. In my experience, varying the threshold slightly affects the absolute number of profiles counted per section but, provided the threshold established when scrutinizing the initial section is maintained throughout, conclusions drawn from subsequent statistical analyses

are unaffected. Quantified results have been presented in terms of numbers of nuclear profiles (i.e., sections through FLI labeled cell nuclei) per animal. This number does not represent the number of FLI immunoreactive nuclei in the sampled tissue, but it provides an index by which relative densities of FLI may be estimated. An excellent technical description of techniques for using profile counts to estimate neuronal densities was published by Coggeshall (1992).

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