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Fos protein-like immunoreactivity in the hypothalamus: Normal development and the effects of cold ambient temperature and early eye opening in the preweanling rat

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City University of New York, 1993

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**FOS PROTEIN-LIKE IMMUNOREACTIVITY IN THE HYPOTHALAMUS: NORMAL
DEVELOPMENT AND THE EFFECTS OF COLD AMBIENT TEMPERATURE AND
EARLY EYE OPENING IN THE PREWEANLING RAT**

by

Myra P. Joyce

**A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the
requirements for the degree of Doctor of Philosophy, the City University of New York.**

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This manuscript has been read and accepted by the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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Abstract

Fos Protein-Like Immunoreactivity in the Hypothalamus: Normal Development and the Effects of Cold Ambient Temperature and Early Eye Opening in the Preweanling Rat

by

Myra P. Joyce

Advisor: Professor Gordon A. Barr

Hypothalamic nuclei mediate diverse physiological and behavioral activities. The cellular activity that mediates behavior occurs at specific times during development. This activity can be associated with changes in gene transcription, metabolic activity, or neuronal depolarization.

Visualization of Fos, the gene product of the *c-fos* proto-oncogene, by immunocytochemical methods was used in this study to examine hypothalamic activity. In the first experiment, the development of Fos in rat hypothalami was investigated. The results showed that Fos-positive labeling initially occurs at specific times during development in different nuclei. This suggests that neuronal activation or gene transcription for hormone synthesis, may be initiated at specific ages.

The second experiment examined the hypothalamic response to cold temperature during development. Rat pups were placed in a 15°C environment for one hour, then immediately sacrificed for Fos immunocytochemistry. Labeled nuclei were first seen in the preoptic area at 10 days and in the anterior hypothalamic nucleus at 21 days. At these ages, the drop in rectal temperatures of the animals after one hour in the cold were reduced. This suggests that these hypothalamic neurons are activated coincidentally with the appearance of a thermogenic response.

The third experiment examined the development of Fos-LI in the suprachiasmatic nuclei (SCN) and compared the appearance of Fos-LI within the SCN to the anatomical development of the nucleus. In addition, the GABA_B receptor agonist, baclofen, was administered to 14-day-old-rats to clarify the functional interactions within the nucleus. At 4-days-of-age Fos-LI labeled nuclei are first seen in the ventrolateral SCN. The labeling

increases and peaks by 10-days-of-age, coincidental with the completion of synaptogenesis. The nuclei in the ventrolateral portion of the caudal SCN are more intensely labeled than those in the dorsomedial region. By 60-days, the density and intensity of Fos-LI nuclei decrease substantially. Surgical eye-opening of 8-day-old pups had no effect on Fos-LI labeling. This suggests that the Fos-positive labeling observed in the ventrolateral SCN may be due to spontaneous activity of retinal ganglion cells. Baclofen administration had no effect on the activity of SCN neurons.

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TABLE OF CONTENTS

	Page
Title Page	i
Approval Page	ii
Abstract	iii
Acknowledgements	v
Table of Contents	vi
List of Tables	vii
List of Figures	viii
General Introduction	1
General Methods	6
Experiment 1 Introduction	7
Methods	13
Results	13
Discussion	34
Experiment 2 Introduction	41
Methods	44
Results	44
Discussion	59
Experiment 3 Introduction	62
Methods	65
Results	66
Discussion	81
General Discussion	84
References	90

LIST OF FIGURES

	Page
Figure 1. Photomicrographs of cresyl violet stained sections of the PVN, AH, arcuate nucleus, VMN, and DMN from a 60-day-old rat.	16
Figure 2. Photomicrographs of Fos-LI labeled sections of the preoptic-anterior hypothalamus of 3- through 60-day-old rats.	19
Figure 3. Photomicrographs of Fos-LI labeled sections of the paraventricular and anterior hypothalamic nuclei of rats 7- through 60-days-of-age.	21
Figure 4. High magnification photomicrographs of Fos-LI labeled sections of the anterior hypothalamic nucleus from 14- and 21-day-old rats.	24
Figure 5. Photomicrographs of Fos-LI labeled sections of the arcuate nucleus of rats 3- through 60-days-of-age.	26
Figure 6. Photomicrographs of Fos-LI labeled sections of the ventromedial nucleus of rats 10- through 61-days-of-age.	29
Figure 7. Photomicrographs of Fos-LI labeled sections of the dorsomedial nucleus of rats 7- through 21-days-of-age.	31
Figure 8. High magnification photomicrographs of Fos-LI labeled sections of the dorsomedial nucleus from 7- and 14-day-old rats.	33
Figure 9. Graph illustrating the average change in rectal temperature of developing rats after one hour in a 15°C environment.	46
Figure 10. Cresyl violet stained section of the hypothalamic sites examined in the cold stress study.	49
Figure 11. Photomicrographs of Fos-LI labeling of the preoptic-anterior hypothalamus of cold stressed and non-stressed control rats during development.	52

	Page
Figure 12. Photomicrographs of Fos-LI labeling of the paraventricular and anterior hypothalamic nuclei of cold stressed and non-stressed control rats during development.	55
Figure 13. Photomicrographs of Fos-LI labeling of the ventromedial nucleus of cold stressed and non-stressed control rats during development.	58
Figure 14. Cresyl violet stained sections of the supra-chiasmatic nuclei from an adult rat taken from rostral, intermediate and caudal levels.	68
Figure 15. Photomicrographs of Fos-LI labeled sections of the supra-chiasmatic nuclei from 3- and 4-day-old rat pups.	70
Figure 16. Photomicrographs of Fos-LI labeled sections of the supra-chiasmatic nuclei from 10-, 14-, and 60-day-old rats.	73
Figure 17. Photomicrographs of Fos-LI labeled sections of the supra-chiasmatic nuclei from 10-day-old rat pups.that underwent surgical eye-opening at 8-days-of-age.	75
Figure 18. Photomicrographs of Fos-LI labeled sections of the supra-chiasmatic nuclei from 10-day-old control rat pups in the eye-opening study.	77
Figure 19. Photomicrographs of Fos-LI labeled sections of the preoptic-anterior hypothalamus from 14-day-old control and baclofen treated rats.	80

General Introduction

The hypothalamus, located in the ventral diencephalon, is comprised of nuclei that are sensitive to internal and environmental stimuli. In Nissl stained tissue, several hypothalamic nuclei, including the paraventricular (PVN), suprachiasmatic (SCN), ventromedial (VMN), and the arcuate nuclei are readily discernible. These nuclei possess distinct boundaries and high cell densities. Other nuclei, such as the lateral preoptic area, lateral hypothalamus and posterior hypothalamus contain more diffuse cell populations. One role of the hypothalamus is perception of internal or environmental stimuli and initiation of physiological and/or behavioral responses. Another role is the mediation of cyclical activities such as reproductive behavior and diurnal rhythmic activities such as circadian temperature fluctuations, sleep-wake and activity cycles. Specialized cells synthesize and secrete hormones that mediate responses to changes in the environment and control diverse physiological and behavioral activities.

Specific nuclei in the hypothalamus have been shown to be sensitive to, and activated by specific types of stimuli, and are involved in mediation of responses to those stimuli. Quite often, more than one hypothalamic nucleus is involved in a single kind of regulatory process. For example, thermoregulatory behaviors are controlled by the efferent activities of the VMN (Holt, Wheal & York, 1987, Amir, 1990b), the preoptic area (POAH) (Carlisle & Laudenslager, 1979), the anterior hypothalamic nucleus (AH) and the posterior hypothalamic nucleus (Refinetti & Carlisle, 1986; Amir, 1990c). Similarly, reproductive behaviors are under the influence of the activity of several hypothalamic nuclei including the VMN (Kennedy & Mitra, 1963; Johnson, Coirini, Ball & McEwen, 1989) and the POAH (Clemens, Smalstig & Sawyer, 1976; Wiegand, Terasawa, Bridson & Goy, 1980).

The behaviors mediated by the hypothalamus are subject to developmental constraints. Specifically, the pathways interconnecting pertinent hypothalamic nuclei and effectors must be complete and functional, and the mechanisms controlling the synthesis

and secretion of the relevant hormones and neurotransmitters must be mature in order to detect the specific stimuli and elicit a response. For example, reproductive activity in rats does not begin until adolescence, and actually, distinct reproductive functions such as receptivity and the ability to reproduce, both hormone mediated events, emerge at different ages (see rev. art., McEwen, 1978).

Several nuclei within the hypothalamus are sexually dimorphic both anatomically and functionally. These sites include the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Simerly, Swanson and Gorski, 1984), the ventromedial nucleus (VMN) (Lenard, Sandor, Hajnal, Jando, Karadi and Kai, 1991; Romano, Mobbs, Lauber, Howells and Pfaff, 1990) and the arcuate nucleus (Argente, Chowen, Zeitler, Clifton and Steiner, 1991). In cresyl violet stained sections, the SDN-POA of male rats is readily discernible as a large, highly dense mass of cell bodies. This characteristic staining is absent in females although a much smaller, dense population of cell bodies can be seen at the same site. This site appears to mediate male reproductive activity since lesions of the SDN-POA abolish male copulatory behaviors (DeJonge, Louwerse, Ooms, Evers, Endert and Van de Poll, 1989).

This thesis examined several aspects of hypothalamic activity. First, an overview of the development of the activity of seven nuclei in the hypothalami of normal, untreated rats was examined, using the appearance of the Fos protein as a marker. The second experiment examined the development of the activity of hypothalamic neurons in response to cold environmental temperatures. The third study was an examination of the normal development of the activity of neurons in the suprachiasmatic nucleus (SCN) and the effects of early eye opening on the activity of SCN neurons. In addition, the GABA_B receptor agonist, baclofen, was administered to examine the intrinsic biochemical interactions between the ventrolateral and dorsomedial SCN. Neuronal activity was determined by the expression of the proto-oncogene *c-fos*, and the expression of the gene

was visualized by immunocytochemical methods using the *c-fos* protein product, Fos, as the antigen.

Fos Background

The proto-oncogene *c-fos* is the cellular homologue of the viral oncogene *v-fos* carried by the FBJ murine osteogenic sarcoma virus (Curran & Teich, 1982). The *c-fos* protein product, Fos, has a molecular weight of 62 kilodaltons (kD) after post-translational modification which involves phosphorylation at serine and threonine residues. The *c-fos* protein product undergoes considerably more post-translational modification than the 55kD *v-fos* product. (Curran, et al, 1982).

As a result of neuronal depolarization, the proto-oncogenes *c-fos* and related proto-oncogenes are transcribed. Fos, Fos-related antigens (Fra's) and the proto-oncoprotein Jun are synthesized in the cytoplasm and translocated to the nucleus, where they associate with a site on the chromatin called the activator protein-1 (AP-1) binding site. It has been suggested that this Fos-Jun-AP-1 complex acts as a promotor of transcription of cell-specific genes (Curran, Rauscher, Cohen & Franza, Jr., 1988). Immunocytochemical methods that permit visualization of the antigen by the binding of a chromagen reveal that the Fos protein is localized within the nucleus of the cell. Currently, many antibodies raised against sequences of the Fos protein recognize Fra's as well. Therefore, even though the notation in the text may read "Fos-like immunoreactivity (Fos-LI)", the implication is that there is some uncertainty regarding the exact identity of the antigen.

Since the late 1980's, a considerable body of literature has emerged on the use of Fos as a marker of cellular, or more specifically, neuronal activation. The early work that provided most of the information on the molecular biology of Fos was done with the neuronally differentiated pheochromocytoma cell line (PC12) (Sambuccetti & Curran, 1986; Morgan & Curran, 1986; Greenburg, Ziff, & Greene, 1986). It was shown that cellular stimulation leads to Ca²⁺ influx and the rapid induction of the *c-fos* gene as

measured by the appearance of *c-fos* messenger RNA (mRNA) (Morgan & Curran, 1986; Greenburg, et. al., 1986). The successful induction of this gene *in vitro* provided the impetus for studies examining the *in vivo* induction of *c-fos*.

The initial *in vivo* studies used induction of seizures as a stimulus for *c-fos* transcription (White & Gall, 1987; Dragunow & Robertson, 1988; Le Gal La Salle, 1988; Dragunow, Robertson & Robertson, 1988; Dragunow & Faull, 1989). These early studies provided valuable information on the time course of *c-fos* induction and the life-span of both the transcript and the Fos protein. These studies showed that concentrations of Fos increased significantly within 30 minutes of stimulation, with peak concentrations attained at two to four hours post-stimulation. In some sites, Fos remained above baseline levels 48 hours after termination of the stimulus. Another important finding was that Fos-induction is site-specific. For example, the severity and extent of seizure activity was predictive of the number and distribution of Fos-immunoreactively labeled nuclei. The larger the area involved in the seizure, the more widespread the Fos-labeling. In addition, partial seizures induced by amygdala kindling resulted in the labeling of only ipsilateral structures, whereas generalized stage 5 seizures resulted in bilateral labeling (Dragunow, et al, 1988).

It became apparent from these seizure studies that the *c-fos* gene could be induced by cellular activation *in vivo*. This body of work paved the way for examination of the effects of other types of stimuli on the induction of *c-fos*. Several studies have focused on the application of noxious peripheral stimuli to a limb and found Fos-LI in cell nuclei in the dorsal horn of the ipsilateral spinal cord (Bullitt, 1989; Menetrey, Gannon, Levine & Basbaum, 1989; Hunt, Pini & Evan, 1987), and in some midline thalamic structures contralateral to the site of stimulation (Bullitt, 1989). Local electrical stimulation of the motor and sensory cortices resulted in the appearance of Fos-LI at sites in which 2-¹⁴C-deoxyglucose accumulated (Sharp, Gonzalez, Sharp & Sagar, 1989). These data suggest that Fos is synthesized not only in neurons that receive direct stimulation, but also in neurons to which those stimulated neurons project.

In addition to direct physical stimulation resulting in the appearance of Fos-LI, several studies have shown that the administration of drugs and hormones similarly cause induction of *c-fos* (Dragunow & Faull, 1990; Gubits, Smith, Fairhurst & Yu, 1989; Chang, Squinto & Harlan, 1988; Robertson, Herrera, Dragunow & Robertson, 1989; Chang & Harlan, 1990; Hoffman, Lee, Attardi, Yann & Fitzsimmons, 1990; Insel, 1990). The drug-induced Fos-LI was observed in the sites known to possess receptor activity for those ligands. In summary, the evidence strongly indicates that neuronal depolarization caused by either externally applied stimuli or drug induced stimulation promotes induction of the *c-fos* gene, followed shortly thereafter by the appearance of the nuclear protein Fos.

Dragunow & Faull (1989) examined the potential usefulness of Fos immunocytochemistry to trace pathways and noted that projection sites distant from the original site of stimulation showed Fos-LI. That study also noted some disadvantages in the use of Fos as a marker of cellular activation and pathways. For example, some mismatches between Fos labeling and 2-deoxyglucose uptake in response to the same stimulus (in this case, pentylentetrazol induced seizures) occurred. In addition, neurons in certain regions did not show an increase in Fos-LI no matter what stimulus was applied, and some sites contained basal levels of Fos-LI that were difficult to distinguish from increased levels due to activation. With these caveats in mind, the investigator must proceed carefully and with a critical eye on the data.

The studies in this thesis are unique in that they use the appearance of Fos-LI labeled nuclei to examine neuronal activity during development. This uniqueness is especially true in the first experiment which examines the baseline appearance of the protein during development in the absence of any applied environmental stimuli. The results of these studies will provide new information on the fluctuations of Fos-LI during development and clarify the role of the proto-oncogene in gene expression and neuronal activation. In addition, a unique perspective on the development of neuronal activity will be provided.

General Methods

Subjects: Since there are biochemical and anatomical differences in the hypothalamus between male and female rats, only male Long-Evans hooded rats pups (*Rattus norvegicus*), born and raised in the Hunter College animal facility served as subjects. Female rats were not used because fluctuations of activity due to estrous cycles would have distorted developmental activity fluctuations. Births were checked for twice daily and day of birth was counted as zero days of age. At 21-days-of-age pups were separated from the dam and sire and housed with littermates in identical plastic tubs. The ambient temperature in the rat colony was maintained at 23°C and the rats were on a 12hr/12hr light/dark cycle with lights on at 7AM. Each rat tub measured 43.5 x 23 x 21 cm and housed one mating pair plus any offspring. Food (Purina Rat Chow) and water were continuously available.

General Procedures for Immunocytochemistry

The animals were sacrificed with an overdose of sodium pentobarbital (0.15 g/kg body weight) IP. They were then perfused transcardially with 0.1M phosphate buffered saline (PBS), pH 7.2 followed by 4% buffered paraformaldehyde, pH 7.2. The brains were carefully removed and placed in fixative for 24 hours and then in 30% sucrose for 48 to 72 hours. After complete infiltration of the brain with 30% sucrose, a block of the brain containing the hypothalamus was sectioned on a cryostat (Hacker Instruments, Fairfield, NJ). Thirty micron frozen sections were collected in 0.1 M PBS, pH 7.2. Three sections were collected per tube, and every fourth section was placed on a microscope slide for cresyl violet staining.

Immunocytochemistry was done with the avidin-biotin HRP method using the Vectastain Elite ABC Kit (Vector Labs, Burlingame CA). All normal serum and antibody dilutions and washes were done with 0.1 M PBS, pH 7.2. The tissue was first incubated for 3 minutes in 3% H₂O₂ to quench endogenous peroxidase activity. It was then washed

twice and incubated for 30 minutes in 10% normal goat serum. The tissue was then incubated overnight at 4°C on a shaker with the primary antibody, rabbit-anti-Fos (Oncogene Sciences, Manhasset, NY) diluted 1:10,000 in PBS with 2% BSA. The primary antibody was an affinity purified polyclonal antibody raised against residues 4 to 17 of human Fos. The tissue was then washed twice and incubated with the biotinylated antibody for 45 minutes. After one wash, the tissue was incubated with the ABC reagent for 30 minutes. The tissue was washed once and incubated for 2 to 5 minutes in the 2,2'-diaminobenzidine-H₂O₂ solution. It was then washed with distilled water and placed on chrom-alum coated microscope slides. After drying thoroughly, the sections were dehydrated through a graded series of ethanol concentrations and xylenes and coverslipped. The sections were examined by light microscopy.

Immunocytochemistry controls were done by omitting the primary antibody from the procedure in some assays and by preadsorbing the primary antibody to the peptide against which it was raised in other assays.

Experiment 1: The Normal Development of Fos Labeling in the Hypothalamus

Neurogenesis in the hypothalamus of rats is nearly complete by the time of birth although postnatal cell proliferation occurs in certain nuclei through 10 days of age (Seress, 1985). While the majority of cells undergoing postnatal mitotic activity are glial cells, neuronal cell division in the hypothalamus is complete prenatally (Ifft, 1972; Altman & Bayer, 1986). The anatomical and biochemical development of the specific hypothalamic nuclei that were examined in this study will be discussed separately.

Preoptic-Anterior Hypothalamus

The preoptic-anterior hypothalamus (POAH) is located partly in the forebrain and extends into the rostral hypothalamus, at the level of the anterior commissure. The POAH defines an area comprised of several distinct nuclei separated by diffuse cell populations.

This site has been intensely studied with respect to its sexual dimorphism (Gorski, Harlan, Jacobson, Shryne and Southam, 1980; Simerly, et. al., 1984; Bleier, Byne, and Siggelkow, 1982). The medial preoptic area contains a bilateral site that is distinctly different in males as compared with females when stained with Nissl stains. This area is called the sexually dimorphic nucleus of the preoptic area (SDN-POA). In both males and females, it contains a greater cell density than the surrounding area, but in males, the volume of increased cell density is larger than in females. Functionally, this site is important for reproductive behavior. Neuronal mitogenesis in the POAH is complete before birth, but synaptogenesis occurs postnatally, largely between 5- and 25-days-of-age (Lawrence and Raisman, 1980). The SDN-POA develops its characteristic sexual dimorphism during the first 10 days of postnatal life (Jacobson, Shryne, Shapiro & Gorski, 1980). Administration of the male hormone, testosterone, during a critical period (postnatal days 2 - 5) , increases the volume of the SDN-POA in castrated male rats and intact females (Rhees, Shryne & Gorski, 1990; Dohler, Coquelin, Davis, Hines, Shryne, & Gorski, 1984).

Other activities that are mediated by POAH neurons include vasoconstriction in response to hemorrhagic shock (Schlaumloffel, Pugh & Bealer, 1990) and thermoregulatory responses (Briese, 1989; Feldberg & Myers, 1965; Lipton, 1968).

Paraventricular Nuclei

The paraventricular nucleus (PVN) is one of the more anatomically complex nuclei of the hypothalamus. It is comprised of distinct subnuclei containing both large (magnocellular) and small cells (parvocellular), as well as distinct distributions of neurochemicals among those cell types (Hatton, Hutton, Hoblitzell & Armstrong, 1976; Armstrong, Warach, Hatton, & McNeill, 1980).

The parvocellular portion of the PVN contains corticotropin releasing factor-immunoreactivity (CRF-IR) (Rho & Swanson, 1987; Jacobson, Sharp & Dallman, 1990)

and thyrotropin releasing hormone-IR (TRH-IR) (Lechan & Jackson, 1982). Cells of the magnocellular divisions are immunoreactive for either oxytocin or vasopressin. Cells containing oxytocin are located in the rostral PVN. In the middle portion of the nucleus, oxytocin and vasopressin cells are mixed, and in the caudal PVN, oxytocin cells predominate (Hou-Yu, Lamme, Zimmerman & Silverman, 1986). This result contrasts with an earlier study reporting that vasopressin neurons were more numerous in the caudal PVN (Swaab, Nijveldt, & Pool, 1975).

Neuronal and secretory cells of the PVN are responsive to specific physiological and environmental challenges. For example, osmotic challenge, induced by the administration of hyperosmotic solutions, results in increased oxytocin mRNA content in magnocellular neurons of the PVN (Van Tol, Voorhuis & Burbach, 1987) as well as increased Fos-IR nuclei in those sites (Ceccatelli, Villar, Goldstein & Hokfelt, 1989; Giovanelli, Shiromani, Jirikowski, & Bloom, 1990). Administration of the peptide, cholecystokinin, known to be important in satiety (Antin, Gibbs, Holt, Young, & Smith, 1975; Crawley & Kiss, 1985; Gibbs, Falasco, & McHugh, 1976; Shillabeer & Davison, 1987) dose-dependently stimulates Fos induction in the PVN, specifically in magnocellular oxytocinergic neurons and parvocellular CRH neurons (Verbalis, Stricker, Robinson, & Hoffman, 1991)

Cells of the PVN are especially sensitive to environmental stressors. As an animal matures, behavioral and physiological responses to stress change, and stimuli stressful to a neonate may not be similarly stressful to an adult. It would, therefore, not be unexpected to see changes in Fos-LI in the PVN during development as the various stress response systems mature and become more or less sensitive to the environment.

Anterior Hypothalamic Nuclei

The anterior hypothalamic nucleus (AH) is a moderately darkly staining collection of cells located ventral to the PVN. Nissl stained tissue shows that the AH is comprised of

a relatively homogeneous population of neurons. Literature on this nucleus is scarce and, therefore, precious little is known about its postnatal development, function and biochemistry.

Afferents of the AH relay visceral, gustatory, and olfactory information and efferents project to the lateral septum and the periaqueductal gray (Swanson, 1987). Local thermal stimulation of the AH activates both autonomic and behavioral thermoregulatory responses (Refinetti & Carlisle, 1986).

Arcuate Nucleus

The arcuate nucleus is located along the ventral midline of the hypothalamus adjacent to the third ventricle. It is bounded rostrally by the retrochiasmatic area and extends caudally to the mammillary nuclei (Paxinos & Watson, 1986). The major secretory factor found in neurons of the arcuate is growth hormone releasing hormone (GHRH) (Sawchenko, Swanson, Rivier & Vale, 1985; Daikoku, Kawano, Noguchi, Nakanishi, Tokuzen, Chihara & Nagatsu, 1986; Horvath & Palkovits, 1988; Argente, et. al., 1991; Rodier, Kates, White & Phelps, 1990). The GHRH cells project to the median eminence and mediate pituitary growth hormone secretion (Merchenthaler, Vigh, Schally & Petrusz, 1984; Wehrenberg, 1986). Cell proliferation of arcuate GHRH neurons peaks on the 14th day of gestation and is complete by the 16th gestational day (Rodier, et al, 1990). Hypothalamic GHRH, measured by RIA, is first detectable on fetal day 18 (Jansson, Ishikawa, Katakami & Frohman, 1987) and GHRH-IR fibers are visible along the ventral surface of the hypothalamus at that time (Ishikawa, Katakami, Jansson, & Frohman, 1986). GHRH immunoreactive perikarya are not seen with immunocytochemical methods until postnatal day 1 in the arcuate nucleus (Ishikawa, et al, 1986; DeGennaro, Redaelli, Locatelli, Cella, Fumagalli, Wehrenberg, & Muller, 1986). These immunoreactive cell bodies were visualized in colchicine treated rats. One of the functions of colchicine is to prevent axonal transport. Prenatal rats did not receive colchicine treatment which may

explain why GHRH-IR was not observed prior to postnatal day 1. In the one-day-old, the number of GHRH-immunoreactive perikarya in the arcuate nucleus is similar to that of the adult, although the size of cell bodies through 20-days-of-age is smaller than that seen in the adult. The intensity of the reaction product of GHRH-immunocytochemistry increases by 10-days-of-age and remains the same through 20 days. Levels of GHRH synthesis, as measured by mRNA concentrations, continue to rise postnatally and attain peak concentrations by 75 days of age.

Other neurochemicals found in neurons of the arcuate nucleus include proopiomelanocortin (Knigge, Joseph, & Nocton, 1981; Kiss and Williams, 1983; Chowen-Breed, Clifton, & Steiner, 1989; Csiffary, Gores & Palkovits, 1990), prolactin (Harlan, Shivers, Fox, Kaplove, Schacter & Pfaff, 1989; Harlan & Schammel, 1991; Shivers, Harlan & Pfaff, 1989), and leuteinizing hormone releasing hormone (LHRH) although there is some disagreement over the presence of LHRH synthesizing cells in the arcuate (Palkovits, Arimura, Brownstein, Schally, & Saavedra, 1974; Witkin, Paden, & Silverman, 1982; Levine & Duffy, 1988; Donoso, Lopez, & Negro-Vilar, 1990).

Ventromedial Nuclei

The VMN is an oval shaped nucleus located in the ventral part of the caudal hypothalamus. This nucleus has been shown to mediate various activities including peripheral sympathetic activation (Saito, Minokoshi & Shimazu, 1989), running (Yokawa, Mitsushima, Itoh, Konishi, Shiota & Takahashi, 1989; Yokawa, Shiota & Takahashi, 1990), feeding and reproductive behaviors (Kennedy & Mitra, 1963). The VMN is functionally sexually dimorphic. For example, kainic acid lesions of the VMN, which destroy cell bodies while leaving fibers of passage intact, cause hyperphagia and weight increases only in female rats (Lenard, et al, 1991). Similarly, estrogen administration results in significant increases in proenkephalin mRNA levels in the ventrolateral VMN in female but not male rats (Romano, et al, 1990).

Many of the behaviors mediated by VMN secretions and efferent activity, such as reproductive activity and thermoregulation, emerge at different stages during development. Therefore, it is not unusual to observe changes in transcriptional activity as the animal matures. Since Fos has been implicated as a promoter of gene transcription, the number and distribution of Fos-like immunoreactive nuclei may change during the periods in which hormonal gene transcription is occurring.

Dorsomedial Nuclei

The dorsomedial nucleus (DMN) is located dorsal to the VMN in the mid to caudal hypothalamus. Morphologically, three distinct subsections can be discerned in Nissl stained tissue. These subsections are distinguished by dense masses of cell bodies separated by regions of low cell density. These subdivisions are particularly evident in the rostral part of the DMN. Specifically, a dorsomedial, ventrolateral, and an intervening band of dense populations of cell bodies are discernable.

Little is known about the development and functions of the DMN except that it is involved in ingestive behavior and weight regulation (Bellinger, Bernardis, & Brooks, 1979). Thyrotropin releasing hormone immunoreactivity has been observed in the dorsal portion of the DMN (Cintra, Fuxe, Wikstrom, Visser, & Gustafsson, 1990), suggesting a role in mediation of metabolic rate and energy balance. Growth hormone releasing hormone neurons have been found scattered throughout the DMN, but lesions of the nucleus do not appear to affect diurnal fluctuations of circulating growth hormone levels (Byrd & Bellinger, 1989).

The normal development experiment was conducted in order to describe the development of neuronal activity in the hypothalamus of normal, untreated rats. The results of this study provided a description of baseline Fos-LI labeling and provided a background upon which to describe the stimulated Fos-LI labeling observed in subsequent experiments.

Subjects and Procedure

Since the hypothalami of males and females differ developmentally, anatomically, biochemically, and functionally, only male rats were used and described in this study. In addition, the fluctuating hypothalamic activity of female rats due to estrous cycles would unnecessarily complicate the results and obscure the goals of this experiment. A total of 53 male rats, ages 3, 4, 7, 10, 14, 21, 28, 35, and 60 days, 6 rats in each of the 3 through 35-day-old age groups, and five 60-day-old-rats were used. Rats were transported from the Hunter College animal facility on the 15th floor to the laboratory on the 6th floor. Rat pups, 21 days and younger, were transported in groups of two to four pups in plastic tubs containing fresh bedding. Rats, 28 days and older had been separated from the dam and sire at 21 days and were transported to the laboratory in their home cages. These older rats were housed one or two to a cage. Upon arrival in the laboratory from the breeding colony, the rats, both pups and adults, were kept in a thermoneutral and quiet environment and were undisturbed until removed for perfusion. The rats were sacrificed between 11:30AM and 12:30PM. Each perfusion took approximately 15 to 20 minutes, therefore, if four pups were being perfused in one session, the last pup was separated from the dam and sire for approximately one hour and isolated for at least fifteen minutes.

Results

Preadsorption of the primary antibody to the 12 amino acid portion of the Fos protein abolished all nuclear labeling but caused high background. Omission of the primary antibody from the immunocytochemistry procedure similarly resulted in the absence of nuclear labeling, although the background labeling was extremely light.

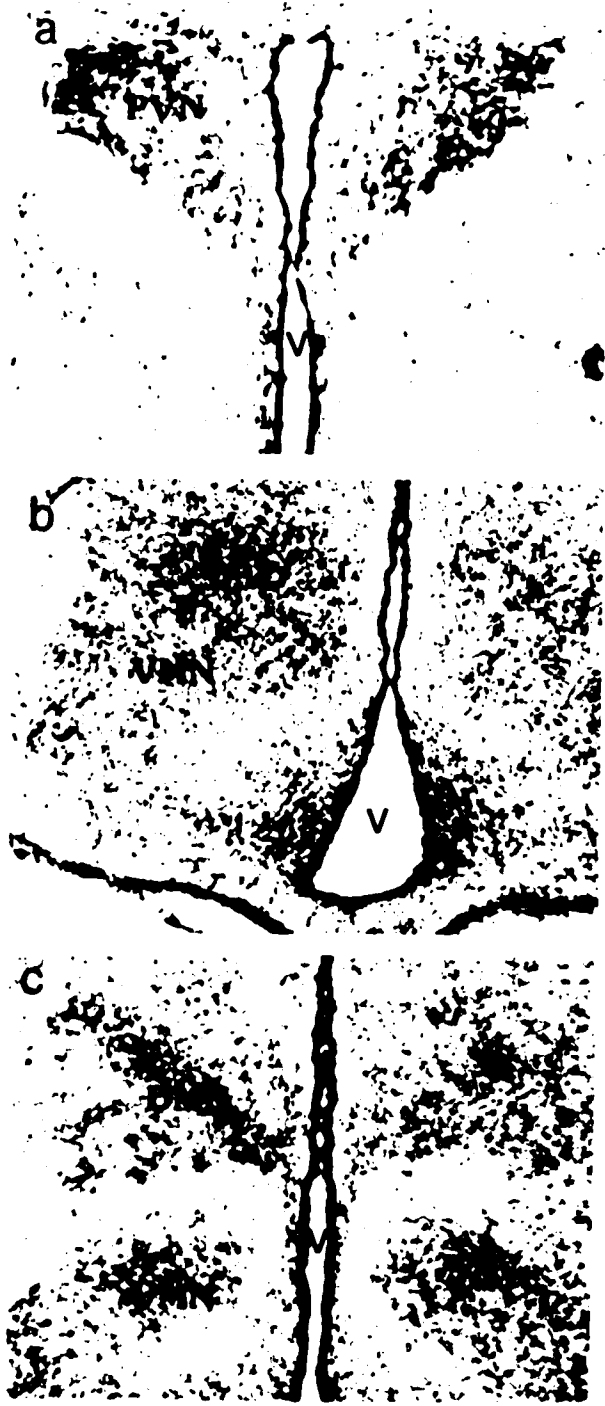
The quantitation of Fos-LI labeling between age groups in the hypothalamic nuclei examined are described in terms of the relative density of labeled nuclei in each hypothalamic site. Often, the changes in density between ages were visually apparent, precluding the necessity of counting labeled nuclei.

Individual differences in Fos protein-like immunoreactive labeling were noted among rats at all ages. One or two pups from each group displayed atypically high or atypically low density of labeling throughout the hypothalamus. The abundance or scarcity of labeled nuclei was usually consistent in a particular animal such that if a rat had low densities of labeled nuclei, that was the case for that rat in several of the hypothalamic sites examined. In some of the hypothalamic nuclei examined in this study, the density of labeled nuclei among siblings of the same age was quite consistent. This was particularly evident in the POAH, VMN, and DMN. In other sites, such as the arcuate nucleus, and slightly less so in the VMN, the density and distribution of labeled nuclei were quite consistent among all animals of the same age group, regardless of familial relationship. The Fos-LI labeling among animals of the same age in the PVN was, in general, less consistent. In this site, the relative density of Fos-positive nuclei showed an order effect among animals of the same age. Four animals were sacrificed in one session that lasted approximately one and one half hours. All four animals were from the same litter. The first two rats to be anesthetized and perfused had low densities of Fos-LI labeled nuclei in the PVN, whereas the last two had slightly higher densities of Fos-LI nuclei.

The development of the appearance of Fos-LI was examined in detail in seven nuclei of the hypothalamus. The changes in nuclear labeling observed at each of these sites during development will be discussed in the following section and Fos-LI labeling of the suprachiasmatic nuclei, will be addressed in the Results section of Experiment 3. Table 1 shows the relative densities of Fos-LI nuclei for each of the hypothalamic nuclei examined at each age. Figure 1, a-c, shows photomicrographs of cresyl violet stained sections from an adult rat. The sections depict five of the hypothalamic sites examined. A photomicrograph of a cresyl violet stained section of the POAH is shown in Figure 10.

Preoptic-Anterior Hypothalamus. Only two sites in the POAH showed consistent nuclear labeling. These are the median preoptic area (MnPOA) and the medial preoptic nuclei

Figure 1. Photomicrographs of cresyl violet stained sections from the hypothalamus of an adult rat. The figure is arranged in a rostral (a), intermediate (b), to caudal (c) direction. Abbreviations: PVN=paraventricular nucleus, VMN=ventromedial nucleus, Ar=arcuate nucleus, DMN=dorsomedial nucleus, V=third ventricle. All magnification bars throughout this thesis = 200 μm .



(POM). Figure 2, a-d show the Fos-LI labeling in the POM and MnPOA of 3-, 10-, 21-, and 60-day-old rats. In pups as young as 3-days-of-age, moderate densities of Fos-positive labeled nuclei per section were present in the MnPOA. The numbers of labeled nuclei in the MnPOA did not vary substantially as the animal matured, although relatively higher densities of Fos-LI labeled nuclei were reached at 10-days-of-age, at which time several rats had very high densities at this site. After 14 days, the density of Fos-LI nuclei in the MnPOA fell to moderate levels.

The POM similarly contained varying densities of Fos-LI labeled nuclei at all ages. At 7-days-of-age, no Fos-positive nuclei were observed in the POM. Low to moderate densities of positively labeled nuclei were in the POM at 3- and, 21-, and 28-days-of-age. At 14- and 35-days-of-age, two brains in each group had slightly higher densities of Fos-positive labeled nuclei, whereas the rest had only sparsely distributed Fos-positive nuclei. In the 60-day-old rats, the numbers of labeled nuclei declined and the densities were low to moderate.

Paraventricular Nucleus. Figure 3, a -d are photomicrographs of Fos-LI labeled sections of the PVN of 7, 21, 36, and 61 day old rats. In the 3-day-old rat pups, Fos-LI labeled nuclei were rarely observed in the PVN. In the rats 7-days-of-age and older, Fos-LI labeled nuclei were distributed among the lateral magnocellular, ventral and medial parvocellular subnuclei. The changes in Fos-LI labeling observed during development will be discussed separately for each of the paraventricular subnuclei.

Lateral Magnocellular PVN. The density of Fos-LI nuclei remained quite consistent throughout development. At every age examined, there were only low to moderate densities of labeled nuclei in the lateral magnocellular PVN. No significant changes were observed in this site during development.

Ventral PVN. With the exception of the three-day-old pups in which labeled nuclei were rarely observed, nearly all of the rats through 10-days-of-age had low densities of

Figure 2. This figure shows Fos-LI labeled sections of the preoptic-anterior hypothalamus from a) 3-day-old, b) 10-day-old, c) 21-day-old, and d) 60-day-old rats. Note the presence of Fos-positively labeled nuclei in the medial preoptic area (POM) in the 3-day-old. This labeling density did not change significantly during development. In the median preoptic area (MnPOA) the Fos-LI labeling is sparse except in the 10-day-old. At 10 days of age, the Fos-LI labeling density in the MnPOA peaked, and then returned to low levels after 14 days of age.

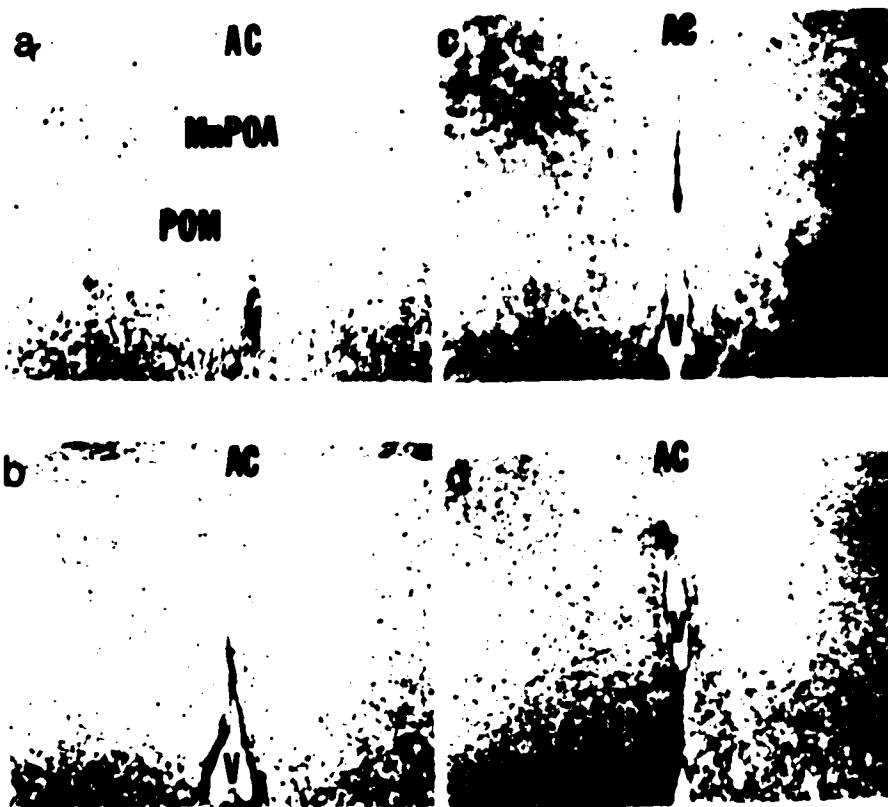


Figure 3. Photomicrographs of Fos-LI labeled sections of the PVN from a) 7-day-old, b) 21-day-old, c) 36-day-old, and d) 60-day-old rats. Most of the labeling observed at each age is in the ventral PVN. Medial parvocellular labeling can be seen in sections from the 21- and 36-day-old rats.



b



d



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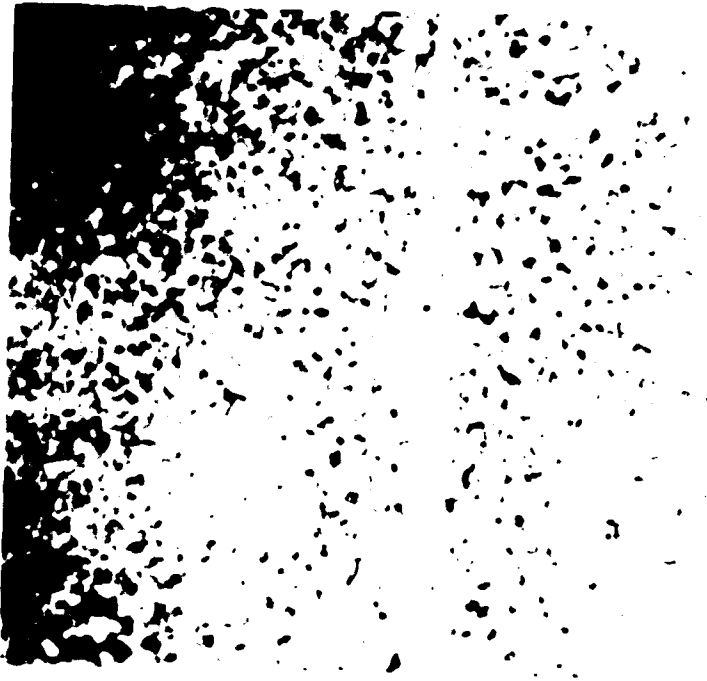
Fos-LI nuclei in the ventral PVN. By 14-days-of-age, the density of Fos-LI increased. Of the five 14-day-old rats examined, one had a high density of Fos-LI nuclei and the other four had low to moderate densities. By 21-days-of-age, moderate densities of Fos-LI were seen in this subnucleus and no additional changes were observed in the older animals.

Medial Parvocellular PVN. With the exception of the three-day-olds and one 7-day-old pup, all of the animals examined at all ages had Fos-positive nuclei in this site. The densities of nuclei, however, were not consistent among rats of the same age, and between different age groups through 14-days-of-age. These fluctuations ranged from low to high densities. By 21-days-of-age, high densities of Fos-LI nuclei were observed consistently in the medial parvocellular PVN and only very minor changes were seen through 35-days-of-age. By 60-days-of-age, the density of Fos-LI labeled nuclei in the medial parvocellular PVN decreased considerably.

Anterior Hypothalamic Nucleus. Figure 4, a and b shows photomicrographs of Fos-LI labeled sections of the AH from 14- and 21-day-old rats. Fos-LI labeled nuclei were rarely observed in the AH in animals 14-days-of-age and younger. Of the 19 rats examined between 3- and 14-days-of-age, only 3 (one 3-day-old, one 10-day-old, and one 14-day-old) had labeled nuclei in the AH and these were few in number. Rats 21-days-of-age and older consistently had moderate to high densities of Fos-LI nuclei present, and the density of labeling remained stable through 60 days. The labeled nuclei were not restricted to any particular portion of the AH and were usually distributed throughout the nucleus.

Arcuate Nucleus. Figure 5, a-c, shows photomicrographs of Fos-LI labeled sections from the arcuate nucleus from 21-, 36-, and 60-day-old rats. Numerous Fos-LI labeled nuclei were observed at all ages throughout the rostro-caudal extent of the arcuate nucleus. Rostrally, most of the labeled nuclei were located in the ventral part of the nucleus. More

Figure 4. High magnification photomicrographs of sections of Fos-LI labeled tissue containing the anterior hypothalamus from a) 14-day-old and b) 21-day-old rats. Prior to 21 days of age, Fos-LI labeled nuclei were rarely observed in the AH. Rats 21 days of age and older consistently had Fos-LI labeled nuclei in the AH.



b



Figure 5. Photomicrographs of Fos-LI labeled sections of the arcuate nucleus from a) 21-day-old, b) 36-day-old, and c) 60-day-old rats. Positively labeled nuclei were observed in the arcuate nucleus at all ages. Peak densities were seen between 21 and 36 days of age. After 36 days, the density of Fos-LI nuclei decreased to moderate levels. At those ages some sections contained extremely high densities of Fos-positive nuclei. In the 60-day-old rats, the density of Fos-positive nuclei decreased to moderate levels.



caudally, the Fos-positive nuclei were found in the lateral part. Each section contained moderately high densities of labeled nuclei except at the ages of 21-, 28-, and 36-days-of-

Ventromedial Nucleus. Figure 6, a-d, shows photomicrographs of Fos-LI labeled sections from the VMN of rats 10, 14, 29, and 61 days of age. The VMN contained considerably fewer Fos-LI labeled nuclei compared to the rest of the hypothalamus. In the rostral part of the VMN, Fos-positive nuclei were nearly totally absent. Occasionally, low densities of labeled nuclei were seen in the caudal VMN, often in the ventral or ventrolateral part of the nucleus. A substantial increase in the number of labeled nuclei was observed at 14- and 21-days-of-age. In the 14- and 21-day-old rats, moderate densities of Fos-LI labeled nuclei were seen in several sections. By 28-days-of-age, the maximum labeling decreased, with some brains containing no labeled nuclei at all. At 35- and 60-days-of-age, the distribution of labeling was similar to that of the 28-day-old animals, except that low densities of Fos-LI nuclei were observed in a section.

Dorsomedial Nucleus. The labeling of Fos-LI nuclei in the DMN showed large individual differences among subjects at all ages (Figures 7 and 8). Some rats had relatively few labeled nuclei throughout the DMN, whereas other animals of the same age had abundant labeled nuclei throughout the rostro-caudal extent of the nucleus. For example, at 7-days-of-age, two pups had no Fos-LI labeled nuclei in the DMN, and the other two pups of the same age had very high densities of labeled nuclei in the DMN. Similarly, at 60-days-of-age, one brain had moderate densities of labeled nuclei per section, two had very high densities, and two had virtually no labeled nuclei in the DMN. With this large variability among subjects, it is very difficult to determine a developmental trend in Fos-LI labeling in the DMN.

Figure 6. Photomicrographs of Fos-LI labeled sections of the ventromedial nucleus from a) 10-day-old, b) 14-day-old, c) 29-day-old rats and d) 61-day-old rats. Note the increase in labeled nuclei in the 14-day-old rat.



Figure 7. Photomicrographs of Fos-LI labeled sections of the dorsomedial nucleus from a) 7-day-old, b) 14-day-old, and c) 21-day-old rats. The most abundant labeling was seen at 7 days of age. After 7 days, the density of Fos-LI labeled nuclei decreased to low to moderate densities.

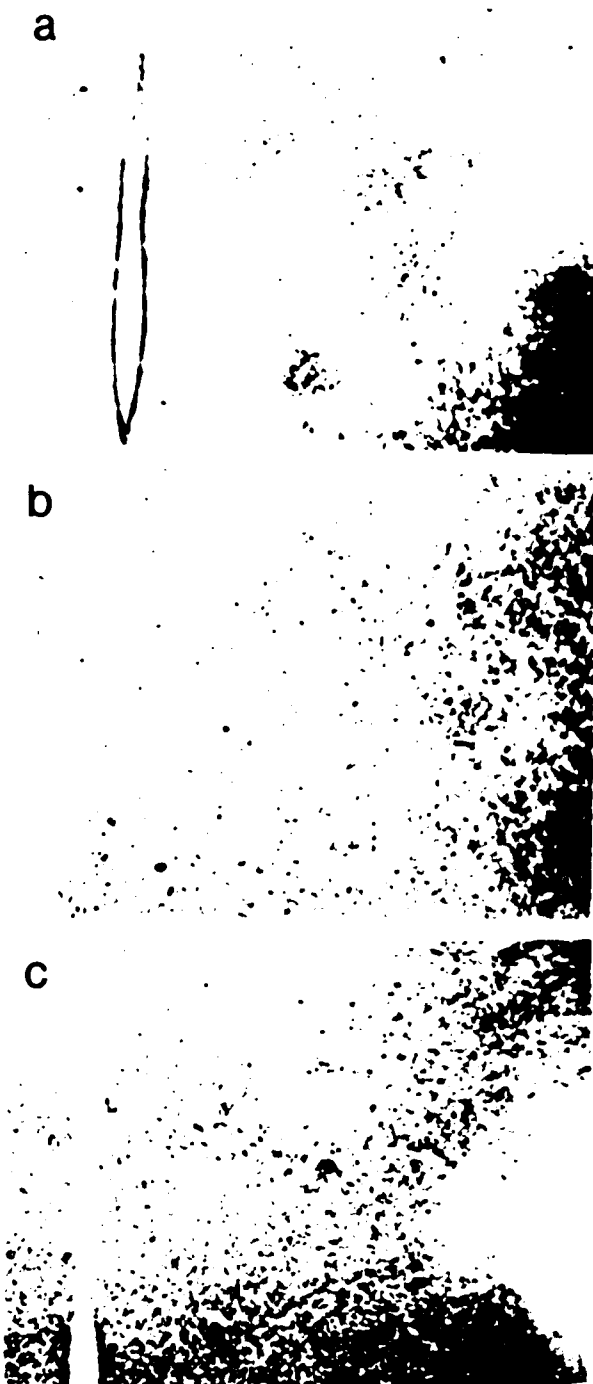


Figure 8. High magnification photomicrographs of Fos-LI labeled sections of the dorsomedial nucleus from a) 7-day-old, and b) 14-day-old rats. Note that in the 7-day-old pup, the high densities of Fos-LI labeling are confined to the subnuclei of the DMN. By 14 days, this labeling pattern is not observed and the Fos-LI labeled nuclei are sparsely scattered throughout the DMN.



Discussion

The densities and the intensities of Fos-LI labeled nuclei changed during development in all of the hypothalamic subnuclei examined. In most cases, during the neonatal period, very few Fos-positive nuclei were present in the hypothalamus. As the animal matured, between 7- and 28-days-of-age, the number of labeled nuclei steadily increased. In most sites, Fos-LI labeling peaked between 21- and 28-days-of-age. After 28-days-of-age, the density of Fos-LI labeling decreased. This age dependent pattern of increasing and then decreasing densities of nuclei was particularly evident in the POAH, the arcuate, VMN, and DMN.

The reasons for the appearance of Fos-LI nuclei in different hypothalamic sites at different times during development are not completely clear. The *c-fos* proto-oncogene is expressed under various conditions that include environmental challenges (Koistanaho & Yang, 1990; Bullitt, 1989) as well as drug and chemical administration (Chang, et. al., 1988; Insel, 1990; Hoffman, et. al., 1990; Dragunow & Faull, 1990). In addition, the presence of nerve growth factor (NGF) in the medium bathing PC12 cells not only causes the cells to differentiate into sympathetic neurons, but also leads to increased *c-fos* mRNA levels (Greenberg, Greene, & Ziff, 1985). This finding suggests that Fos has a role in neuronal differentiation. During early postnatal development, synaptogenesis and cell growth are occurring in many hypothalamic sites (Laemle, Repke, Hawkes, & Rice, 1991; Lawrence & Raisman, 1980; Moore & Bernstein, 1989). The fluctuating numbers of positively labeled nuclei observed during development may reflect the anatomical maturation of the hypothalamus. Fos may be involved in promoting the transcription of genes for cytoskeletal proteins. An AP-1 recognition sequence has been found upstream from the gene for the promotor of the β -amyloid precursor protein gene (Beyreuther, Weidemann, Salbaum, Dyrks, Multhaup, Fischer, Hilbich, Konig, Beer, Bunke, Monning, Simms, Martins, Rumble, & Masters, 1988). The β -amyloid precursor protein

is a cytoskeletal component that, when overproduced, is implicated as a contributing factor in the neuronal degeneration observed in Alzheimer's disease. Under normal developmental conditions, a growing cytoskeletal matrix is required for both cell growth and axonal elongation (Bhattacharya, Saha, & Sarkar, 1991; Bhattacharya, & Sarkar, 1991; Lazarini, Deslys, & Dormont, 1991).

Several studies have shown that *c-fos* induction by conditions such as seizure activity (White & Gall, 1987) or injection of an inflammatory substance into a limb (Draisci & Iadarola, 1989) is followed closely in time by the appearance of cell specific proteins, for example, enkephalin, following seizure activity. It has become clear that Fos promotes gene transcription, and it may therefore, stimulate the synthesis of hypothalamic hormones. The hormonal mediation of many physiological and behavioral activities is subject to developmental constraints.

The preoptic area of the hypothalamus contains specific nuclei that contribute to various activities and homeostatic responses. The maturation of these activities occurs at different stages of development and can be detected by measuring the activation of POAH neurons or by observing the successful induction of the physiological response. Two sites in particular that showed consistent Fos-LI labeled nuclei within groups of animals were the median preoptic nucleus (MnPOA) and the medial preoptic nucleus (POM).

In the rats examined in this study, there were no noticeable fluctuations in the density of labeled nuclei in the SDN-POA during development. Since this site is so intimately associated with male reproductive activity (DeJonge, et. al., 1989) it would not be unreasonable to expect to see increased cellular activity during the critical periods of sexual maturation. A recent investigation (Robertson, Pfaus, Atkinson, Matsumura, Phillips, & Fibiger, 1991) demonstrated a substantial increase in Fos-LI labeled nuclei in the medial POA and the bed nucleus of the stria terminalis of the male rat following sexual behavior. The labeled nuclei were not exclusively restricted to the SDN-POA, but were distributed rather widely throughout the medial POA. This distribution is in agreement with

the observed distribution of Fos-LI labeled nuclei in the present study, although the baseline Fos-positive nuclei were distributed more sparsely. One phenomenon that does seem to correlate with the development of Fos-LI in the POM in general is the fluctuation of the concentrations of circulating testosterone during development. Peak densities in Fos-LI labeled nuclei were present in the POM in the 14- and 35-day-old rat, suggesting increased neuronal activity. Between 15- and 17-days-of-age, testosterone levels peak, and then between 21- and 34-days-of-age, testosterone levels decline but, at 35-days-of-age, the concentration increases significantly (Dohler & Wuttke, 1975). This relationship between the fluctuations of the density of Fos-LI in the POM and serum testosterone levels during development is compelling, but it would be important to know if those cells that are expressing *c-fos* during those particular ages possess receptor activity for testosterone.

The MnPOA showed only very small differences in Fos-positive labeling among groups of animals of the same age. Considerable numbers of labeled nuclei were present in pups as young as 3-days-of-age, and the Fos-LI labeling reached a peak at 21 days. Unfortunately, the significance of the changes in Fos-LI observed in the MnPOA during development is unclear, since the functional and biochemical characteristics of this site have not been well described in the literature.

The PVN, on the other hand, has been intensely studied both biochemically and anatomically. It is comprised of distinct subpopulations of cells that differ functionally and morphologically. Two morphologically distinct cell types can be discerned in Nissl stained tissue. These cell types include large cells, comprising the magnocellular portions of the PVN, and smaller cells, forming the parvocellular subnuclei (Armstrong, et. al., 1980; Hatton, et. al., 1976).

In addition to possessing distinct cell types, PVN nuclei contain similar appearing cells segregated into specific subnuclear sites. Immunocytochemical methods have shown that the subnuclear organization of the PVN is additionally distinguished by the distribution of various neurochemicals. Thyrotropin-releasing hormone (TRH) is found in the anterior

and medial parvocellular portions of the PVN (Lechan & Jackson, 1982). During development, TRH concentrations progressively increase as the animals mature, although diurnal variations in concentration are not evident until between 10- and 17-days-of-age (Martino, Bambini, Bartelena, Aghini-Lombardi, Breccia, Baschieri, & Pinchera, 1986). Between 17- and 23-days-of-age, peak TRH concentrations were measured at the beginning of the dark period, 1800 h. In the 31-day-old and older rats, peak TRH concentrations were measured five hours into the lights on period, 1200 h. Twelve noon is approximately the time of day that the rats in this study of Fos-LI were sacrificed for immunocytochemistry. Considerably more Fos-positive nuclei were observed in the medial parvocellular portion of the PVN in rats 21-days-of-age and older as compared with younger animals. Even though mid-day peak concentrations of TRH were not observed in rats younger than 23 days, the noon-time TRH concentrations in general in the 23-day-old animal were higher than those of younger rats. Double-labeling or TRH stimulation studies, would provide more information on the biochemical nature of the cells that contained the Fos-LI nuclei observed in the medial parvocellular PVN.

Another neurochemical specific to the parvocellular subdivision of the PVN is corticotropin-releasing factor (CRH). Perikarya labeled positively for CRH are abundant in the dorsal medial parvocellular subnucleus of the PVN (Rho & Swanson, 1987). This site partially overlaps the TRH containing regions of the PVN, although the CRH-positive neurons extend further dorsally. CRH is secreted in response to internal or environmental challenges, and several studies have examined the effects of various stressors on Fos-immunoreactivity in the PVN. Immobilization stress or injections of hypertonic solutions resulted in increased Fos-IR in PVN neurons that were additionally immunoreactive for CRH (Ceccatelli, et. al., 1989). Adrenalectomy (Jacobson, et. al., 1990), a procedure that eliminates circulating corticosteroids, short-circuits a negative feed-back control of CRF cells; and cholecystokinin administration (Verbalis, et. al., 1991) similarly results in the induction of *c-fos* in CRF-characterized neurons. In the untreated rats in the normal

development study, Fos-LI was often, although, inconsistently observed in the dorsal medial parvocellular PVN. Quite frequently, if several animals were sacrificed for immunocytochemistry on the same day, the last one or two rats had considerably more Fos-LI nuclei in the dorsal medial parvocellular PVN than the first one or two rats sacrificed. Removal from the home cage, exposure to an alien environment, and loss of cage-mates are potential stressors. In addition, the pups were removed from sources of nourishment, both liquid and solid. Therefore, all of these factors combined to place the animal in a stressful situation and the large numbers of Fos-LI nuclei observed in CRF-positive sites might not be unexpected. This was especially true for the younger rats. As noted in the Results section, in the older rats, particularly the 60-day-olds, considerably fewer Fos-positive nuclei were observed in the PVN. The older rats were subjected to less stress, since they were usually housed one or two to a cage, and kept in their home cage while awaiting sacrifice. Food and water were always available. Of all the sites in the PVN where Fos-labeling was observed, it is quite possible that the Fos-LI in the dorsal medial parvocellular PVN was most probably restricted to CRF synthesizing neurons and the expression was due to the environmental stress that the rats experienced.

The magnocellular portions of the PVN contain neurons that are immunoreactive for oxytocin and vasopressin (Swaab, et. al., 1975; Hou-Yu, et. al., 1986). Administration of hypertonic solutions leads to the elevation of oxytocin mRNA levels (Van Tol, et. al., 1987) and increased Fos-LI labeling in magnocellular PVN neurons that are immunoreactive for oxytocin (Ceccatelli, et. al., 1989; Giovanelli, et. al., 1990). Oxytocin seems to participate in the control of body fluid osmolarity. Vasopressin, on the other hand, is important for maintaining the appropriate fluid volume. The Fos-LI labeling in the magnocellular portion of the PVN in normal rats during development was rather inconsistent among animals of the same age, and no developmental trend was observed. It is therefore not possible at this time to define a developmental pattern for Fos-LI in magnocellular PVN neurons. The application of a specific stimulus for those hormone

synthesizing cells would provide a clarification of the biochemical nature of the Fos-LI nuclei observed.

The arcuate nucleus is adjacent to the third ventricle in the ventral portion of the caudal half of the hypothalamus. Several hormones have been localized to arcuate neurons. Probably the most intensely studied of all the neurochemicals in the arcuate is growth hormone-releasing hormone (GHRH; Daikoku, et. al., 1986; Merchenthaler, et. al., 1984; Sawchenko, et. al., 1985). This regulator stimulates the secretion of growth hormone from the pituitary. The distribution of GHRH-immunoreactive neurons in the arcuate is similar to the pattern of Fos-LI observed in that nucleus. In the rostral arcuate, GHRH-positive neurons are found in the ventral portion of the nucleus, and more caudally, the laterally located cells stain positively for GHRH. This pattern is similar to the Fos-LI labeling observed in this study.

Proliferation of GHRH cells is complete prior to birth (Rodier, et. al., 1990), although fluctuations in the concentrations of the peptide and its mRNA are observed throughout postnatal development. In general, hypothalamic GHRH content increases slowly between one and 20-days-of-age (DeGennaro, et. al., 1986; Jansson, et. al., 1987) and then markedly increases between 20- and 50-days-of-age (Jansson, et. al., 1987), or between 35- and 45-days-of-age (Gabriel, Millard, Koenig, Badger, Russell, Maiter, & Martin, 1989). Results of studies examining the mRNA levels confirm the results of the assays for the peptide (Argente, et. al., 1991). After 50-days-of-age, only minor increases in GHRH were measured in the hypothalamus. This developmental pattern of GHRH concentration fluctuations resembles the developmental changes observed in the density of Fos-LI nuclei in the arcuate. Between 3- and 14-days-of-age, the density of labeled nuclei remained relatively stable and low. By 21-days-of-age, high densities of Fos-positive nuclei were observed in the arcuate and remained high through 35-days-of-age. By 60-days-of-age, the densities of labeled nuclei dropped to levels similar to those observed in prepubertal rat pups. Based on the distribution of Fos-LI labeled nuclei and the

developmental pattern observed, it may be tentatively concluded that at least part of the Fos-LI observed in the arcuate may participate in induction of the GHRH gene.

Another protein found in cells of the arcuate nucleus is proopiomelanocortin (POMC; Chowen-Breed, et. al., 1989; Chowen-Breed, Fraser, Vician, Damassa, Clifton, & Steiner, 1989). POMC is cleaved into several neuroactive peptides including β -endorphin, α -melanotropin, β -lipotropin, and adrenocorticotropin (ACTH). In the rostro-caudal direction, the intermediate level of the arcuate contains many cells that are positive for ACTH. These cells are especially abundant in the lateral aspects of the nucleus (Knigge, et. al., 1981; Kiss & Williams, 1983). This distribution is similar to that observed for Fos-LI labeling. Parvocellular CRH neurons of the PVN project to cells of the median eminence (Rho & Swanson, 1987; Swanson, 1987), which is rich in ACTH cells as well as Fos-LI nuclei. This pathway, the hypothalamo-pituitary-adrenal axis, is crucial for mediation of stress responses and it is therefore probable that at least some of the Fos-LI observed in the arcuate and adjoining median eminence is attributable to the stresses imposed.

In the arcuate nucleus, several genes may be the targets for Fos stimulated induction. These include the genes that code for POMC and GHRH. The transcription of POMC may be an environmentally mediated event in which the presence of a stressful situation is the precipitating factor. The synthesis of GHRH, on the other hand, appears to be a developmentally regulated event.

The VMN has been shown to mediate many behaviors including feeding and reproductive activity (Kennedy & Mitra, 1963), locomotion (Yokawa, et. al., 1989; Yokawa, et. al., 1990), and autonomic control of thermoregulation (Nijjima, Rohner-Jeanrenaud, & Jeanrenaud, 1984). Most of these activities are responses that are stimulated by environmental factors. It was not surprising, therefore, to observe very low levels of Fos-LI in the VMN of untreated rats not exposed to appropriate stimuli.

Based on the results of this study, it is quite possible that the appearance of Fos-LI in the hypothalamus at specific stages during development reflects both neuronal growth

and differentiation as well as the stimulation of the transcription of genes for hormones and neurotransmitters.

Experiment 2: Development of Fos expression in response to cold ambient temperatures

The thermoregulatory responses of adult animals to cold ambient temperatures include a variety of physiological and behavioral adjustments. Behaviorally, the response may be as simple as thermotaxis, i.e., moving toward a warmer location. The physiological mechanisms for thermogenesis include activation of brown adipose tissue (BAT), peripheral vasoconstriction and shivering.

Young mammals, especially altricial preweanlings, are inefficient at generating and maintaining heat. Behavioral thermoregulatory responses to cold ambient temperatures predominate. In young rat pups, huddling with the dam and littermates provides warmth, and changing position within the huddle, termed "pup flow" (Alberts, 1978), provides a mechanism for obtaining more or less heat. It was originally believed that very young rat pups, for example, around three days of age, were unable to respond to temperature fluctuations when outside of the nest. Recently, measurable behavioral responses to cold environmental temperatures have been observed in these very young animals. For example, cold exposure elicited increased rates of ultrasounding in pups less than 10 days of age (Allin & Banks, 1971; Oswalt & Meier, 1975). This "distress" signal may be used to alert the dam that the pup has been displaced from the nest and that she should retrieve it. Even though these young rats rely on the dam to return them to the nest, pups as young as three days of age display thermotaxic behavior (Johanson, 1979; Kleitman & Satinoff, 1982). When placed in a thermal gradient, the pups were able to move along the gradient and settle at a position that provided sufficient warmth to maintain optimal body temperature. These reports suggest that young rats are capable of detecting and behaviorally responding to environmental temperatures that are out of their thermoneutral zone, that is, the temperature range within which metabolic rate is unaltered.

GDP binding assays in interscapular brown adipose tissue (BAT) demonstrate that BAT is strongly activated in response to cold environmental temperatures between four and seven days of age (Nedergaard, Connolly, & Cannon, 1986). The basal activity of BAT actually decreases after 10-days-of-age to levels below those observed in the one-day-olds, except in animals raised in cold environments (Himms-Hagen & Park, 1984). In addition, O₂ consumption, which is used as a measure of metabolic activity, including the heat generating activity in BAT, increases in rat pups between one- and six-days-of-age in response to exposure to temperatures below 35°C. Brown adipose tissue activity is stimulated by the sympathetic nervous system, and subcutaneous injections of norepinephrine increase O₂ consumption in six-day-old pups (Thompson & Moore, 1968). This suggests that the mechanisms mediating thermogenesis in BAT are functional in these young animals. In contrast, shivering in response to cold is not apparent until nine to 12-days-of-age (Gulick, 1937). Literature on the development of peripheral vasoconstriction as a thermogenic mechanism is quite scarce. There are no data on the age of the onset of this thermogenic response.

The central nervous system sites activated by fluctuations in ambient temperature and mediate physiological and behavioral responses to those fluctuations are located mostly in the hypothalamus (Morimoto, Nakamori, Ono, Watanabe, Sakai, & Murakami, 1986; Dean & Boulant, 1989). Manipulation of several hypothalamic sites, either chemically, electrically, and lesioning can either stimulate or inhibit thermoregulatory responses. When the connections between the preoptic-anterior hypothalamus (POAH) and the medial forebrain bundle are severed, rats are unable to maintain body temperature in a 5°C environment (Gilbert & Blatteis, 1977). The POAH contains cells that are not only thermosensitive but respond specifically to only warm or cool local stimuli. Upon being stimulated, these thermosensitive cells activate other hypothalamic nuclei such as the PVN (Matsumura, Nakayama & Ishikawa, 1983), or the VMN (Imai-Matsumura, Matsumura, Tsai & Nakayama, 1988). Preoptic area stimulation has also been shown to initiate

thermoregulatory responses and cause elevation or depression of body temperature (Carlisle & Laudenslager, 1979; Murakami & Morimoto, 1982). Animals with POAH lesions show increased body temperature in the heat even though they are able to bar press to escape the heat (Lipton, 1968). This suggests that the POAH lesions disrupt autonomic thermoregulatory responses while leaving the behavioral thermoregulatory responses intact.

Activity of the VMH seems to affect directly the activity of BAT, the main source of internally generated heat in nonshivering thermogenesis. In intact animals placed in cold environments, the discharge rates of nerves innervating the interscapular BAT increase. Animals with VMH lesions do not show this temperature dependent increase in discharge rate (Niiijima, et. al., 1984). Injections of the excitatory amino acid glutamate into the VMH results in a dose-dependent increases of the temperature of BAT (Amir, 1990b). Similarly, injections of glutamate into the PVN (Amir, 1990a) and the SCN (Amir, Shizgal & Rompre, 1989) result in dose-dependent increases of BAT temperatures. Refinetti and Carlisle (1986) showed that thermal stimulation of the anterior hypothalamic nuclei (AH) stimulates both autonomic and behavioral thermoregulatory responses, whereas thermal stimulation of the posterior hypothalamus stimulates only behavioral thermoregulatory responses.

Thermoregulation is, therefore, a complex, multilevel process mediated by several sites within the hypothalamus. The evidence indicates that some of the physiological responses necessary for thermogenesis do not appear until several weeks of age. The question that was addressed in the second study is, what is the developmental time course for the hypothalamic components of the thermogenic response? Specifically, I asked if the neurons of the hypothalamic nuclei that mediate thermogenesis are responsive to cold ambient temperatures prior to or coincidental with the appearance of the thermogenic response? Neuronal responsiveness was determined by the appearance of Fos-LI nuclei in the thermogenic nuclei of the hypothalamus of cold-stressed animals.

Subjects and Procedure

The subjects in this study were Long Evans hooded rats, 3, 7 to 10, 14, 21, 28, and 35 days of age. Table 2 shows a breakdown of the number of rats in each group. The experiment was conducted using groups of four siblings per session. The rats were weighed and then placed in individual plastic containers (4cm x 4cm x 8cm high) with approximately one inch of wood shavings covering the bottom. The containers were then placed for one hour in a temperature controlled refrigerated chamber cooled to 15°C. Rectal temperatures were measured (Physitemp Instruments Inc., Clifton, NJ) immediately before placing the rats in the cold environment and immediately upon removal from the cold. Non-cold stressed control rats that were 10-days-of-age and younger were placed in similar plastic containers with bedding in an incubator warmed to 37°C, and older rats were maintained at room temperature, 23°C. To evaluate the effects of another type of stressful environment on Fos-LI labeling in the hypothalamus stressed control rats ("shaker stress") were placed in containers and put on a slowly moving shaker for one hour. The rectal temperatures of the non-stressed control rats and the shaker stressed rats were similarly taken both before and after treatment.

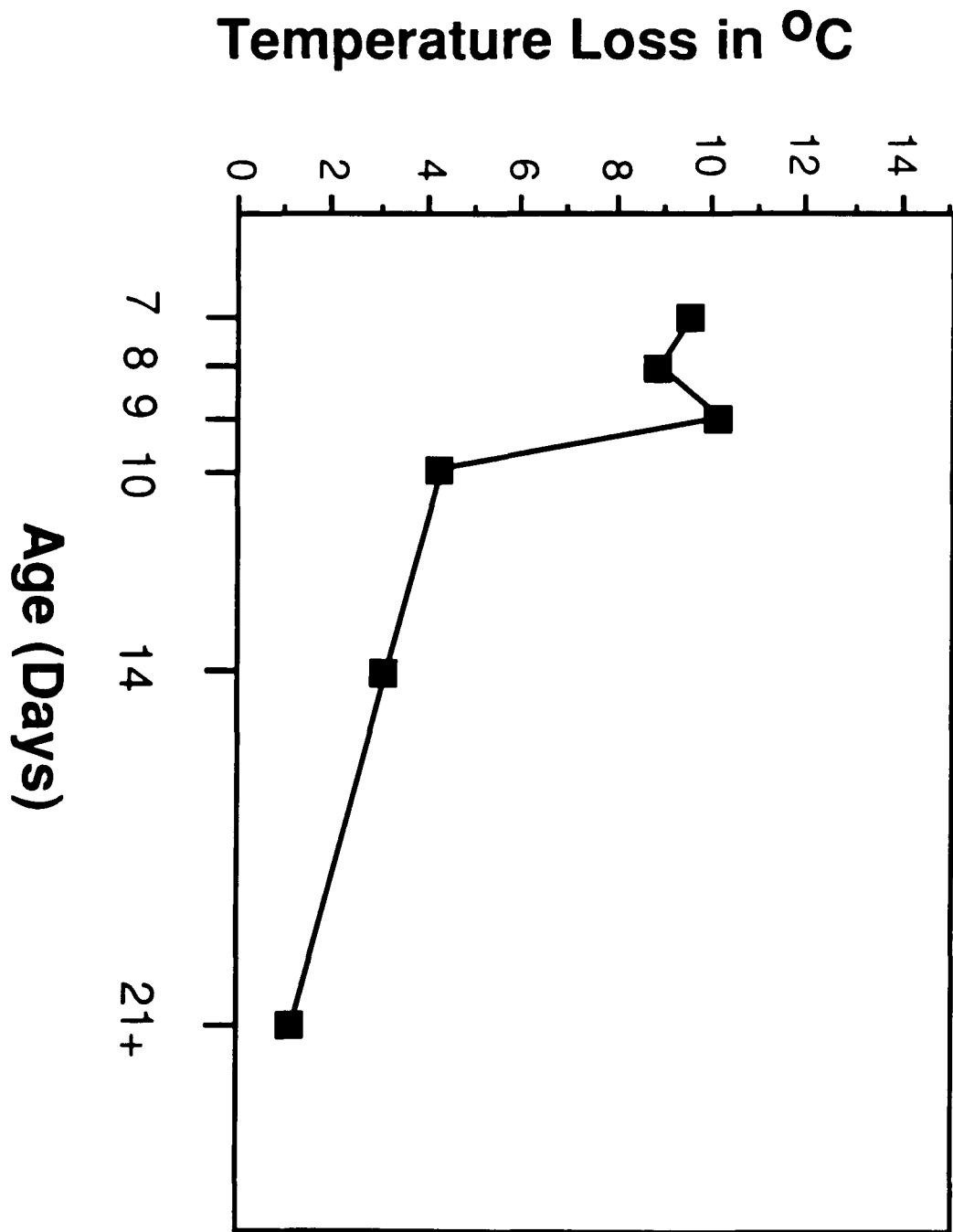
Immediately, one, two, or three hours after the final temperature determination, the rats were given an overdose of sodium pentobarbital, perfused and used for immunocytochemistry as described in the General Methods. Antibodies raised in both rabbits (Oncogene Sciences), and sheep (Cambridge) were used.

RESULTS

Rectal temperature

Figure 9 graphically illustrates the rectal temperature loss after one hour at 15°C in rat pups 7- to 21-days-of-age. Seven, eight, and nine day old pups showed an average

Figure 9. This graph depicts changes in the average rectal temperature of rats 7 to 21 days of age after one hour in the 15°C environment.



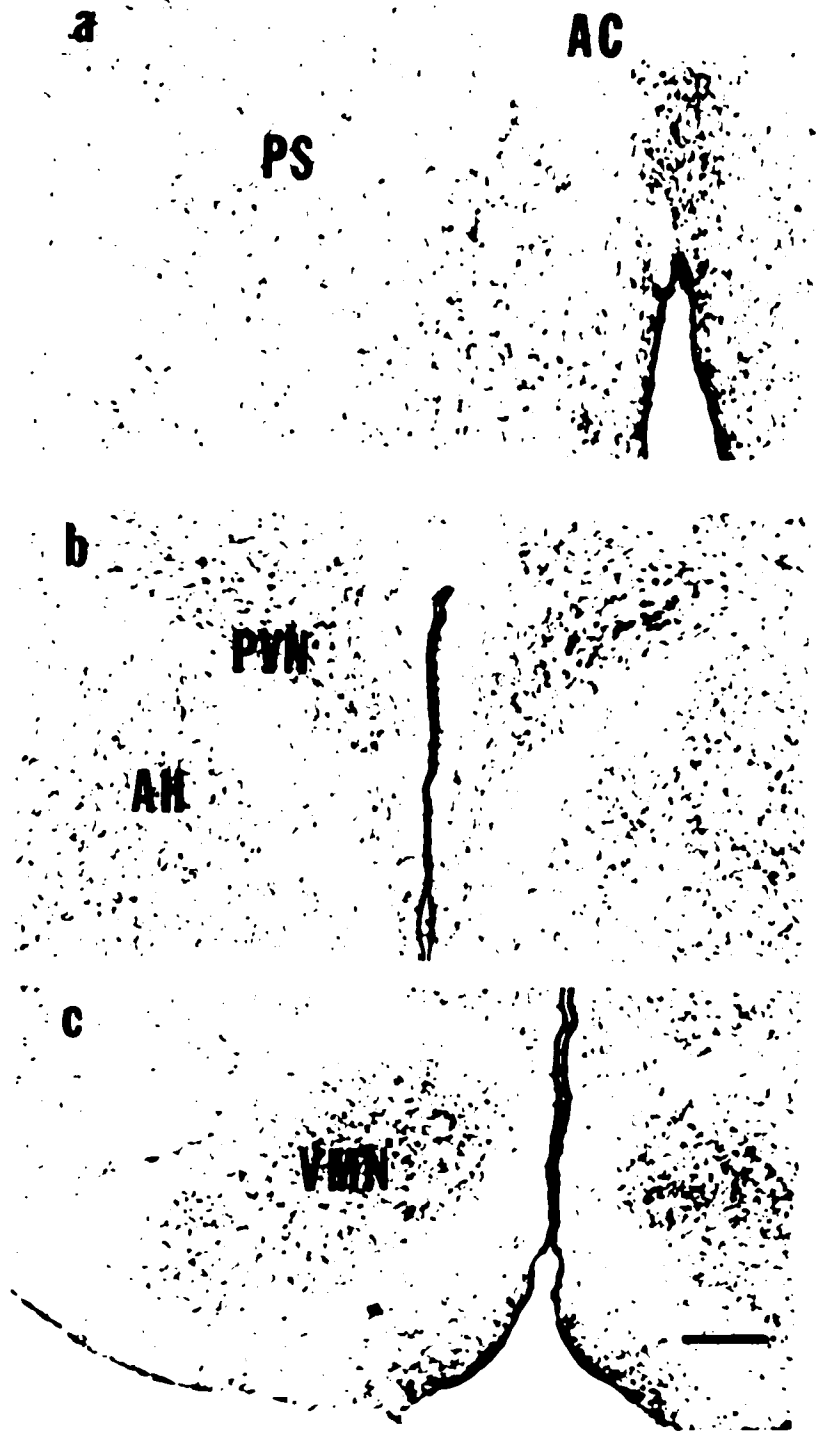
drop in rectal temperature of 9.6°C. No significant differences in rectal temperature depression were noted between seven, eight and nine days of age. By 10 days of age, the decrease in rectal temperature averaged 4.3°C. Fourteen day old pups had an average of 3.18°C drop in rectal temperature and rats 21 days and older showed a temperature drop of 1.25°C after one hour in the cold.

Fos Staining

Preadsorption of the primary antibody to the 12 amino acid sequence of the Fos protein abolished all nuclear labeling. The assays with the sheep antibody indicated a time dependent expression of Fos. In the assays with the primary antibody raised in sheep, the numbers of Fos-like immunoreactive (Fos-LI) nuclei and the intensities of labeling were greater in animals sacrificed two hours after removal from the cold, as compared with the animals that were sacrificed immediately upon removal from the cold. Very little change was observed in the Fos-LI labeling in rats sacrificed three hours after removal from the cold as compared with the rats sacrificed two hours after removal from the cold. When the immunocytochemistry was done with the primary antibody raised in rabbit, the Fos-positive nuclei were as numerous and as darkly stained two hours after removal from the cold as when the animals were sacrificed immediately upon removal from the cold. In some animals, by three hours after removal from the cold, the numbers of Fos-positive nuclei and the intensity of labeling appeared to decrease. Since the labeling was considerably more intense and consistent with the rabbit than with the sheep antibody, all of the results reported in the following are from immunocytochemistry with the primary antibody raised in rabbit and from those rats sacrificed immediately after one hour in the cold.

Fos-LI nuclear labeling was consistently observed in several hypothalamic and extra-hypothalamic nuclei at all ages and under all conditions. Labeled nuclei were especially numerous in the paraventricular nucleus of the thalamus. Other sites that contained Fos-positive nuclei under all conditions included the median preoptic area, the

Figure 10. Cresyl violet stained sections of the hypothalamic sites that were examined in the cold-stress study. a) POAH, b) PVN and AH, c) VMN. Abbreviations: AC=anterior commissure, PS=parastrial nucleus of the POAH.



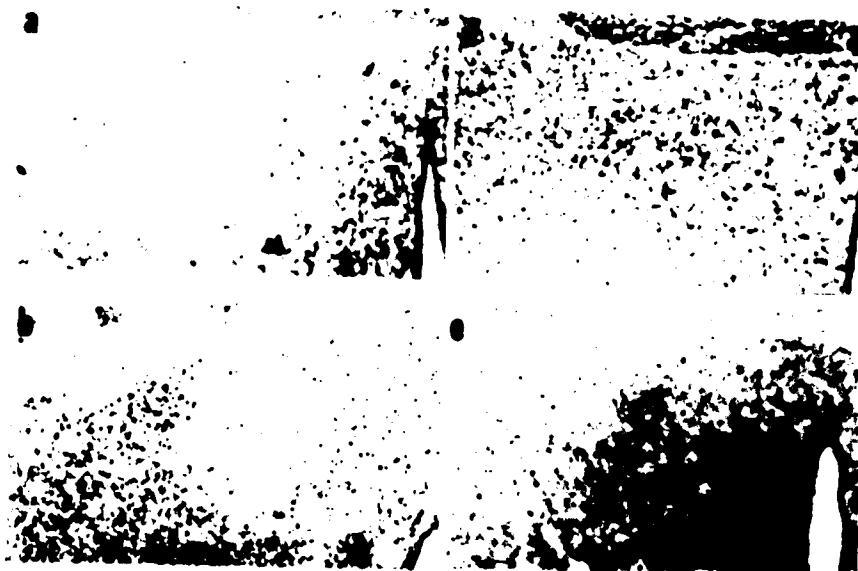
nucleus of the diagonal band, and the suprachiasmatic nucleus. The following describes the appearance of Fos-LI in four of the hypothalamic nuclei that have been previously shown to participate in the thermogenic response system. Figure 10 shows cresyl violet stained sections of the POAH, PVN and AH and the VMN.

Non-stressed Controls: Prior to 10-days-of-age, Fos-LI nuclei were rarely observed in the POAH, PVN, AH and VMN. Fos-positive nuclei were present at 10-days-of-age in the control animals, although there were considerably fewer as compared with the cold-stressed animals. By 28-days-of-age, the numbers of nuclei positively labeled for Fos were maximal in the non-stressed control animals. (Data not shown.)

Cold Stressed Pups

Preoptic-Anterior Hypothalamus: Figure 11, a-e, shows Fos-LI nuclear labeling in the POAH of non-stressed control and cold stressed rat pups. Fos-LI nuclei were first seen in the POAH at nine-days-of-age in response to cold temperatures. At this age, small numbers of Fos-LI nuclei were seen sparsely distributed in the dorsal POAH in the cold stressed rat pups. Labeled nuclei were rarely observed in either nonstressed controls or shaker stressed rats. In the 10-day-old cold stressed rats, substantial numbers of Fos-LI nuclei were concentrated ventral to the anterior commissure in a preoptic area subnucleus, the parastrial nucleus. Nonstressed control rats had very few Fos-positive nuclei, whereas shaker stressed rats had many Fos-positive nuclei scattered unevenly throughout the preoptic area at 10-days-of-age. The numbers of Fos-LI nuclei increased through 21-days-of-age in the cold stressed animals and became more homogeneously distributed. Nonstressed control and shaker stressed animals over 10-days-of-age had moderate numbers of Fos-LI nuclei sparsely distributed in the POAH.

Figure 11. Photomicrographs of Fos-LI labeled sections of the POAH from a) 9-day-old, b) 10-day-old, c) 28-day-old cold stressed rats, d) 10-day-old and e) 28-day-old rats non-stressed control rats. A dramatic increase in Fos-LI labeled nuclei in the parastrial nucleus of the POAH occurred in the 10-day-old cold stressed pup. This labeling in the POAH remained similar in rats 10 days of age and older.

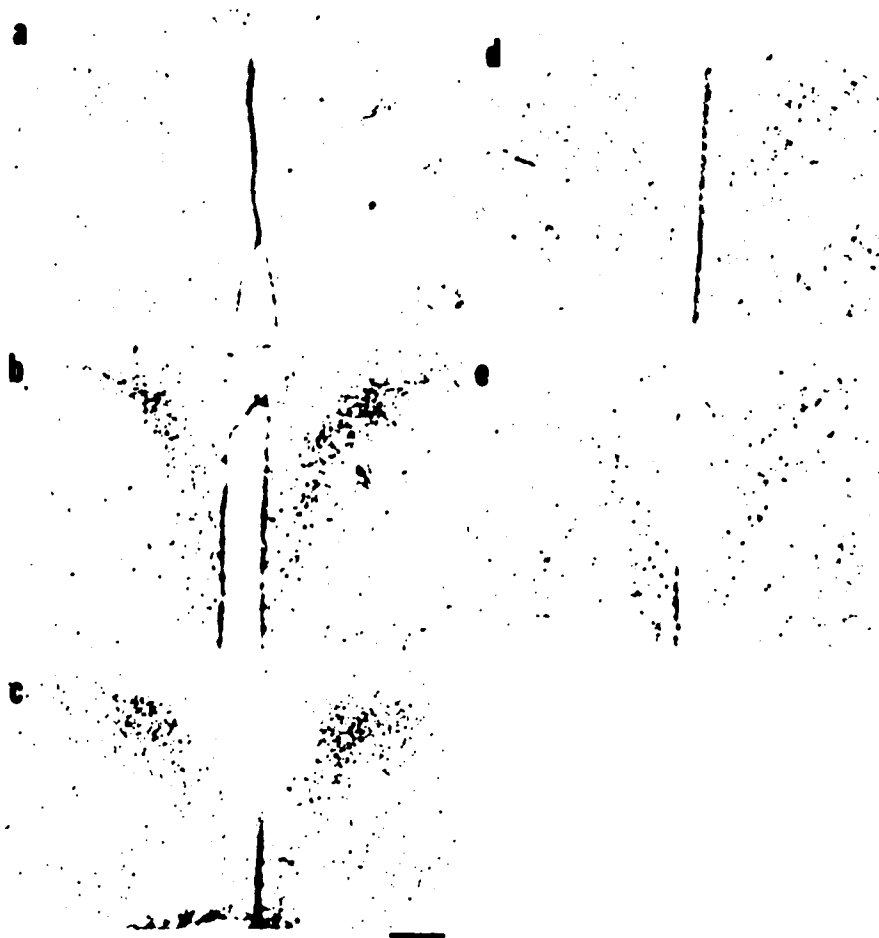


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Paraventricular Nucleus: Figure 12, a-e, shows Fos-positive labeling the PVN and AH of nine-, 10- and 21-day-old cold stressed rats and 9- and 10-day-old non-stressed control rats. Fos-positive nuclei were first observed in the PVN at eight-days-of-age in cold stressed, nonstressed control, and shaker stressed animals. In the cold stressed and shaker stressed animals, the nuclei were lightly labeled and sparsely distributed throughout the magnocellular and parvocellular PVN. In nonstressed control rats, labeled nuclei were similarly seen, but most were confined to the magnocellular portion of the PVN. In cold-stressed nine-day-old pups, the distribution of labeled nuclei was similar to that of eight-day-old cold stressed animals although the majority of labeled nuclei were localized primarily to the magnocellular PVN. In the 10-day-old cold-stressed pup, not only was the staining more intense than that observed in the nine day old, but in general, the numbers of Fos-LI nuclei were considerably greater in all of the 10-day-olds tested, including the non-stressed and shaker-stressed controls. Most of the Fos-positive nuclei were confined to the parvocellular portion of the PVN. The magnocellular PVN was nearly devoid of labeling in all of the treatment groups. By 21 days of age, both the parvocellular and the magnocellular portions of the PVN were consistently labeled, with cold stressed rats showing slightly more Fos-LI nuclei than the nonstressed control rats.

Anterior Hypothalamic Nucleus: The AH of Fos-LI labeled sections of cold-stressed and non-stressed control rats can be seen in Figure 12. Fos-LI nuclei were not seen in the AH under any of the experimental and control conditions until 21-days-of-age. At 21 days, large numbers of Fos-LI nuclei were observed to be scattered throughout the AH in the cold stressed rats. The 21-day-old and older nonstressed control rats consistently had several Fos-positive nuclei in the AH although there were always considerably fewer nuclei in those groups than were observed in the cold stressed animals.

Figure 12. Photomicrographs of Fos-LI labeled sections of the PVN and AH from a) 9-day-old, b) 10-day-old, c) 21-day-old cold stressed rats, and d) 9-, and e) 10-day-old non-stressed control rats. Fos-LI labeling was present at all ages in the cold stressed rats and increased during development. Most of the labeling can be seen in the medial parvocellular portion of the PVN. In the AH, Fos-positive labeling is not observed until 21 days of age and continues to be seen in older rats.



Ventromedial Nucleus: Figure 13, a-e, shows Fos-positive labeling in the VMN of cold stressed animals nine-, 10-, and 28-days-of-age and non-stressed control rats 9- and 10-days-of-age. Fos-LI nuclei were seen in cold stressed pups as young as three-days-of-age. The number of labeled nuclei increased only slightly through 10-days-of-age and subsequently declined in number as the animal matured. Most of the labeled nuclei were confined to the ventromedial portion of the posterior VMN. At all ages, the nonstressed control and shaker stressed animals had a few Fos-LI nuclei in the VMN.

Figure 13. Photomicrographs of Fos-LI labeled sections of the VMN from a) 9-day-old, b) 10-day-old, c) 28-day-old cold stressed rats, and d) 9-, and e) 10-day-old non-stressed control rats.



Discussion

The results of this study suggest that each of the four hypothalamic sites examined is activated initially by cold environmental temperatures at specific times during development. The ages at which neural activation, as indicated by the presence of Fos-LI nuclei, is first observed may coincide with the appearance of autonomic thermogenic responses that are mediated by the specific hypothalamic nuclei.

The most profound developmental change observed was the appearance of Fos-LI nuclei in the POAH in the 10 day old rat pup. In pups nine days of age and younger, Fos-LI labeled nuclei were essentially absent from this site. At 10 days of age, large numbers of darkly stained nuclei were observed to be densely clustered in an area of the POAH termed the parastrial nucleus. This Fos expression appears to be specifically activated in response to the cold stress since the non-stressed controls and the shaker stressed animals did not show such labeling in the POAH. The change in rectal temperature of the rat pups after one hour in the cold indicates that some thermoregulatory developmental transitions occur between nine and 10 days of age. There is substantial evidence indicating that the POAH is important in generating both cooling and warming responses in adult animals. The thermoregulatory responses that are mediated by the POAH appear to be physiological rather than behavioral (Lipton, 1968). Animals with POAH lesions retained their behavioral thermoregulatory capabilities to an extent comparable to those of non-lesioned control animals, but physiological thermoregulatory responses were significantly diminished. Individual cells in this nucleus were responsive to either only warm or only cool local stimuli, with small numbers of cells sensitive to both warm and cool local stimuli (Carlisle & Laudenslager, 1979; Dean & Boulant, 1989; Imai-Matsumura, et. al., 1988; Matsumura, et. al., 1983). It is not clear, though, which thermogenic response or responses are mediated by the POAH. There is evidence that suggests that both shivering and peripheral

vasoconstriction are the physiological events initiated by this hypothalamic site in the adult. When the connections between the POAH and the medial forebrain bundle or between the POAH and the rest of the hypothalamus were severed, cutaneous vasoconstriction upon exposure to a 5°C environment did not occur, and it was noted in this study that such lesions had no significant effects on shivering (Gilbert & Blatteis, 1977). In another study (Murakami & Morimoto, 1982), however, it was shown that local cooling of the POAH produced marked shivering in rats.

One of the earliest autonomic thermogenic mechanisms shown to be functional in the young rat is BAT activation. Rat pups as young as three days of age show evidence of the presence of functional, heat generating BAT stores (Nedergaard, et. al., 1986; Thompson & Moore, 1968). For example, Moore & Donne (1984) showed that four day old mice increased oxygen consumption, indicative of increased metabolic activity in BAT, in response to cold ambient temperatures or injections of norepinephrine. In the present work, at three days of age, the only hypothalamic site that showed increased numbers of Fos-LI labeled nuclei in response to cold environmental temperatures was the VMN. This nucleus has been shown in adults to be important for mediating BAT-dependent thermogenesis. Electrical or chemical stimulation of the VMN, for example, elevated BAT temperature (Amir, 1990b; Holt, et. al., 1987) and accelerated conversion of radioactive glucose to a lipid fraction in interscapular BAT (Shimazu, 1984). Prior treatment with propranolol, a β -noradrenergic receptor blocker, attenuated glutamate induced increases in BAT and body temperatures. This suggests that the VMN mediates sympathetic activation of BAT thermogenesis. Lesions of the VMN were followed by decreased spontaneous discharge rates of fibers innervating BAT and also abolished the increase in discharge rates normally invoked by exposure to cold ambient temperatures (Nijjima, et. al., 1984). The evidence is, therefore, consistent with the hypothesis that the VMN is responsible for BAT activation in three day old animals. The Fos-LI labeling observed in this site in very young

animals may reflect neural activation that stimulates BAT thermogenesis in response to the cold ambient temperature.

The Fos-LI labeled nuclei observed in the PVN may be a response to stressors of several kinds, rather than to cold ambient temperatures in particular. Fos-positive nuclei were consistently observed in the PVN under all conditions, although there were subtle differences in density and distribution among ages and groups. In spite of the evidence showing that glutamate injections into the PVN stimulate BAT thermogenesis (Amir, 1990a), without further experimentation it cannot be concluded with certainty that the neuronal activation observed in the PVN was due specifically to the exposure of the rat pup to the cold environment.

The anterior hypothalamic nucleus is another site that mediates responses to fluctuations in ambient temperatures. Injections of serotonin (5-HT) or epinephrine into the AH of cats increased and decreased body temperature respectively (Feldberg & Myers, 1965). In the present study, this hypothalamic nucleus also had large numbers of Fos-LI nuclei following cold stress in animals 21-days-of-age and older. Younger rats did not show any Fos-positive nuclei in the AH, and non-stressed control rats and shaker stressed rats also rarely had labeled nuclei in this site before 21-days-of-age. It appears, therefore, that the Fos-LI nuclear labeling observed in the AH was in response to the cold environmental temperatures. It has been suggested that the mechanisms by which the AH increases body temperature include both shivering and peripheral vasoconstriction (Feldberg & Myers, 1965). Shivering in response to cold temperature has been shown to occur as early as nine days of age (Gulick, 1937). The age at which vasoconstriction participates in the thermogenic response is not known. The activation of the AH may be involved with the process of vasoconstriction. Coincidental with the appearance of Fos-positive nuclear labeling in the AH between 14- and 21-days-of-age, the change in rectal temperature after one hour in the cold decreased from an average of 3.18°C at 14-days-of-

age to an average of 1.25°C in rats 21 days and older. Whether this nucleus contributes to mechanisms that mediate the observed temperature changes and the nature of the contribution remain to be determined.

The primary question in this developmental study was whether the cells of the thermoregulatory sites in the hypothalamus respond to temperature fluctuations prior to or coincidental with the appearance of the physiological thermogenic response. Visualization of the nuclear protein Fos was used to determine if cellular activation had been achieved and to determine the location and extent of the response. The results of the study showed that neurons at several hypothalamic sites are activated by cold environmental temperatures, and that this activation is subject to developmental constraints. Particularly noteworthy is the effect observed in 10-day-old rat pups, both in terms of Fos expression and the thermogenic responses. This result in particular, suggests that the neural activation observed is developmentally coincidental with the physiological thermogenic response, at least in the POAH.

Experiment 3: The Development of Fos-LI in the SCN and Effects of Early Eye Opening and Baclofen Administration on Fos-LI in the SCN

The SCN is a small, bilateral, ovoid nucleus located just dorsal to the optic chiasm and adjacent to the wall of the third ventricle. This nucleus receives visual system projections from retinal ganglion cells via the retinohypothalamic tract (RHT; Millhouse, 1977; Shibata, Oomura, Hattori & Kita, 1984) and the ventral lateral geniculate nucleus and intergeniculate leaflet via the geniculohypothalamic tract (GHT; Pickard, 1982). The majority of RHT and GHT projections, as well as other SCN afferents, terminate primarily in the ventrolateral portion of the caudal SCN (Campbell & Ramaley, 1974; Mason Sparrow, & Lincoln, 1977; Shibata, et. al., 1984). There is overwhelming evidence that cells of the SCN mediate circadian rhythms. It has been shown that SCN cells maintained *in vitro* or isolated by deafferentation of the SCN, maintain circadian metabolic activity

(Green & Gillette, 1982). Similarly, animals visually deprived by either bilateral enucleation or maintained in total darkness retain their circadian rhythms of neuronal activity in cells of the dorsomedial SCN, but rhythmicity disappears in the ventrolateral SCN (Shibata, Liou, Ueki, & Oomura, 1984). Although circadian activity fluctuations are evident in visually deprived animals, the cycle deviates from the 24 hour photoperiod (Deguchi, 1975). This evidence suggests that the cells of the dorsomedial SCN possess an intrinsic mechanism for the generation of rhythmic activity.

The full expression of some of the cyclically mediated activities is constrained by anatomical and biochemical development and therefore does not occur until specific ages. For example, in the rat, circadian fluctuations of CRH secretion do not occur until postnatal day18 (Campbell and Remaley, 1974) and diurnal temperature rhythms do not acquire the adult pattern until around 40-days-of-age (Kittrell & Satinoff, 1986).

Several maturational events must occur before the SCN can mediate cyclical activities. One is the development of the ability of the SCN neurons to display metabolic activity fluctuations independent of extrinsic stimulation. It has been shown that this characteristic is present in prenatal animals by embryonic day 19 (Reppert & Schwartz, 1984). Another requirement for the mediation of diurnal rhythms is the integrity of the input from the visual system, i.e., the RHT and the status of eye opening of the animal. Although isolated cells of the SCN cycle around an approximate 24 hour period, it is the visual system input that entrains the cycle around the 24 hour photoperiod. Moore and Bernstein (1989) reported that synapses characteristic of retinohypothalamic tract terminations are not observed until four days of age, and adult numbers of all types of synapses in the SCN are present at 10 days of age. In another study, RHT terminations in the SCN were not found before 4- to 7-days-of-age, and peak terminal density occurred between 15- and 21-days-of-age (Mason, et. al., 1977). The rat pup normally opens its eyes on day14.

Several recent studies have shown that manipulation of visual input affects the Fos activity of neurons in the SCN. Rats subjected to bright light (Rea, 1989; Rusak, Robertson, Wisden, & Hunt, 1990; Aronin, Sagar, Sharp, & Schwartz, 1990), sacrificed during the light portion of the light/dark cycle, or maintained in constant light (Earnest, Iadarola, Yeh & Olschowka, 1990) showed increased activity in the ventrolateral SCN, as indicated by the presence of the nuclear protein Fos. In this same study, the numbers of Fos positive cells in the dorsomedial SCN increased relative to the numbers of Fos-positive cells observed in light exposed animals when the rats were sacrificed during the dark portion of the light/dark cycle or kept in a constantly dark environment. It seems apparent that these two subdivisions of the SCN respond differentially to light stimulation. A study of the intrinsic anatomy of the SCN has shown that, in general, the majority of the afferents to the SCN terminate in the ventrolateral portion of the nucleus, and neurons of the ventrolateral SCN project to the dorsomedial part of the SCN (Van Den Pol, 1980). The projection from the ventrolateral to the dorsomedial SCN may be inhibitory and therefore, in the dark, the effect may be one of disinhibition resulting in activation of neurons in the dorsomedial SCN.

In the third study, the normal development of cellular activity in the SCN was investigated in rats three- through 60-days-of-age. The appearance of the nuclear protein Fos was used as a marker of neuronal activity. In addition, the effects of early eye opening were examined to determine the effects of light on the activity of SCN neurons. The eye opening study was done in order to determine if increasing visual input to the SCN would enhance the inhibitory interactions between the ventrolateral and dorsomedial portions of the nucleus. The expectation was that, if the retina is able to transduce light into neural signals at 10-days-of-age, the increased activity in the ventrolateral SCN, as relayed by the retinal ganglion cells, would inhibit the activity of the neurons of the dorsomedial SCN. The result should be decreased Fos-LI labeling in the dorsomedial SCN. The GABA release blocker, baclofen, was administered in the third part of this study to provide

information on possible inhibitory interactions between the ventrolateral and dorsomedial SCN neurons.

Subjects and Procedures

Eye Opening Surgery: At 8 days of age, pups were brought from the animal facility to the laboratory and placed in an incubator warmed to 33°C while awaiting surgery. Surgery was usually performed between five and 30 minutes from the time of arrival to the laboratory. The pups were deeply anesthetized by methoxyflurane inhalation (Metofane, Pitman-Moore). Their faces were then carefully disinfected with Betadine. The eyelids were opened by stretching the lids and carefully cutting along the margin (which is quite prominent) with a sterile scalpel. The procedure produces little or no bleeding and takes about 1 to 2 minutes (Kenny & Turkewitz, 1986). Antibiotic ophthalmic ointment (bacitracin-neomycin-polymyxin [Neobacimyx, Scheering Corp]) was applied to all pups (in the eyes of experimentals and on the lids of controls) on days 8 through 10. The pups were allowed to recover from the anesthesia in the 33°C incubator, and when they were awake and moving about they were returned to their home cage. Experimental pups' lids were checked daily. If they resealed, they were reopened by applying warm water and gently pulling them apart. Control pups were handled in a manner as similar as possible to the experimental pups, short of doing the actual surgery.

At 10 days of age, the pups were transported from the animal facility to the laboratory and given an IP injection of an overdose of sodium pentobarbital. The animals were then perfused, and immunocytochemistry was performed as described above.

Baclofen administration. At 14-days-of-age, pups were given an intraperitoneal injection of baclofen (Research Biochemicals, Inc., Natick, MA) diluted in 0.1M PBS, pH 7.2 (10mg/kg body weight) or vehicle. Two hours after injection, the pups were injected with an overdose of sodium pentobarbital and perfused for immunocytochemistry as described in the General Methods section.

RESULTS

Preadsorption of the primary antibody to the Fos-protein fragment abolished all nuclear labeling otherwise observed in Fos immunocytochemical procedures. The distribution pattern of Fos-positive nuclear labeling divides the SCN into three morphologically distinct rostral to caudal divisions. Cresyl violet stained tissue in Figure 14, a-c, shows sections taken at rostral, intermediate and caudal levels of the SCN and is representative of the three levels of Fos labeled SCN tissue shown in the subsequent figures.

3-days-of-age: In the 3-day-old pups, Fos-positive nuclei were sparsely distributed throughout the SCN. At all three rostro-caudal levels, most of the labeled nuclei were located in the dorsomedial portion of the nucleus. At the caudal level of the SCN, Fos-positive nuclei were rarely seen in the ventrolateral SCN. (Figure 15.)

4-Days-of-Age: By 4-days-of-age, the numbers of Fos-LI labeled nuclei increased dramatically throughout the SCN. The ventrolateral portion of the caudal SCN clearly showed a greater number of positively labeled nuclei than that observed in the 3-day-olds, although the numbers of Fos-LI labeled nuclei were still quite low. (Figure 15.)

10-Days-of-Age: Between 4- and 10-days-of-age, the overall number of Fos-LI labeled nuclei increased substantially, especially in the ventrolateral SCN. In addition, at 10-days-of-age, the intensity of the reaction product was the maximum observed as compared to both younger and older animals. A pattern of differential labeling intensity was seen in the caudal SCN with the nuclei of the ventrolateral SCN more darkly labeled than those of the dorsomedial SCN. (Figure 16, a-c.)

Figure 14. Photomicrographs of cresyl violet stained sections of the suprachiasmatic nuclei at a) rostral, b) intermediate, and c) caudal levels. Abbreviation: OC=optic chiasm.

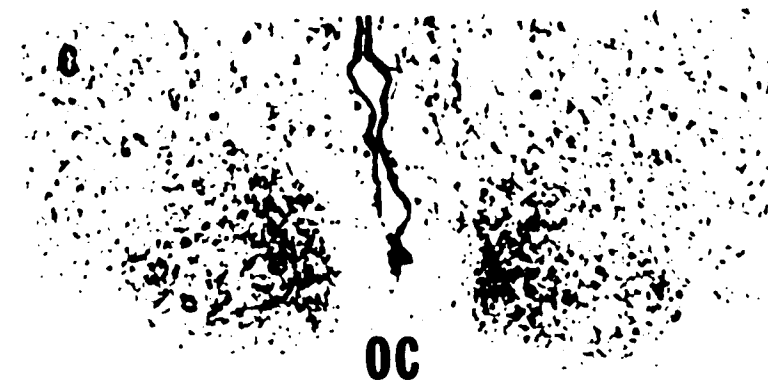
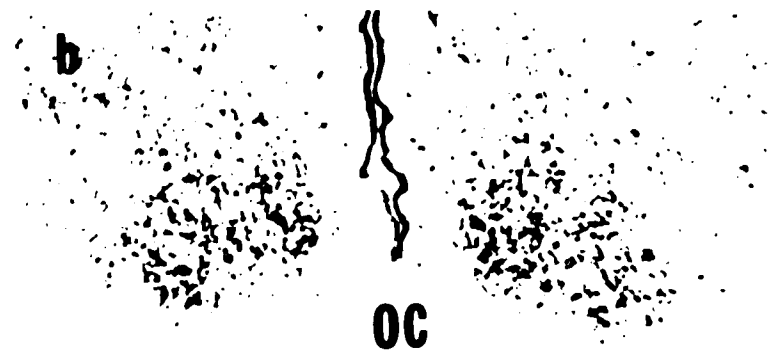
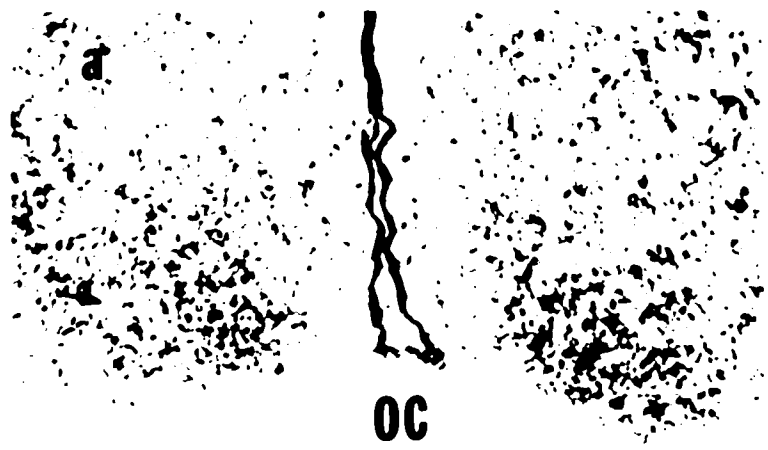
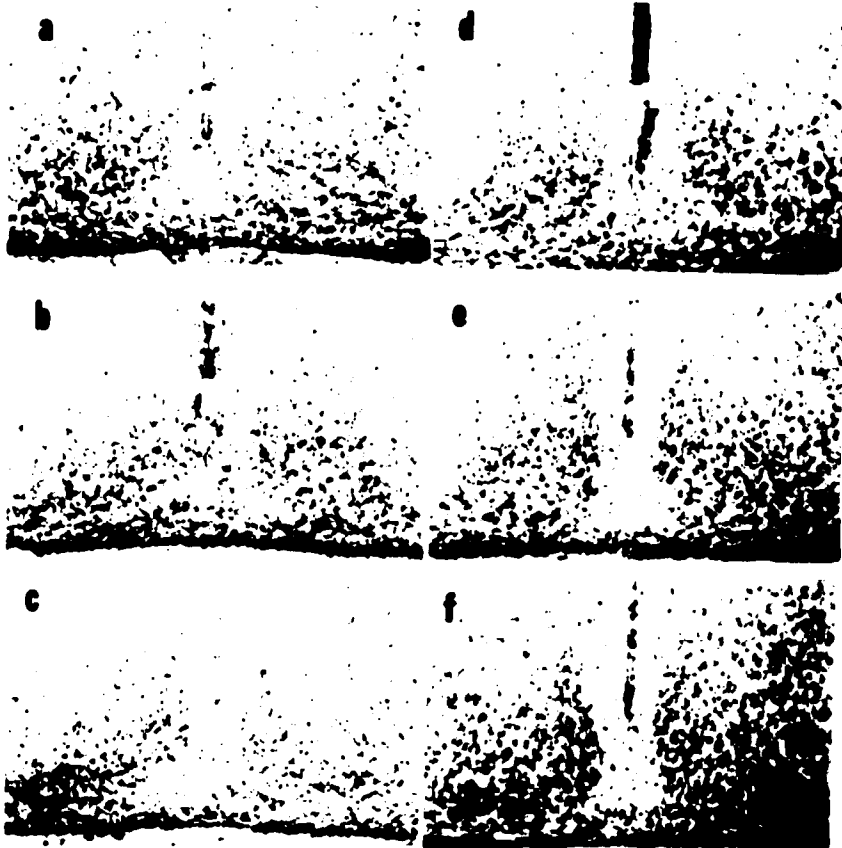


Figure 15. Photomicrographs of Fos-LI labeled sections of the SCN from a-c) 3-day-old, and d-f) 4-day-old rat pups from a and d) rostral, b and e) intermediate, and c and f) caudal levels. The density of labeled nuclei increases at every rostral to caudal level of the SCN between 3 and 4 days of age. In addition, at 4 days of age, labeled nuclei are first observed in the ventrolateral portion of the caudal SCN.



14-Days-of-Age: Between 10- and 14-days-of-age the number of Fos-positive nuclei did not change considerably, although the intensity of the reaction product diminished. The major changes observed occurred in the caudal SCN. At this level, the numbers of labeled nuclei in the ventrolateral SCN increased to the adult level, while the density and staining intensity of nuclei in the dorsomedial SCN decreased. (Figure 16, d-f.)

21- through 36-Days-of-Age: No changes in Fos-LI were observed in either the rostral or caudal SCN between 14- and 36-days-of-age, except that, at the intermediate level, the numbers of labeled nuclei were considerably greater in the 21-day-olds. The density and distribution of Fos-LI in the SCN of 28- and 36-day-old rats is similar to that of 21-day-olds. (Figures not shown.)

60-Days-of-Age: Between 36- and 60-days-of-age, the total numbers of Fos-positive nuclei decreased substantially throughout the SCN. The ventrolateral portion of the caudal SCN is the only site where darkly labeled nuclei are consistently observed. Very few Fos-LI nuclei are present within the boundaries of the SCN although many labeled nuclei are seen just outside the periphery of the nucleus. (Figure 16, g-i.)

Eye Opening Study. The density and intensity of Fos-LI labeling observed in the SCN of pups that underwent surgical eye-opening was virtually identical to that of both control animals and 10-day-old pups in the normal development study. Figures 17 and 18 show rostral and caudal sections of Fos-LI labeled tissue from 10-day-old rats that underwent either surgical eye-opening (Figure 17) or control conditions (Figure 18).

Baclofen results. Injections of baclofen in the 14-day-old pups did not appear to have any effect on Fos-LI in the SCN. Both experimental and control animals showed similar densities of Fos-LI nuclei and the distribution and intensity of the reaction product was

Figure 16. Photomicrographs of Fos-LI labeled sections of the SCN from a-c) 10-day-old, and d-f) 14-day-old and g-i) 60-day-old rats a, d and g) rostral, b, e and h) intermediate, and c, f and i) caudal levels. The density of Fos-LI labeled nuclei peaks at 10 days of age and diminishes thereafter. By 60 days of age, the labeled nuclei in the SCN is quite sparse.

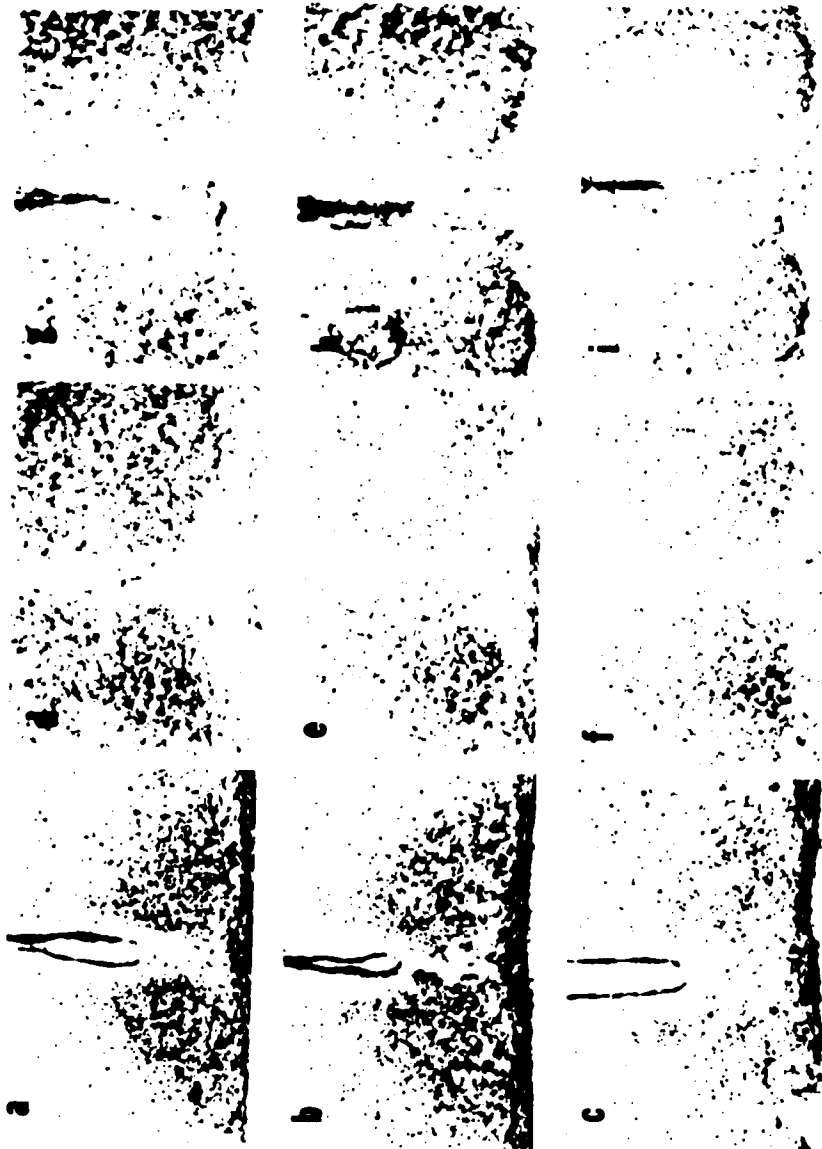


Figure 17. Photomicrographs of Fos-LI labeled sections of the SCN at a) rostral and b) caudal levels of a 10-day-old rat that underwent surgical eye-opening at 8 days of age. The Fos labeling in the SCN of treated rats is similar to the control animals in density, intensity, and distribution.



Figure 18. Photomicrographs of Fos-LI labeled sections of the SCN at a) rostral and b) caudal levels from a control rat in the early eye opening study.

a



b

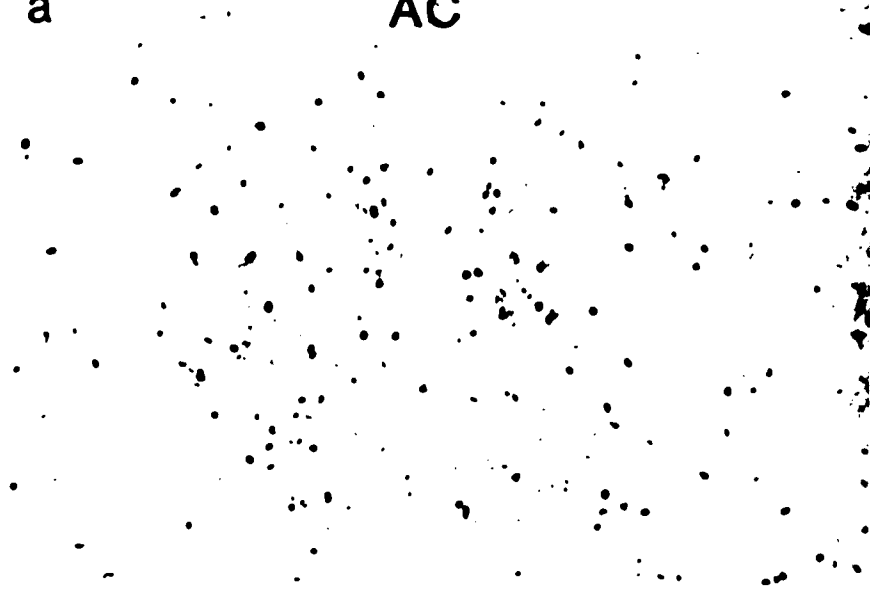


similar between groups. Labeling differences were noted, though, in the POAH. In particular, considerably more nuclei were labeled in the MnPOA in the baclofen treated rats (see figure 19). This observation implies that baclofen administration did affect neuronal activity in at least part of the hypothalamus, although no effect was seen in the SCN.

Figure 19. Photomicrographs of Fos-LI labeled sections of the POAH of 14-day-old a) control and b) baclofen treated rats. Most notable is the apparent increase in labeling in the MnPOA of the baclofen treated rat.

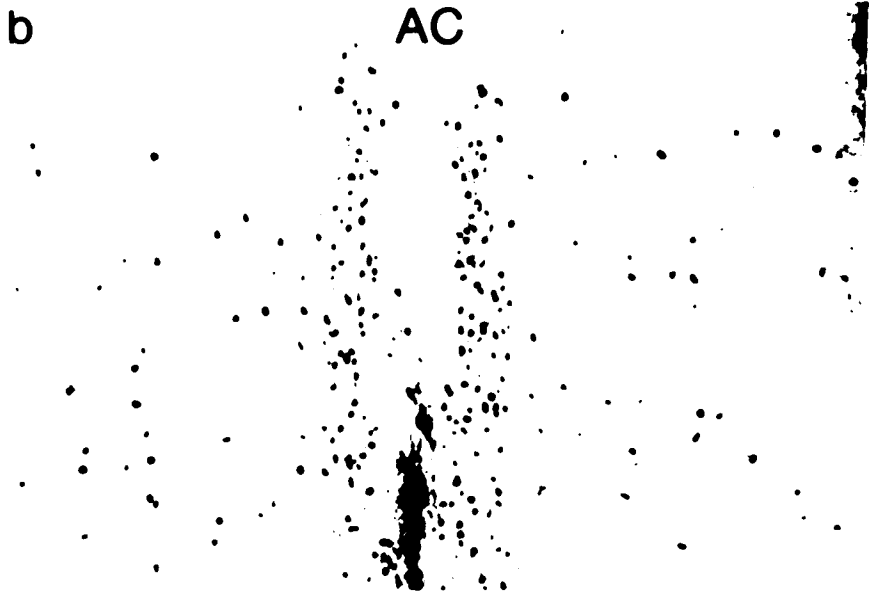
a

AC



b

AC



Discussion

This study examined the appearance of Fos protein-like immunoreactivity during normal development in the SCN of rats. Fos-LI was used as a marker to indicate cellular activity. The changes in Fos-LI in the SCN during development may be related to the activity of developing SCN afferents, intrinsic synaptogenesis, changes in transcription of cell specific genes, intrinsic metabolic activity, or to a combination of these.

Fos-LI labeled nuclei were consistently observed in the dorsomedial SCN of rat pups prior to the appearance of synapses arising from afferent projections or significant synaptogenesis. The RHT does not enter the SCN until four-days-of-age (Campbell & Remaley, 1974; Mason, et al, 1977) and the numbers of synapses in the whole SCN at two to four days is 13% of the adult number (Moore & Bernstein, 1989). Despite the absence of extrinsic neuronal stimulation and significant intrinsic transynaptic communication, SCN neurons are metabolically active and, at three-days-of-age, this activity may be reflected in the appearance of Fos-positive nuclei. It is not unreasonable to hypothesize, though, that the small number of Fos-LI nuclei present at three-days-of-age is indicative of the small number of functional synapses observed at that age. Therefore, transynaptic activity cannot be ruled out, although without the benefit of afferent input, activity fluctuations of the SCN neurons are probably intrinsically mediated.

Some of the developmental changes observed, such as the number, distribution, and intensity of labeled nuclei, can be correlated with various anatomical and biochemical changes that have been previously reported to occur in the maturing SCN. The absence of Fos-LI nuclei in the ventrolateral SCN at three-days-of-age, and the subsequent appearance of labeled nuclei at four-days-of-age, coincide with the reported development of the RHT. The appearance of Fos-positive nuclei at this age suggests that synaptic activity may be occurring and ultimately leading to depolarization of postsynaptic SCN neurons. The presence of Fos-LI labeled nuclei in the four-day-old pups provides evidence for either the

occurrence of synaptogenesis or the presence of a functional retino-hypothalamic pathway. The projecting retinal ganglion cells may be spontaneously active, or the retina may be capable of transducing light stimuli received through closed eyelids into neural impulses. In the rat, cells of the retina are not mature until between the 7th and 12th postnatal day (Brackevelt & Hollenberg, 1970). Therefore, light driven excitation of SCN neurons is quite unlikely. Another indication of this lack of light stimulated Fos-induction in the SCN is that the early eye opening procedure did not result in a difference in either the intensity or distribution of Fos-LI. Alternatively, even in the absence of a functionally mature visual system, retinal ganglion cells display spontaneous activity (Chalupa, 1988). Therefore, transsynaptic activity between the RHT and the SCN should not be unexpected and result in the appearance of Fos-LI nuclei.

Through 10-days-of-age, the numbers of Fos-LI labeled nuclei increase throughout the entire SCN. In addition, the intensities of the reaction products are greatest in the 10-day-old, as compared with both younger and older rats. During this period, synaptogenesis is proceeding throughout the entire SCN and the input from the visual system to the ventrolateral portion of the caudal SCN is similarly occurring. During synaptogenesis, the protein synapsin I is found in the vicinity of the newly forming synapse (DeCamilli, Cameron, & Greengard, 1983; DeCamilli, Harris, Huttner, & Greengard, 1983). The high levels of Fos-LI, as indicated by the large numbers of labeled nuclei and the intense staining, may reflect Fos-promoted expression of the synapsin I gene. Alternatively, Fos expression may be occurring to promote transcription of a gene for a trophic factor that provides axonal guidance or the transcription of genes for cytoskeletal proteins, both of which are important factors that are present during neuronal growth and synaptogenesis.

Another possibility is that the high level of Fos-LI observed in the 10-day-old pup reflects either transsynaptic activity or intrinsic metabolic activity that, if examined at different times during the 24 hour cycle, may show variations in the numbers of Fos-

positive nuclei and the intensity of the labeling of those nuclei. This question was not addressed in this study.

The difference in the intensity of Fos-LI labeling between the ventrolateral SCN and the dorsomedial SCN first becomes apparent at 10-days-of-age in the caudal SCN. In rats 14-days-of-age and older, the nuclear labeling in the ventrolateral part of the caudal SCN is clearly more intense than that seen in the remainder of the nucleus. Reports describing the intrinsic anatomy of the SCN (Van Den Pol, 1980) indicate that the caudal ventrolateral SCN communicates with the rest of the nucleus by way of local circuit neurons. Since activity of the ventrolateral SCN seems to inhibit the activity of the dorsomedial SCN, it is quite possible that these connections are inhibitory. Activation of ventrolateral neurons by visual system inputs essentially shuts off activity in the dorsomedial portion of the nucleus. The SCN contains large amounts of GABA (Michels, Morin, & Moore, 1990; Francois-Bellan, Kachidian, Dusticier, Tonon, Vaudry, & Bosler, 1990; Van den Pol & Tsujimoto, 1985; Vincent, Hokfelt, & Wu, 1982), a neurotransmitter associated primarily with inhibitory activity. This interaction between the visual system and the efferent portion of the SCN may play an important role in either entraining the circadian rhythm or in resetting the endogenous clock.

Several studies have shown that manipulation of GABA activity in the SCN disrupts the circadian timing systems. Diazepam, a drug that enhances GABA activity, blocks light induced phase advances in hamsters although it does not block light induced phase delays (Ralph & Menaker, 1986). Conversely, the GABA antagonists, bicuculline (Ralph & Menaker, 1985) and picrotoxin (Smith, et. al., 1990) block phase delays but not advances. Apparently, a significant inhibitory interaction between the visually driven portion of the SCN and the efferent portion of the nucleus is present. The pattern of Fos-LI labeling seen in the 14-day-old animal illustrates this functional interaction and suggests that it is anatomically and biochemically mature at this age.

Baclofen was administered to determine if the differential labeling intensity observed between the dorsomedial and ventrolateral SCN was indeed due to GABA-mediated inhibition between these two sites. Baclofen is a GABA_B receptor agonist. Activation by baclofen of GABA_B receptors, which are autoreceptors, inhibits the release of GABA. Baclofen administration essentially results in disinhibition of post-synaptic GABA receptive neurons. In hamsters, baclofen administration reduces the effects of phase-shifting in response to light (Ralph & Menaker, 1989), suggesting that the drug is pharmacologically active in the SCN. No differences in Fos-LI labeling were observed in the SCN of the 14-day-old rats that were administered baclofen and the control animals. However, differences in labeling were seen in the median preoptic area between the experimental and control groups. The animals that had been administered baclofen showed very dense Fos-LI labeled nuclei in the dorsal and ventral median preoptic nuclei, whereas the Fos-LI labeling in the control animals was relatively sparse. This therefore indicates that the dose administered was sufficient to obtain a physiological response. The absence of Fos-positive nuclei in the SCN of the rats after baclofen administration suggests that either these disinhibited neurons require an additional source of stimulation or that the presynaptic GABA neurons of the SCN in rats do not possess GABA_B receptors or are not yet developed in the 14-day-old rat. To test further the functional intrinsic anatomical relationships within the SCN, other GABA antagonists, such as bicuculline or picrotoxin, should be administered.

General Discussion

The goals of this thesis were to determine the efficacy of using *c-fos* expression as a marker of normal neuronal activity during development, examine the development of neuronal activity in seven hypothalamic nuclei, and to assess the hypothalamic responses to specific environmental stimuli during development. The results of these experiments provided some answers to these questions and opened these issues to further avenues of investigation.

Neurons are identified as expressing the *c-fos* proto-oncogene if increases in the concentration of the mRNA is detected by Northern blot (Morgan & Curran, 1986; Sonnenberg, Macgregor-Leon, Curran, & Morgan, 1989), if the Fos protein is isolated by gel electrophoresis and identified by immunoblot analysis (Franza, Jr., Rauscher, Josephs & Curran, 1988), or if the Fos protein is visualized by immunocytochemical methods in the cell nucleus (Hunt, et. al., 1987; Koistinaho & Yang, 1990; Hoffman, et. al., 1990). Immunocytochemistry was used in the present study to detect the presence and determine the anatomical localization of the *c-fos* proto-oncogene protein product.

Detection of *c-fos* mRNA or Fos protein has been used as a marker of neuronal activation in response to a variety of exteroceptive perturbations. For example, environmental stimuli, such as light (Rea, 1989), somatic stimuli such as tactile pressure or the injection of irritants (Bullitt, 1989; Menetrey, et al, 1989) or the administration of convulsant drugs such as pentylenetetrazol (Dragunow & Robertson, 1988) or hormones such as estradiol (Insel, 1990), which increases the activity in some hypothalamic nuclei, have been shown to induce the transcription of the *c-fos* proto-oncogene. Following transcription of the gene, the mRNA is translocated from the cell nucleus to the cytoplasm where it is translated into the Fos protein. Prior to entry into the nucleus, the protein undergoes post-translational processing that includes phosphorylation at serine and threonine residues (Morgan & Curran, 1991). Upon entering the nucleus, the Fos protein forms a heterodimeric complex with the protein product of the *c-jun* proto-oncogene. These two molecules bind at leucine residues and this interaction is called the "leucine zipper." The Fos/Jun complex recognizes a specific nucleotide sequence on the DNA to which it binds, the activator protein (AP-1) recognition sequence. Upon binding, this AP-1 complex promotes transcription of the gene associated with the AP-1 binding site (Curran & Franza, 1988). Several time course studies have shown that shortly after the appearance of the *c-fos* mRNA or the Fos protein, other cell specific proteins are detected (White & Gall, 1987; Draisci & Iadarola, 1989).

Several hypotheses have been proposed to explain the appearance of Fos-LI. The ones that are particularly relevant to the results obtained in this study are that Fos may be involved in cell growth and differentiation, Fos is expressed upon neuronal depolarization, and Fos is expressed in response to intrinsic metabolic activity. All of these factors ultimately result in one singular effect: the Fos-stimulated promotion of the expression of target genes.

The various behaviors that are mediated by hormonal secretions and neuronal efferent activity of the hypothalamus emerge at different stages of development. The synthesis of the secretory factors that stimulate these behaviors is dependent upon gene expression, and this expression must commence and terminate at the appropriate stages of development. The *c-fos* proto-oncogene protein product, along with the product of the proto-oncogene *c-jun*, acts as a promoter of target genes. The normal development study was carried out with the aim of correlating the appearance of Fos with the synthesis of hypothalamic secretory factors. This premise is based on the contention that Fos will be synthesized as part of the molecular pathway ultimately resulting in the synthesis of the pertinent secretory factors. This premise additionally suggests that a mechanism for *c-fos* induction other than transynaptic stimulation is operating.

A major portion of the present work examined the changes that occur in Fos expression in the hypothalamus of the untreated developing rat. In several hypothalamic sites, Fos-LI nuclei were present throughout development and the numbers of nuclei either changed or remained relatively constant, depending upon the site, as the animal matured. Other hypothalamic sites may or may not have Fos-positive nuclei at specific stages of development. This age dependent variability of Fos-LI may reflect the changing expression of hypothalamic hormones that occurs during development. During development, the cellular machinery in the central nervous system undergoes many morphological and metabolic changes. All occur during specific stages of maturation and all are reflected in qualitative and quantitative changes in gene transcription. The appearance of Fos-LI in

several areas of the hypothalamus is related to the induction of specific genes, the products of which fluctuate as the animal matures. In this case, the stimulus for Fos induction is the changing endogenous environment encountered during development rather than the external environment. This may be the case, for example, for the Fos-LI observed in the putative GHRH cells of the arcuate nucleus.

External environmental stimuli, on the other hand, appear to contribute an even greater effect on the induction of *c-fos*. Developmental patterns in the appearance of Fos-LI in response to environmental stress were observed, and this suggests that specific molecular machinery must be responsive and functional in order to perceive and elicit a response at the cellular level. It was reported that Fos-positive nuclei were not observed in the POAH in response to cold stress until 10-days-of-age. This finding suggests that prior to 10-days-of-age, neurons in this site are either unresponsive to decreases in environmental temperature or are unable to set in motion the required genetic activation to elicit a response to the stimulus. Coincidental with the appearance of Fos-LI in the POAH at 10-days-of-age, the drop in rectal temperature after one hour in the cold was more than half that measured in rat pups 9-days-of-age and younger. This result suggests that the *c-fos* induction may be part of the pathway involved in eliciting a thermogenic response. This compelling evidence, at first glance, suggests that these particular POAH neurons first become responsive to cold temperatures at 10-days-of-age. An alternative explanation may be that the cells are responsive to the stimulus prior to 10-days-of-age, but the transcriptional machinery that mediates *c-fos* transcription or a factor inherent in the gene itself, may not be functional until 10-days-of-age. In this case, the appearance of Fos-LI does not reflect the depolarizing effect of the stimulus but the mediation of the response to the stimulus. The known mechanisms that stimulate *c-fos* induction suggest, though, that this alternative explanation is less likely since most evidence points to the need for a depolarizing stimulus to induce *c-fos* transcription. In this case the depolarizing stimulus is the cold environmental temperature.

The Fos-LI observed in the SCN presents an even greater conundrum. Is the appearance of Fos-LI due to the promotion of components required for growth and synaptogenesis, intrinsic metabolic activity, transsynaptic stimulation or environmental stimulation? A case can be made for all of these factors. Through 10-days-of-age, the numbers of Fos-LI nuclei and intensity of the reaction product progressively increase, and they peak at 10 days. It is during that same period that cell growth and synaptogenesis are proceeding rapidly. The synthesis of cytoskeletal proteins is required for both of these processes, and Fos may be instrumental for promoting the transcription of those genes.

The question of the appearance of Fos-LI as a result of intrinsic metabolic activity has not been directly addressed in this study. Therefore, for the present, it will remain unsettled. Environmental and transsynaptic stimulation are intimately related, since one usually results in the other. The pattern of Fos-LI observed in the SCN is particularly reflective of the activity of the afferent environmental input as well as the intrinsic activity of the nucleus. Increases in Fos-LI in the ventrolateral SCN, the site of visual system terminations, occurs concomitantly with decreased Fos-LI throughout the rest of the SCN. The Fos induction in the ventrolateral SCN is possibly due to excitatory transsynaptic activity mediated by the projecting retinal ganglion cells, while the absence of Fos-LI throughout the rest of the SCN may be due to intrinsic inhibitory projections. Previous work has shown that rats sacrificed during the dark portion of the light/dark cycle show more Fos-positive nuclei in the dorsomedial SCN and less in the ventrolateral SCN, as compared to rats sacrificed during the lights on period or exposed to light during the lights off period.

In conclusion, it has been shown that the occurrence of some developmentally mediated events is under the control of a series of molecular steps including the induction of the proto-oncogene *c-fos*. The occurrence of any or all of these steps is subject to the constraints of development. In addition, the ability to respond, either physiologically or behaviorally, to an environmental stimulus, appears to require a mechanism within the

molecular or genetic machinery of the cell that promotes synthesis of the necessary neurochemical factors and ultimately elicits a response.

The results of these experiments provide a preliminary indication of the efficacy of the use of Fos-LI as a marker of neuronal activity during development both in untreated and environmentally stimulated rats. During normal development, the density and distribution of Fos-LI labeled nuclei change and these changes are suggestive of changes in neuronal activity, quite possibly transcriptional activity, as the animal matures. The functional significance of the appearance of Fos-LI is still unclear, but further analysis such as double-labeling experiments and time-course studies would provide clues as to the role of *c-fos* induction.

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