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Mechanisms Underlying Prolactin-Induced Activation of Tuberoinfundibular Dopaminergic Neurons

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MECHANISMS UNDERLYING PROLACTIN-INDUCED ACTIVATION OF TUBEROINFUNDIBULAR DOPAMINERGIC NEURONS

By

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ABSTRACT

MECHANISMS UNDERLYING PROLACTIN-INDUCED ACTIVATION OF TUBEROINFUNDIBULAR DOPAMINERGIC NEURONS

By

Kenneth Hentschel

It has been more than ten years since investigators discovered that activation of tuberoinfundibular dopamine (TIDA) neurons by prolactin was biphasic, consisting of early tonic and delayed inductive components. While the tonic component has been studied extensively, little is known about the inductive component. The purpose of this dissertation was to characterize mechanisms underlying prolactin-induced activation of TIDA neurons and to identify mediators of the actions of prolactin. To this end, a combination of anatomical, immunohistochemical, molecular, neurochemical and pharmacological experimental approaches were employed to address the following specific aims: to characterize the temporal effects of prolactin on neurochemical estimates of TIDA neuronal activity; to characterize the temporal effects of prolactin on immediate early gene expression in anatomically and neurochemically distinct subpopulations of TIDA neurons; and to characterize the role of neurotensin as a mediator of the effects of prolactin on TIDA neurons.

First, the inductive activation of TIDA neurons by prolactin was temporally characterized using neurochemistry (i.e. via analysis of concentrations of the dopamine metabolite 3,4-dihydroxyphenylacetic acid in the median eminence where these neurons terminate). Results of neurochemistry experiments confirmed that prolactin activates TIDA neurons after a delay of 6-12 h. Immunoneutralization studies further indicated that

a period greater than 3 h but less than 6 h of hyperprolactinemia is required for its initiation, and that hyperprolactinemia is required to sustain the response.

Secondly, the temporal effects of prolactin on immediate early gene expression in subpopulations of TIDA neurons was characterized using neural activity mapping with inducible transcription factors combined with dual immunohistochemistry. Prolactin differentially regulated the expression of Fos-related antigens (FRA), a cellular marker of genomic activity, in TIDA neurons in different arcuate nucleus subdivisions. Following the induction of hyperprolactinemia transient increases in FRA expression were first observed in TIDA neurons of the ventrolateral arcuate nucleus (1.5 h) followed by those of the dorsomedial arcuate nucleus (3 h). Additionally, prolactin affected FRA expression in a group of non-catecholaminergic neurons in the arcuate nucleus. These studies demonstrated that prolactin elicits genomic effects prior to the delayed activation of TIDA neurons, identified a novel group of prolactin-responsive non-catecholaminergic neurons and suggested a temporal sequence of activation.

Finally, the role of neurotensin as a mediator of the effects of prolactin on TIDA neurons was characterized. The first approach utilized neural activity mapping with inducible transcription factors and dual immunohistochemistry for the detection of FRA and neurotensin. By 1.5 hours after injection, prolactin increased the percentage of neurotensin neurons expressing FRA and the increased genomic activity of these neurons was maintained for 12 h. In addition, prolactin increased total numbers of neurotensin neurons in the arcuate nucleus after 6-12 h. Taken together, prolactin modulated gene expression in neurotensin and other neurons, stimulated neurotensin synthesis and recruited these neurons into an activated pool.

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Another approach utilized neurochemistry and the neurotensin receptor antagonist SR-48692 to demonstrate a role for neurotensin receptors in the inductive activation of TIDA neurons by prolactin. The neurotensin receptor antagonist had no effect on basal TIDA neuronal activity or plasma prolactin concentrations. However, the neurotensin receptor antagonist blocked the delayed stimulatory effects of prolactin on TIDA neurons. The results demonstrated that neurotensin receptors mediated the delayed, but not tonic stimulatory effects of prolactin on TIDA neurons.

In conclusion, the inductive activation of TIDA neurons by prolactin was characterized by a temporal delay of 6-12 h, was dependent upon a period of elevated prolactin for greater than 3 h for initiation and required hyperprolactinemia for maintenance of the response. The experiments also lent insight into the mechanisms underlying prolactin-induced activation of TIDA neurons. Such mechanisms involved prolactin-dependent genomic changes in TIDA and neurotensin neurons in the arcuate nucleus hours prior to the time of TIDA neuronal activation. The role of neurotensin in the delayed inductive activation of TIDA neurons by prolactin was also characterized. It was observed that prolactin caused early and prolonged increases in the genomic activity of existing neurotensin neurons and of those neurons recruited by prolactin to synthesize neurotensin. Moreover, results demonstrated that neurotensin receptors mediated the delayed stimulatory effects of prolactin on TIDA neurons.

To my loving wife, Alicia,

my beautiful daughters, Brynn and Kaylin,

and my wonderful families.

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3V

AAA

ABC

AP-1

ARC

BSA

Ca-Ca

cAMP

CaRE

CC

CPM

CRE

CREB

DA

DAB

DM

DMN

DMSO

DOPA

DOPAC

EDTA

FRA

LIST OF ABBREVIATIONS

3V	third ventricle
AAAD	aromatic L-amino acid decarboxylase
ABC	avidin biotin complex
AP-1	activator protein-1
ARC	arcuate nucleus
BSA	bovine serum albumin
Ca-CamK	calcium calmodulin-dependent kinase
cAMP	adenosine 3,5-monophosphate
CaRE	calcium response element
CC	corpus callosum
CPM	counts per minute
CRE	cAMP response element
CREB	cAMP response element binding protein
DA	dopamine
DAB	3,3-diaminobenzidine tetrahydrochloride
DM	dorsomedial
DMN	dorsomedial nucleus
DMSO	dimethyl sulfoxide
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
EDTA	disodium ethylenediamine tetraacetic acid
FRA	fos-related antigens

GAB

h

HIPP

icv

IEG

im

ip

IR

iv

LH

LRG

MAO

ME

min

MLD

NA

ND

NHP

NID

NM

NR

NS

NT

GABA	gammabutyric acid
h	hours
HIPP	hippocampus
icv	intracerebroventricular
IEG	immediate early gene
im	intramuscular
ip	intraperitoneal
IR	immunoreactivity
iv	intraventricular
LH	lateral hypothalamus
LRG	late response gene
MAO	monoamine oxidase
ME	median eminence
min	minutes
MLDA	mesolimbic dopamine
NA	nucleus accumbens
ND	non-detectable
NHPP	National Hormone and Pituitary Program
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NMDA	N-methyl D-aspartate
NRS	normal rabbit serum
NSDA	nigrostriatal dopamine
NT	neurotensin

NTR

opt

PBS

PC

PKA

PKC

PRL

PRL-A

q

RIA

s

sc

SEM

SRE

SRF

ST

TBS

TH

TI

TX

TYR

VL

VMN

NTR	neurotensin receptor
opt	optic tracts
PBS	phosphate buffered saline
PC	parvocellular
PKA	protein kinase A
PKC	protein kinase C
PRL	prolactin
PRL-AB	prolactin antiserum
q	every
RIA	radioimmunoassay
s	second
sc	subcutaneous
SEM	standard error of the mean
SRE	serum response element
SRF	serum response factor
ST	striatum
TBS	tris-buffered saline
TH	tyrosine hydroxylase
TI	tuberoinfundibular
TX	Triton-X100
TYR	tyrosine
VL	ventrolateral
VMN	ventromedial nucleus

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1. GENERAL INTRODUCTION

A. STATEMENT OF PURPOSE

Much of what is known about the mechanisms underlying prolactin-induced activation of tuberoinfundibular (TI) dopamine (DA) neurons is based upon neurochemical estimates of neuronal activity. Since these methods rely on changes in neurochemistry in termini of TIDA neurons, this index provides a composite estimate of activity of all neurons projecting to this region. While neurochemistry has been useful and informative, the advent of new techniques (e.g. neural activity mapping using inducible transcription factors coupled with dual immunohistochemistry) allows the estimation of neuronal activity at the cellular level, and hence the investigation of differentially-responsive subpopulations of TIDA neurons.

The overall aim of this proposal is to characterize mechanisms underlying prolactin-induced activation of TIDA neurons and to identify mediators of the actions of prolactin. To this end, a combination of anatomical, molecular, neurochemical and pharmacological approaches are employed to address the following specific aims: (1) To characterize the temporal effects of prolactin on neurochemical estimates of TIDA neuronal activity, (2) to characterize the temporal effects of prolactin on immediate early gene expression in anatomically and neurochemically distinct subpopulations of TIDA neurons, and (3) to characterize the role of neurotensin as a mediator of the effects of prolactin on TIDA neurons.

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B. ANATOMY AND FUNCTION OF CENTRAL DOPAMINERGIC NEURONS

The mammalian brain contains several different dopaminergic neuronal systems classified alphanumerically by the locations of their perikarya and axon termini (See **Figure 1.1**; Dahlström and Fuxe, 1964). The largest and most studied is the meso-telencephalic system, comprised of nigrostriatal dopamine (NSDA) and mesolimbic dopamine (MLDA) neurons. NSDA neurons have perikarya in the substantia nigra pars compacta (A_8-A_9) and axons that terminate in the striatum. These neurons regulate sensorimotor integration and motor movement. Dysfunction of NSDA neurons is involved in the pathogenesis of Parkinson's disease (Chase and Oh, 2000). MLDA neurons have perikarya in the ventral tegmental area (A_{10}) and axons that terminate in limbic structures including the nucleus accumbens, olfactory tubercles, septum, amygdala, and various cortical regions. These neurons regulate motivational behavior, and their dysfunction has been implicated in the pathogenesis of schizophrenia (Snyder et al., 1974; Matthyssee, 1980).

TIDA neurons have perikarya in the arcuate nucleus (ARC; A_{12}) and axons that terminate in the external layer of the median eminence. Dopamine released in the median eminence from TIDA neurons travels in the hypophysial portal blood to the anterior pituitary where it tonically inhibits prolactin secretion (Ben-Jonathan, 1985). The primary focus of this dissertation is the regulation of TIDA neurons by prolactin. NSDA and MLDA systems are incorporated to highlight differences in the regulation of TIDA neurons compared to "classical" mesotelencephalic neurons.

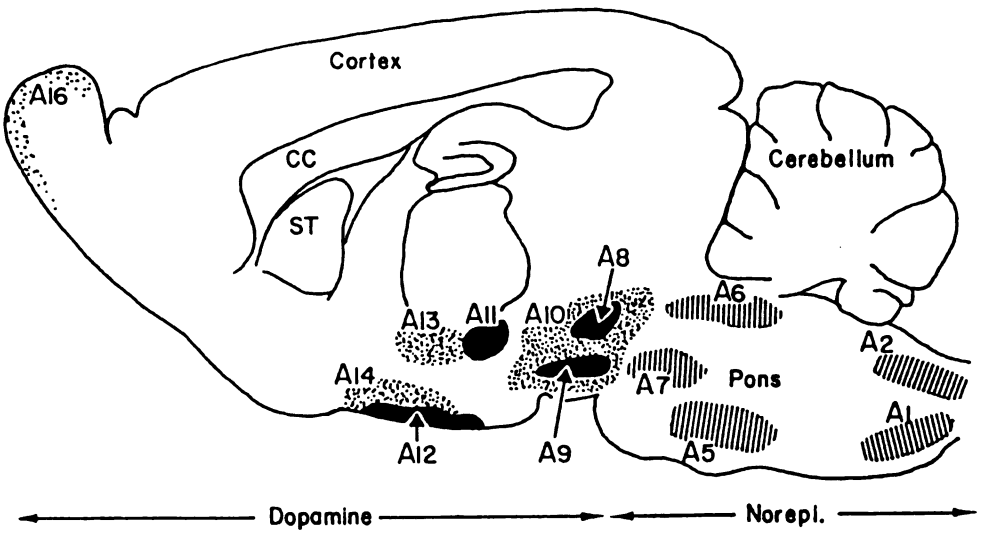

Figure 1.1 Schematic saggital view of the rat brain demonstrating the distribution of catecholamine-containing perikarya (Moore, 1987a). Cell groups A_1-A_7 are noradrenergic and groups A_8-A_{16} are dopaminergic (Dahlstrom and Fuxe, 1964). Abbreviations: CC, corpus callosum; HIPP, hippocampus; Norepi, norepinephrine; ST, striatum.

C. BIOCHEMISTRY OF CENTRAL DOPAMINERGIC NEURONS

Current knowledge of the synthesis, storage, release and metabolism of dopamine has been predominantly derived from studies of NSDA neurons. However, the majority of neurochemical events occurring in MLDA and TIDA nerve termini are similar to those described for NSDA neurons. As schematically depicted in **figure 1.2,** dopamine synthesis begins when dietary tyrosine is actively transported into the nerve terminal (Guroff et al., 1961) and converted by the rate limiting enzyme, tyrosine hydroxylase (TH) to 3,4-dihydroxyphenylalanine (DOPA). DOPA is rapidly decarboxylated by aromatic L-amino acid decarboxylase (AAAD) to form cytosolic dopamine (Nagatsu, 1973). The majority of newly synthesized cytosolic dopamine is packaged into synaptic vesicles and stored for release while a fraction remains free in the cytosol where it may inhibit the synthesis of additional dopamine by end product inhibition of TH, and be metabolized by monoamine oxidase to 3,4-dihydroxyphenylacetic acid (DOPAC).

Stored dopamine in NSDA and MLDA nerves is released into synaptic clefts of forebrain structures where it can act at pre-synaptic autoreceptors or post-synaptic receptors. Activation of pre-synaptic autoreceptors inhibits further synthesis and release of dopamine in these neurons (Christiansen and Squires, 1974; Roth et al., 1975). Stimulated TIDA nerves release dopamine into the hypophysial portal vasculature in the external layer of the median eminence, rather than into a classical synapse like mesotelencephalic neurons. Dopamine released there acts at post-effector receptors in the anterior pituitary. TIDA neurons also differ from mesotelencephalic neurons in that they lack pre-synaptic autoreceptors (Gudelsky and Moore, 1976; Demarest and Moore, 1979; Lookingland and Moore, 1984).

Figure 1
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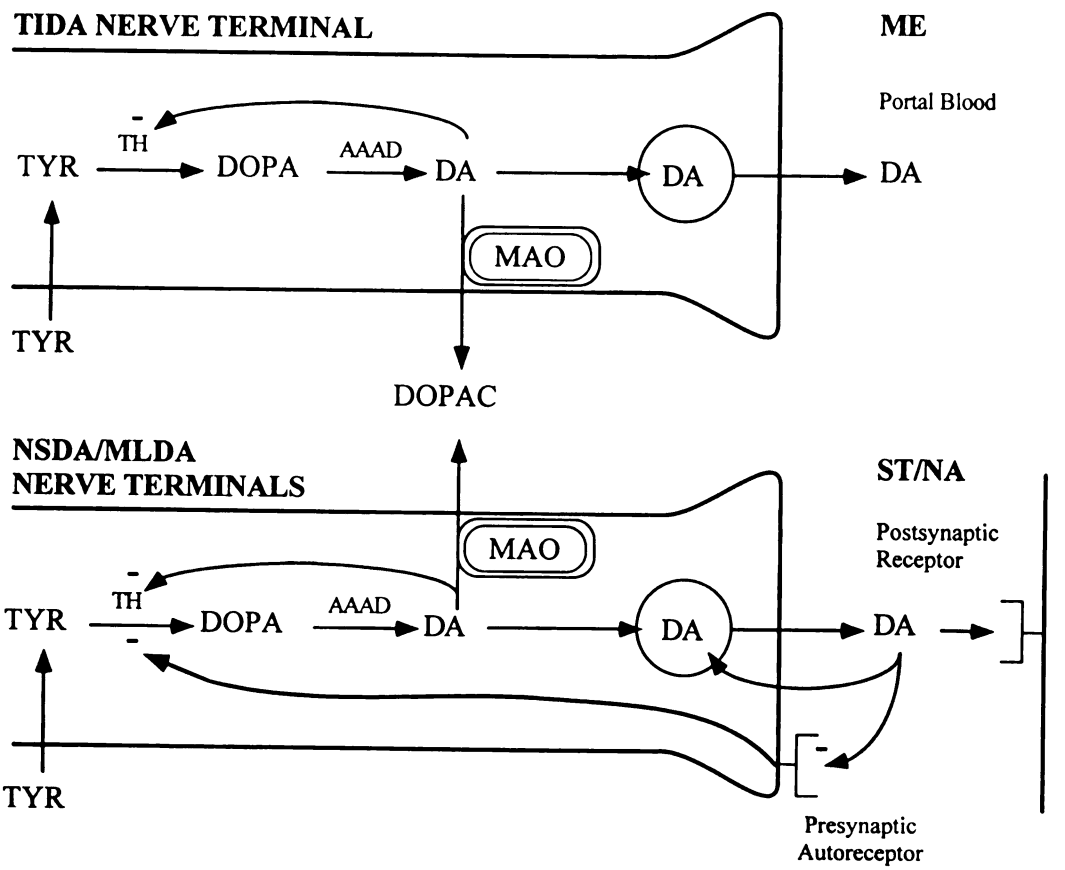

Figure 1.2 Schematic representation of differences in neurochemical and metabolic pathways in TIDA (ABOVE) and NSDA or MLDA (BELOW) nerve terminals (modified from Moore, 1987a). Abbreviations: AAAD, aromatic L-amino acid decarboxylase; DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; MAO, monoamine oxidase; ME, median eminence; NA, nucleus accumbens; ST, striatum; TH, tyrosine hydroxylase; TYR, tyrosine.

The synaptic actions of dopamine are terminated in NSDA and MLDA nerves predominantly via high affinity uptake pumps in the pre-synaptic nerve termini. Once in the nerve terminal, recaptured dopamine can be repackaged into synaptic vesicles for re-release or be metabolized by mitochondrial monoamine oxidase to DOPAC. Non-vesicular cytosolic dopamine transported from the synapse may also feed back to inhibit the activity of TH, and hence the synthesis of dopamine.

TIDA nerves differ from the mesotelencephalic system in the removal of dopamine from the synapse. Presynaptic TIDA nerves have a dopamine uptake pump, however, it is of low affinity and questionable relevance (Demarest and Moore, 1979; Annunziato et al., 1980). Since TIDA nerves release dopamine in the median eminence with significant concentration gradient imposed by the fenestrated portal vasculature, it is likely that the low affinity uptake system in TIDA nerves does not have an important role. Consequently, in TIDA neurons little to no dopamine is recaptured and metabolized to DOPAC. Therefore, DOPAC concentrations in TIDA neurons are due to the metabolism of newly synthesized dopamine (Lookingland et al., 1987a; 1987b). DOPAC may diffuse out of NSDA, MLDA and TIDA nerves where it may be metabolized by extraneuronal catechol-O-methyltransferase to form homovanillic acid. A minority of extraneuronal dopamine that evades uptake may be directly methylated by catechol-O-methyltransferase to form 3-methoxytyramine, and subsequently oxidized to homovanillic acid.

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D. NEUROCHEMICAL INDICES OF DOPAMINERGIC NEURONAL ACTIVITY

The rate limiting enzyme of dopamine synthesis, TH is intricately regulated on multiple levels: transcription of mRNA, multiple mRNA splice variants, differential RNA stability, translation, differential (protein) stability, allosteric modulation, phosphorylation state, end-product inhibition and inhibitory pre-synaptic autoreceptors (Kumer and Vrana, 1996). The net effect of this diverse and redundant system of regulation, is a tight coupling of dopamine synthesis, release and metabolism. As a consequence of this coupling, steady state concentrations of dopamine remain fairly constant within the neuron despite alterations in rates of release (Moore, 1987a). Accordingly, *in vivo* neurochemical estimation of TH activity has been used as an index of dopaminergic neuronal activity (Roth et al., 1976; Lookingland et al., 1987a; Lindley et al., 1988; Tian et al., 1991).

In the past, neurochemical estimates of dopaminergic neuronal activity have been made following inhibition of dopamine synthesis. For example, α-methyltyrosine inhibits TH and causes an exponential decline in dopamine concentrations at a rate proportional to the level of neuronal activity (Brodie et al., 1966; Fuxe et al., 1969; Lofstrom et al., 1976; Lookingland and Moore, 1984). Similarly, 3-hydroxybenzylhydrazine inhibits AAAD and causes accumulation of DOPA at a rate proportional to the level of neuronal activity (Carlsson et al., 1972; Murrin and Roth, 1976; Demarest et al., 1979; Demarest and Moore, 1980). In most cases these indices provide an accurate and reliable estimate of neuronal activity. However, these drugs require 30 min or more for product decline, or

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accumulation, respectively, and are therefore not well suited for the estimation of acute changes in dopaminergic neuronal activity.

Additionally, neurochemical methods involving inhibition of dopamine synthesis are not ideally suited for the estimation of TIDA neuronal activity. Since these drugs block dopamine synthesis and newly synthesized dopamine is preferentially released (Gudelsky and Porter, 1979), dopamine release is blocked and the secretion of prolactin from the pituitary is disinhibited. This precludes the concomitant measurement of TIDA neuronal activity and the functional correlate of TIDA neuronal activity, plasma prolactin concentrations. Furthermore, the resulting hyperprolactinemia complicates the interpretation of such experiments because control groups have been exposed to elevated prolactin for 30 min or more which may influence neuronal activity.

Estimation of the activity of TIDA neurons by measuring changes in concentrations of the metabolite DOPAC in terminal regions of these neurons overcomes the disadvantages of neurochemical procedures employing TH inhibition. Briefly, the use of DOPAC as an index of neuronal activity is based upon the following rationale. Activity-induced dopamine release is accompanied by an increase in cytoplasmic concentration of dopamine synthesized *de novo*. A percentage of this cytoplasmic non-vesicular dopamine pool is metabolized to DOPAC by monoamine oxidase at a rate proportional to *de novo* synthesis (Lookingland et al., 1987a). Hence, procedures which alter dopaminergic neuronal activity produce proportional changes in DOPAC concentrations.

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E. NEURAL ACTIVITY MAPPING WITH INDUCIBLE TRANSCRIPTION FACTORS

Ten years after initial discovery, neural activity mapping with inducible transcription factors is now widely utilized and accepted. This technique has led to the localization and neurochemical identification of neurons within specific brain regions that are uniquely responsive to a variety of stimuli (Sheng and Greenberg, 1990; Pennypacker et al., 1995; Chaudhuri, 1997; Harris, 1998). Neural activity mapping with inducible transcription factors is based upon the following rationale. Neurons respond to extracellular stimulation by modulating the expression of certain immediate early genes encoding transcription factors. The basal expression of these genes in quiescent cells is low. Upon stimulation, genes encoding transcription factors are rapidly and transiently transcribed (Greenberg et al., 1985; Morgan and Curran, 1986; Bartel et al., 1989). These transcription factors regulate the expression of late response genes whose expression is modulated over a time frame of hours (Merlie et al., 1984; Castellucci et al., 1988; Goldman et al., 1988). The products of the late response genes are thought to serve more specific effector functions in the neuronal response.

One of the best studied immediate early genes is *c-fos*. In the last 10 years a great deal has been learned about the molecular mechanisms underlying the induction of *c-fos* and the role of its protein product Fos in expression of late response genes. As shown in **figure 1.3**, external stimuli (such as neurotransmitters or membrane depolarization) that cause increased concentrations of intracellular cAMP or calcium result in phosphorylation of cAMP response element binding protein (CREB) and its activation as a transcription factor. This phosphorylation is mediated by protein kinase A (PKA) for

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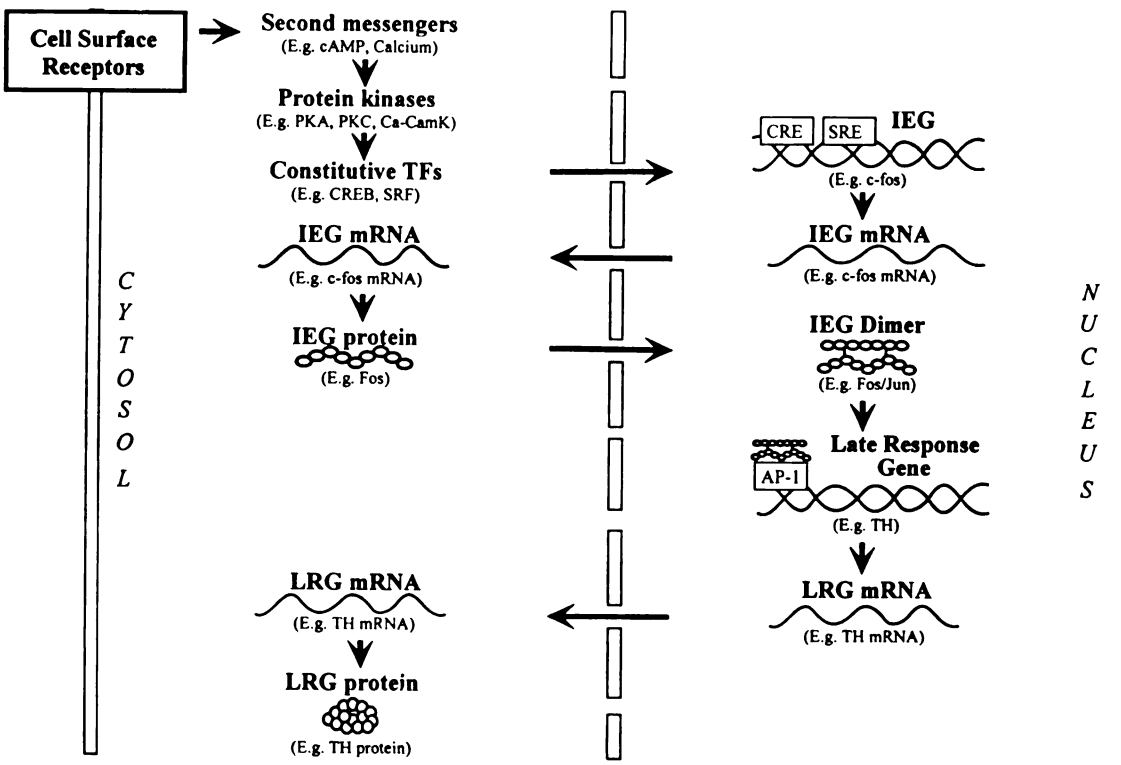

Figure 1.3 Schematic representation of extracellular signal-mediated long-term changes in gene expression in a neuron (modified from Hughes and Dragunow, 1995). Abbreviations: AP-1, activator protein-1 site; Ca-CamK, calcium-calmodulin-dependent kinase; CRE, cAMP response element; CREB, cAMP response element binding protein; IEG, immediate early gene; LRG, late response gene; PKA, protein kinase A; PKC, protein kinase C; SRE, serum response element; SRF, serum response factor; TFs, transcription factors.

the cAMP pathway, and via unidentified calcium/calmodulin kinase (CAMK) for the calcium pathway. Phosphorylated CREB interacts with calcium response elements (CaRE) and cAMP response elements (CRE) within the promoter of immediate early genes like *c-fos* to stimulate initiation of transcription. Transcription of *c-fos* may also be induced by various signaling pathways that converge upon activation of protein kinase C (PKC). PKC phosphorylates and thereby activates a serum response factor (SRF). Phosphorylated dimeric SRF interacts with the serum response element (SRE) in the promoter of the gene to stimulate its transcription. *C-fos* mRNA may be transiently detected 15-30 minutes following stimulation of the neuron (Greenberg et al., 1985; Morgan and Curran, 1986; Bartel et al., 1989; Hoffman and Murphy, 2000). The transient expression of *c-fos* mRNA is due to its inherent instability (10-15 minute half-life) and end product inhibition. *C-fos* mRNA is translocated to the cytosol where it is translated into Fos protein. Generally, these proteins appear 60-90 minutes after stimulation and persist for 2-5 h (Hoffman et al., 1992; Hoffman and Murphy, 2000).

Fos proteins translocate to the nucleus and heterodimerize with constitutively expressed Jun-related transcription factors (Morgan and Curran, 1989; Hughes and Dragunow, 1995). These heterodimers bind AP-1 elements (Sheng and Greenberg, 1990) in late response gene promoters (Kumer and Vrana, 1996) and modulate the transcription rate of these genes (e.g. TH: Icard-Liepkalns et al., 1992; Guo et al., 1998, and neurotensin: Kislauskis and Dobner, 1990; Bullock et al., 1994). The effect of the Fos/Jun heterodimer on transactivation rate of the late response gene depends upon the combination of Fos- and Jun-related transcription factors composing the dimer. For example, c-Fos/c-Jun greatly enhances transcription rate, whereas c-Fos/Jun-B represses

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it. Ultimately, over a time frame of hours, the late response gene products are synthesized by a protein synthesis-dependent mechanism.

Neural activity mapping using inducible transcription factors has limitations that should be recognized to avoid erroneous conclusions. The first consideration is termed cell type expression specificity and is based on the observation that not all cells produce all transcription factors. Thus, negative results demand careful interpretation (Chaudhuri, 1997; Harris, 1998). Another complexity of neural activity mapping using inducible transcription factors is due to the convergence/redundancy of multiple signaling pathways leading to stimulus-coupling uncertainty. Stimulus-coupling uncertainty can make a causal link between changes in gene expression and a stimulus difficult to establish and highlights the importance of appropriate controls (Chaudhuri, 1997; Harris, 1998). Chronically stimulated neuronal systems exemplify stimulus-coupling uncertainty and are not suited to neural activity mapping using inducible transcription factors. It should also be recognized that the disappearance of Fos expression does not reliably indicate signal termination. Fos signal offset is complicated by the time course of Fos *per se*, stimulus-coupling uncertainty and staining parameters (Hoffman and Murphy, 2000). The induction of Fos does not always indicate that a neuron's firing rate has increased since growth factors and other neuromodulators that influence levels of second messengers may influence Fos expression without concurrently affecting firing rate of the cell (Hoffman and Murphy, 2000). In an effort to avoid the common pitfalls of neural activity mapping with inducible transcription factors, this dissertation conservatively considers only positive data after acute stimulation and not causal molecular relationships.

On the other hand, neural activity mapping using transcription factor products (i.e. proteins) like Fos, instead of its mRNA is particularly advantageous. For example, Fos proteins have greater stability and longer half lives than the mRNA which allows a greater window for detection. Since Fos synthesis has a latency of at least 30 minutes, animal handling artifact is minimized (Hoffman and Murphy, 2000). In the TIDA system, the use of antiserum directed at the conserved region of Fos proteins (i.e. the leucine zipper) that recognizes Fos and related antigens (FRA) has been shown to increase sensitivity, and allows the detection of decreases in immediate early gene expression since some FRA may have prolonged expression, unlike Fos (Hoffman et al., 1992). Major advantages of this technique are its superior cellular resolution and nuclear localization of signal. Since FRA proteins are localized within the nucleus dual immunohistochemistry can be utilized to chemically identify the neuron (via detection of a key synthetic enzyme in the cytosol) and determine its responsiveness to particular stimulus. Therefore, the technique may be applied to study subpopulations of neurons that are functionally unique and intermingled with chemically similar neurons. Furthermore, the pattern of Fos expression in these neurons can suggest a mechanism for recruitment of inactive neurons into an activated pool. Accordingly, immuno-histochemical detection of neurons expressing immediate early gene products represents an early neurochemical marker of responsiveness and permits the identification of functional heterogeneity in subpopulations of chemically similar neurons within discrete brain regions.

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F. SUBPOPULATIONS OF TIDA NEURONS

There is anatomical and histochemical evidence to divide TIDA neurons into subpopulations in the ARC. As shown in **figure 1.4**, the ARC may be divided into dorsomedial (DM) and ventrolateral (VL) regions by extending a line dorsolaterally at an angle of 30-50° from the ventrolateral aspect of the third ventricle. The magnocellular DM-ARC contains TH-immunoreactive (IR) perikarya of TIDA neurons whereas the parvocellular DM-ARC, located in the ventromedial aspect of the DM-ARC, is devoid of TH-IR and hence TIDA neurons (Halasz et al., 1985). TIDA neuronal perikarya in the DM-ARC are smaller and have more intense TH-IR staining than those of the VL-ARC. Processes from neurons in the DM-ARC are oriented dorsoventrally and terminate in the medial aspect of the median eminence (Kobayashi and Matsui, 1969; Rethelyi, 1985; Daikoku et al., 1986) in contrast to those from the VL-ARC which course mediolaterally and terminate in the lateral aspect of the median eminence (Kobayashi and Matsui, 1969; Fuxe et al., 1986).

In addition to anatomical differences, there is a discrepancy in TH- and AAAD-IR between the DM and VL subdivisions of the ARC. TIDA neurons in the DM-ARC contain both TH-IR and AAAD-IR, whereas those in the VL-ARC contain only TH-IR (Skagerberg et al., 1988; Misu et al., 1996). Therefore, neurons in the DM-ARC contain the full complement of enzymes necessary to synthesize dopamine, whereas those in the VL-ARC lack AAAD and produce only DOPA (Meister et al., 1988a; Komori et al., 1991; Misu et al., 1996; Skagerberg et al., 1988). The different characteristics of subpopulations of TIDA neurons probably reflects functional heterogeneity.

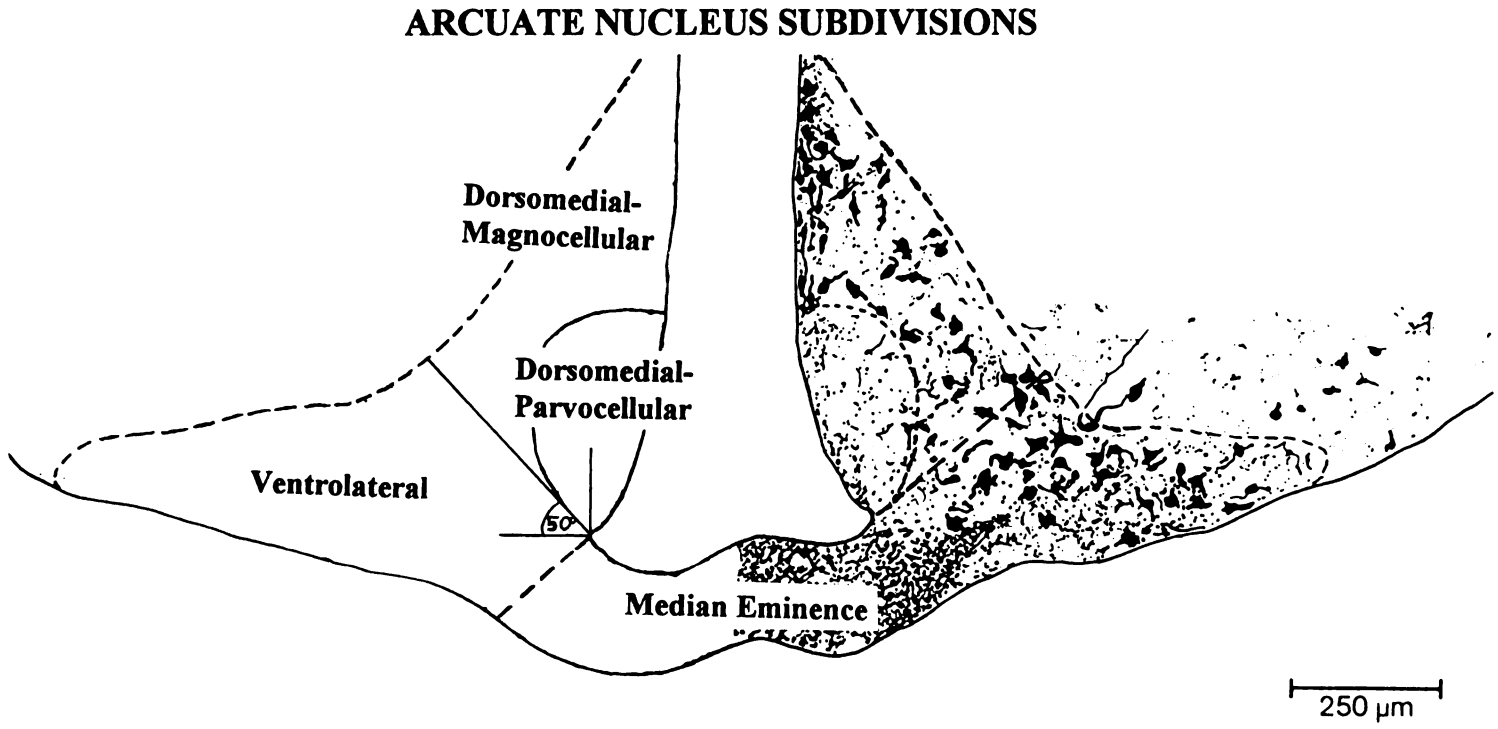

Figure 1.4 Drawing of a frontal section through the middle ARC depicting characteristic TH-IR in the ARC subdivisions. RIGHT: Drawing of TH-IR (black profiles; 1:1000 TH antiserum, PAP technique) from a photomontage of photomicrographs of the stained section (modified from Fuxe et al., 1985). LEFT: ARC subdivisions. The ARC was subdivided into dorsomedial and ventrolateral regions by extending a line dorsolaterally at an angle of 30-50° from the ventrolateral aspect of the third ventricle. The dorsomedial ARC was further subdivided into magnocellular and parvocellular aspects based upon the absence of large TH-IR neurons in the dorsomedial-parvocellular subdivision.

In support of functionally distinct TIDA neuronal populations, neurons from different subdivisions demonstrate differential responsiveness to selected stimuli. For example, activity of TIDA neurons in the DM-ARC, but not VL-ARC, is decreased after ovariectomy (Cheung et al., 1997), suggesting an estrogen-associated reproductive function. In the studies described herein, responsiveness of subpopulations of TIDA neurons is described by ARC subdivision for the detection of functional heterogeneity in the context of hyperprolactinemia.

G.

G. REGULATION OF CENTRAL DOPAMINERGIC NEURONS

1. Dopamine receptor-mediated mechanisms

The activity of central dopaminergic neurons is tightly regulated by dopamine receptor-mediated mechanisms. However, there are significant differences in the regulation of mesotelencephalic and TIDA neurons. Traditional non-selective dopamine receptor agonists (e.g. apomorphine, bromocriptine) inhibit NSDA and MLDA neuronal activity by acting at presynaptic dopamine autoreceptors and/or postsynaptic dopamine receptors on neurons that feed back to inhibit these systems. Conversely, non-selective dopamine receptor antagonists (e.g. haloperidol, raclopride) stimulate NSDA and MLDA neuronal activity by acting at these same receptors.

In contrast, the activity of TIDA neurons is not acutely affected by non-selective dopamine receptor agonists and antagonists (Gudelsky and Moore, 1976; Demarest and Moore, 1979; Moore, 1987a; 1992). For example, for up to 2 h after the administration of haloperidol, neurochemical estimates of the rates of dopamine synthesis, turnover and metabolism are increased in termini of NSDA and MLDA, but not TIDA neurons (Gudelsky and Moore, 1977). These data are consistent with the presence of dopamine autoreceptors on NSDA and MLDA, but not TIDA neurons. Although TIDA neurons are not directly responsive to dopamine receptor antagonists, studies show increased dopamine synthesis (Demarest et al., 1984; Moore, 1987a), turnover (Gudelsky and Moore, 1976) and metabolite formation 12 h after haloperidol administration (Gudelsky and Moore, 1977) as a result of their ability to increase circulating concentrations of prolactin (Moore and Lookingland, 1995). Hence, until recently, it was thought that mesotelencephalic, but not TIDA neurons are acutely regulated by dopamine receptor-

mediated mechanisms.

Yet with the advent of selective "second generation" dopamine agonists and antagonists, studies have revealed that TIDA neurons are also acutely regulated by dopamine receptor-mediated mechanisms that act independently of prolactin. TIDA neurons are regulated by inhibitory D_1 dopamine receptors (Durham et al., 1998). However, this D_1 dopamine receptor-mediated regulation is activity dependent; i.e. selective D_1 agonists have little effect on basal activity of TIDA neurons, but inhibit activated TIDA neurons (Berry and Gudelsky, 1990). On the other hand, selective D_2 dopamine receptor agonists stimulate TIDA neuronal activity (Berry and Gudelsky, 1991; Eaton et al., 1993a; 1993b) via an afferent mechanism involving disinhibition of tonically active dynorphinergic interneurons (Durham et al., 1996). However, there is little endogenous dopamine tone at these receptors under basal conditions since selective D_2 dopamine receptor antagonists have no acute effect on TIDA neurons *per se* (Eaton et al., 1993b). The opposing actions of inhibitory D_1 and stimulatory D_2 dopamine receptors could account for previous reports of lack of net effect of non-selective dopamine receptor agonists on TIDA neurons (Durham et al., 1998).

2. Prolactin feedback

As depicted in **figure 1.5**, dopamine released from TIDA neurons in the median eminence travels in the portal blood to the pituitary gland where it tonically inhibits the secretion of prolactin from anterior pituitary lactotrophs. Prolactin, in turn, feeds back to activate TIDA neurons through an unknown mechanism. Feedback activation of TIDA neurons by prolactin is comprised of interrelated tonic and inductive components (Demarest et al., 1984; 1986). In female rats, the tonic component determines the basal rate of prolactin secretion by regulating short-term changes in TIDA neuronal activity in response to acute changes in circulating prolactin concentrations. This component sets the basal level of activity of TIDA neurons with a short latency of 2-4 h.

The inductive component involves a change in the capacity of TIDA neurons to respond to prolonged periods of hyperprolactinemia (Moore et al., 1987b) and is observed in both males and females. The inductive component stimulates TIDA neuronal activity to levels 2-3 times basal (Demarest et al., 1985a; Selmanoff, 1985) 12 h following systemic (Hokfelt and Fuxe, 1972; Selmanoff, 1981; Demarest et al., 1984; 1986) or central (Annunziato and Moore, 1978) administration of exogenous prolactin, or after experimental manipulations which result in endogenous hyperprolactinemia (Moore et al., 1985). This inductive component of prolactin feedback regulation of TIDA neurons is dependent upon protein synthesis (Johnston et al., 1980) and is associated with increased expression of TH mRNA in the ARC (Arbogast and Voogt, 1991; Selmanoff et al., 1991).

Both components are interdependent in that the induction component is expressed via the tonic component (thus its expression requires elevated prolactin levels), and the

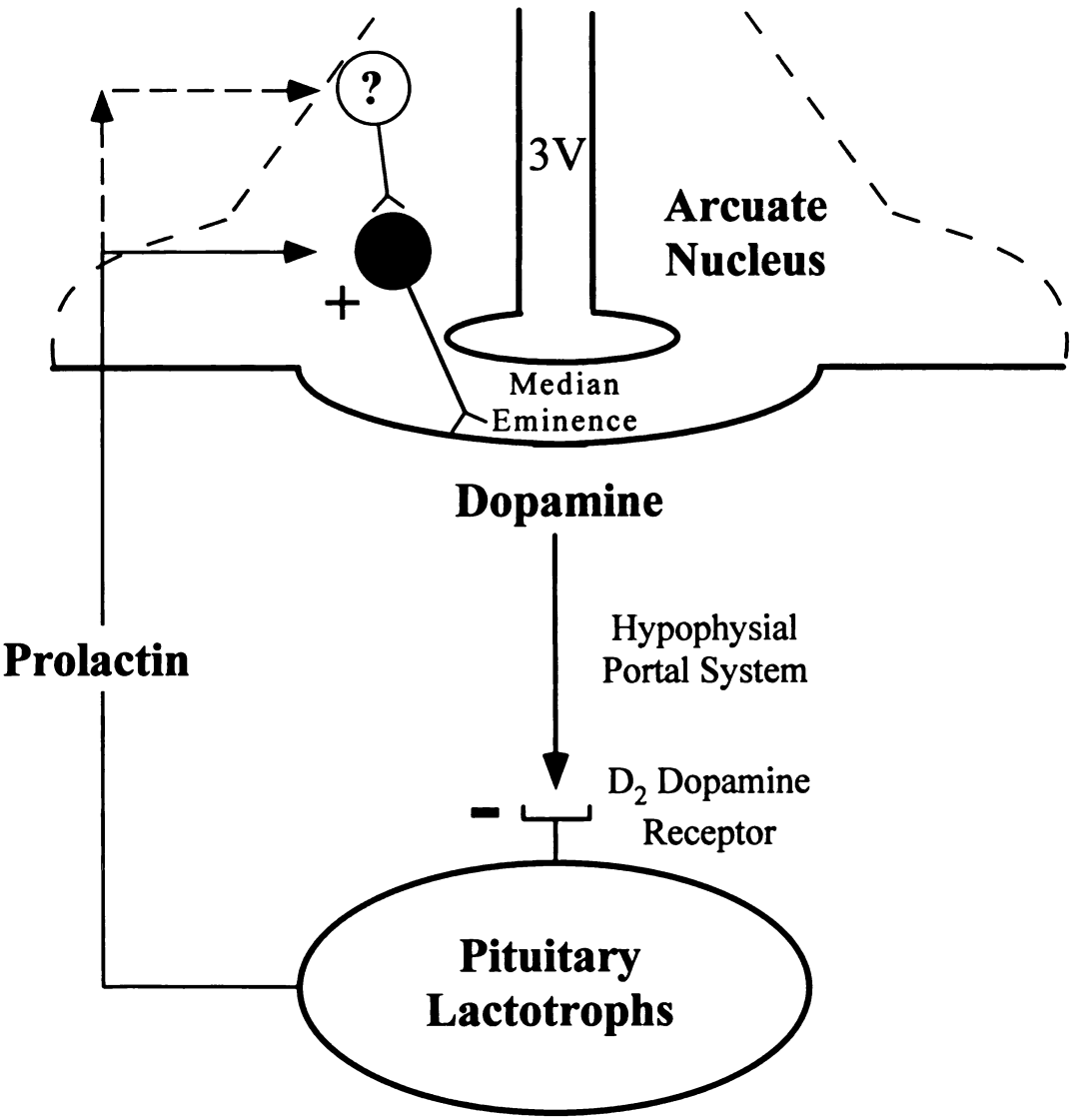

Figure 1.5 Schematic representation of the feedback regulation of TIDA neuronal activity and prolactin secretion (modified from Moore, 1987a). Abbreviations: 3V, third ventricle.

magnitude of the change in TIDA neuronal activity in response to acute changes in circulating prolactin is modulated via the inductive component by the preceding history of prolactin exposure (Demarest et al., 1984; 1986).

3. Afferent regulation of TIDA neurons

The basal tonic activity of TIDA neurons is acutely regulated by a wide variety of afferent neuronal inputs. TIDA neuronal activity is acutely inhibited during selected physiological states such as stress and lactation. Following immobilization stress the activity of TIDA neurons in female rats is acutely inhibited in a neuronal circuit involving cholinergic and 5-hydroxytryptaminergic mediators (Moore, 1987a; Moore and Lookingland, 1995). Similarly, in the lactating rat suckling rapidly inhibits TIDA neuronal activity (Demarest et al., 1983) and decreases dopamine content in the hypophysial portal blood (Ben-Jonathan et al., 1980). While the mechanism of suckling-induced inhibition of TIDA neurons is largely unknown, there is evidence that this circuit involves the endogenous mu opioid enkephalin (Callahan et al., 1996), 5-hydroxytryptamine (Demarest and Moore, 1981a) and acetylcholine (Moore and Lookingland, 1995).

Neuropeptides and neurotransmitters also acutely inhibit TIDA neuronal activity. In male rats, the endogenous kappa opioid dynorphin tonically inhibits TIDA neuronal activity (Manzanares et al., 1991; 1992), whereas the peptide galanin elicits activity-dependent inhibition of TIDA neurons, affecting only activated TIDA neurons (Gopalan et al., 1993). The excitatory amino acid neurotransmitter glutamate, acting through AMPA receptors, tonically inhibits TIDA neuronal activity via a circuit involving $GABA_A$ receptors (Wagner et al., 1994a; 1994b). Like $GABA_A$ receptors, activation of the $GABA_B$ receptor isoform also inhibits TIDA neuronal activity, yet this inhibition is not tonic (Wagner et al., 1994c).

On the other hand, TIDA neuronal activity is acutely stimulated by other

neuropeptides and neurotransmitters. For example, the peptides bombesin (Manzanares et al., 1991; Toney et al., 1992a; Lin and Pan, 1993), neurotensin (Gudelsky et al., 1989; Pan et al., 1992; Lin and Pan, 1993) and alpha-melanocyte stimulating hormone (Lindley et al., 1990a) each acutely stimulates TIDA neuronal activity. Additionally in female rats, glutamate acts via NMDA receptors to tonically stimulate TIDA neurons (Wagner et al., 1993). However, there exist no data regarding the role of afferent neurotransmitters or neuropeptides in the delayed inductive activation of TIDA neurons by prolactin.

H. NEUROTENSIN

1. Synthesis

Twenty five years ago the tridecapeptide neurotensin was isolated from bovine hypothalamus (Carraway and Leeman, 1973), sequenced (Carraway and Leeman, 1975a) and synthesized (Carraway and Leeman, 1975b). The biological activity of this peptide resides predominantly in the carboxyl terminal hexapeptide region (i.e. residues 8-13) whose amino acid identity is highly conserved across vertebrates (Granier et al., 1982).

Synthesis of neurotensin commences with transcription of neurotensin mRNA from its respective gene. Since the fourth exon codes for the entire neurotensin precursor, the elaboration of splice variants is precluded. Regulation of neurotensin gene expression depends upon interactions of various transcription factors with the promoter region of the gene. The promoter includes consensus sequences for several cis-acting elements, including an AP-1 site. Mutations of the AP-1 site vastly inhibit the expression of neurotensin mRNA following the application of known inducers (Kislauskis and Dobner, 1990) suggesting that AP-1 complexes have a role in the regulation of this gene.

Similar to other neuropeptides, neurotensin is synthesized as part of a larger precursor protein (170 amino acids). A single copy of neurotensin exists near the carboxyl terminus (Dobner et al., 1987; Kislauskis et al., 1988). Proneurotensin is proteolytically cleaved during axonal transport, rather than in the neuronal cell body (Nicot et al., 1995). A consistent product is derived from processing without numerous post-translational variants.

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2. Central distribution

Neurotensin exhibits heterogeneous regional distribution (Jennes et al., 1982; Uhl et al., 1976, 1977). Neurotensin-IR is abundant in the central nervous system (Carraway and Leeman, 1976; Goedert et al., 1985). Neurotensin-IR is found in particularly high levels in the hypothalamus, and the majority of neurosecretory cells capable of neurotensin synthesis are located in the ARC (Merchenthaler and Lennard, 1991). The neurotensin-IR fibers from these neurons densely ramify over the neurohemal zone of the median eminence (Jennes et al., 1982; Ibata et al., 1984; Kiss et al., 1987).

Immunohistochemical colocalization studies suggest the existence of two functionally distinct groups of neurotensin neurons located in different sudivisions of the ARC. In the DM-ARC, neurotensin is colocalized with TH (Hokfelt et al., 1984; Everitt et al., 1986; Meister and Hokfelt, 1988b), galanin and GABA (Ibata et al., 1983; Hokfelt et al., 1984; Everitt et al., 1986; Meister and Hokfelt, 1988b; Ciofi et al., 1993). In the VL-ARC, neurotensin may be colocalized with growth hormone-releasing hormone (Everitt et al., 1986; Meister and Hokfelt, 1988b; Niimi et al., 1991) and/or TH (Hokfelt et al., 1984; Everitt et al., 1986; Meister and Hokfelt, 1988b) but not AAAD (Meister et al., 1988b; Okamura et al., 1988a). There are conflicting reports concerning the extent to which TH and neurotensin coexist in the DM-ARC. Some studies report coexistence is extensive (Hokfelt et al., 1984; Everitt et al., 1986) while others report it is rare (Meister and Hokfelt, 1988b).

3. Receptors

Radioligand binding experiments have revealed two classes of neurotensin binding sites, low and high affinity (Mazella et al., 1983; Kitabgi et al., 1985). The low affinity sites selectively bind levocabastine, whereas the high affinity sites are relatively levocabastine-insensitive (Kitabgi et al., 1987; Schotte et al., 1988).

The high affinity neurotensin binding sites have been classified as NTR_1 (Mazella et al., 1985). Cloning and expression studies demonstrate that this receptor belongs to the superfamily of G-protein-coupled receptors and is linked to multiple signal transduction pathways. Known NTR_1-associated second messenger systems include PKC-dependent intracellular calcium mobilization (Berry and Gudelsky, 1992), formation of cAMP (Yamada et al., 1993), phosphatidyl inositol hydrolysis (Watson et al., 1992) and the formation of nitric oxide (Marsault and Frelin, 1992). The low affinity levacobastine-sensitive neurotensin binding sites, also belonging to the superfamily of G-protein-coupled receptors, have been classified as NTR_2 (Chalon et al., 1996).

Neurotensin receptors are distributed throughout the hypothalamus in moderate density (Rostene and Alexander, 1997). In the hypothalamus, the NTR_2 isoform is predominantly expressed by glial cells, whereas the NTR_1 isoform is predominantly expressed by neurons (Schotte et al., 1988). NTR_1 is distributed throughout the mediobasal hypothalamus on both nerve termini and perikarya (Dana et al., 1989; Boudin et al., 1996). Within the mediobasal hypothalamus, NTR_1 mRNA has been localized within neurons of the DM-ARC (Goedert et al., 1984; Meister et al., 1989), including TIDA neurons *per se* (Alexander, 1997).

4. Central actions

Neurotensin stimulates central dopaminergic systems (e.g. NSDA and MLDA systems), both as a neuromodulator and neurotransmitter. Neurotensin modulates the effects of other transmitters on neuronal firing rate even at concentrations too low to influence basal activity (Baldino et al., 1985a). For example, neurotensin promotes repetitive firing of cholinergic neurons of the basal forebrain (Alonso et al., 1994) by abrogating the prolonged calcium-dependent, potassium-mediated hyperpolarization following stimulation (Farkas et al., 1994). Neurotensin also facilitates basal and potassium-induced dopamine release in central dopaminergic systems (Okuma and Osumi, 1982).

Within the ARC of hypothalamic slices, neurotensin produces excitatory electrophysiological effects that persist in calcium-free medium *in vitro* (Herbison et al., 1986; Lin and Pan, 1993). Furthermore, *in vivo*, administration of neurotensin produces dose-dependent activation of TIDA (Widerlov et al., 1982; Gudelsky et al., 1989; Pan et al., 1992), NSDA (Pinnock, 1985) and MLDA (Widerlov et al., 1982) neurons.

Given the distribution of neurotensin and its receptors within the mediobasal hypothalamus and the stimulatory effects of this peptide on TIDA neurons, it is possible that neurotensin has a role in the delayed inductive effects of prolactin on TIDA neurons.

I. THESIS OBJECTIVE

The studies described herein aim to test the hypothesis that neurotensin mediates the delayed inductive activation of TIDA neurons by prolactin. If neurotensin mediates the delayed inductive activation of TIDA neurons by prolactin, then: prolactin will elicit temporally-dependent neurochemical effects and changes in immediate early gene expression in TIDA neurons; the neurotensin receptor antagonist SR-48692 will block the delayed-inductive activation of TIDA neurons by prolactin; and prolactin will elicit temporally-dependent changes in immediate early gene expression in neurotensin neurons of the mediobasal hypothalamus.

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2. MATERIALS AND METHODS

A. ANIMALS

All experiments were performed using gonadally-intact male and female Long-Evans rats weighing 200-225 g at time of purchase from Harlan Laboratories (Indianapolis, IN). Animals of the same gender were housed 1-3 animals per cage, maintained in a temperature- (22 ± 1°C), humidity- (30 - 60%) and light-controlled (illuminated between 06:00 - 18:00 h) environment, and provided with food (Harlan Teklad; Bartonville, IL) and tap water *ad libitum*. In experiments involving female rats, estrous cycles were monitored daily by microscopic examination of epithelial cytology of vaginal lavage, and only those animals displaying a vaginal smear consistent with the first day of diestrus after two consecutive cycles were used. In order to minimize the effects of stress, animals were allowed to accommodate to their housing for an average of 3-7 days before use, were handled daily in a manner similar to that required of the day of experimentation and were decapitated in a room separate from yet adjacent to their housing.

B. DRUGS

The dose of haloperidol (Sigma Chemical Co., St. Louis, MO) was calculated as its free base and dissolved in 0.3% tartaric acid (Mallinckrodt Chemical Works, St. Louis, MO). All other doses of drugs were calculated as the appropriate salt. 2- (1- (7-Chloro-4-quinolinyl) -5- (2,6-dimethoxy-phenyl) pyrazol-3-yl) carbonylaminotricyclo (3.3.1.1.$^{3.7}$) decan-2-carboxylic acid (SR-48692) was kindly supplied by Dr. Danielle Gully (Sanofi Recherche; Toulouse, France) and solubilized in 0.9% saline containing 10% dimethyl

sulfoxide (DMSO; Sigma). Distilled water was used to dissolve biological rat prolactin (NIDDK-rPRL-B-7; Lot # AFP-6452B) generously supplied by Dr. A.F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Hormone and Pituitary Program (NHPP).

The anesthetic Equithesin was utilized in two instances: (1) prior to intracerebroventricular cannulation surgery, and (2) minutes prior to transcardial perfusion and fixation for immunohistochemistry experiments. Equithesin was prepared by stirring 42.51 g chloral hydrate (Sigma), 9.72 g sodium pentobarbital (Sigma) and 21.26 g magnesium sulfate in 443 ml of warmed 1,2-propanediol (propylene glycol). After these compounds were completely dissolved, 120 ml of 95% ethanol was added and the total volume was brought to 1000 ml with double distilled water. The final solution was measured into 100 ml aliquots and stored in amber drug bottles at room temperature.

Antiserum to rat prolactin (rPRL-B-6; NIDDK NHPP) was generated previously in rabbits. Prolactin was dissolved in normal saline, emulsified in complete Freund's adjuvant (Sigma) in a ratio of 1:2 (v/v), and 1 mg equivalents were injected s.c. at multiple sites in a total volume of 1 ml. Booster injections of 200 μg prolactin emulsified in saline and incomplete Freund's adjuvant were repeated monthly until a high antibody titer was obtained.

Prolactin antiserum specificity and titer were determined by radioimmunoassay. Specificity was determined by incubating prolactin antiserum (1:6,000) and radiolabeled rat prolactin with various amounts (0-1 μg) of unlabeled rat prolactin, growth hormone, thyroid-stimulating hormone and luteinizing hormone (NIDDK NHPP). Unlabeled rat prolactin competed on an equimolar basis with radiolabeled prolactin for binding to the

prolactin antiserum with a lower limit of sensitivity of 50 pg/tube. In contrast, prolactin antiserum did not bind with up to 100 ng/tube of growth hormone or 1 μg/tube of thyroid-stimulating hormone or luteinizing hormone (Toney et al., 1992b).

Doses and routes of administration of drugs, biological rat prolactin and prolactin antiserum are indicated in the legends of the appropriate figures.

C. SURGICAL MANIPULATIONS

1. Intracerebroventricular cannulation and injections

Intracerebroventricular (i.c.v.) injections were administered to freely moving rats via cannula guides which were implanted 3-14 days prior to the experiment. Rats were anesthetized with Equithesin (3 ml/kg, i.p.) and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set at 2.4 mm below the horizontal plane (Paxinos and Watson, 1986). A 23-gauge stainless steel guide cannula, 10 mm in length, was implanted 1.4 mm right lateral to bregma and 3.2 mm below the dura mater (Paxinos and Watson, 1986), and anchored to the skull with stainless steel screws (Small Parts Inc., Miami Lakes, FL) and dental cement (Dentsply, York, PA). Following surgery rats were injected (0.2 ml/rat, i.m.) with Combiotic (200,000 U/ml procaine penicillin G and 250 μg/ml dihydrostreptomycin sulfate; Pfizer Inc., New York, NY) per AUCAUC guidelines and a stainless steel stylet (10 mm in length) was inserted in the guide cannula to maintain its patency. Male rats were allowed 3-5 days for recovery prior to the experiment. Female rats were utilized after two regular estrous cycles following the surgery (i.e. 8-14 days recovery). At the time of the experiment, prolactin or vehicle was injected into freely moving rats over a minute interval in a volume of 3 μl. The volume

was delivered with a 10 µl Hamilton microsyringe (Reno, NV) connected by a 30 cm length of PE-10 tubing (Clay Adams/Beckton-Dickinson, Sparks, MD) to a 30-gauge stainless steel injector which protruded 1 mm beyond the tip of the guide cannula and into the lateral ventricle. Cannula placement was verified post-mortem in appropriate frontal brain sections with the aid of a dissecting microscope and only those animals with the tip of the cannula tract in the right lateral cerebral ventricle were used in the study.

2. Intravenous (external jugular) catheterization and injections

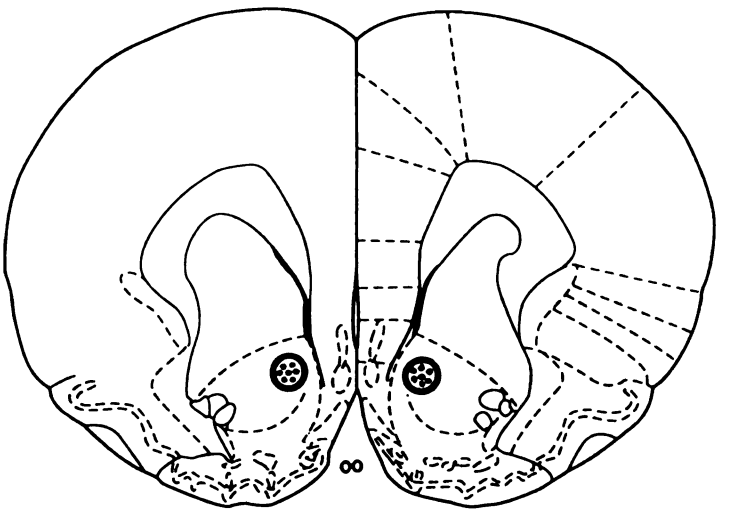
Intravenous injections of prolactin antiserum or its normal rabbit serum vehicle were administered to conscious, freely moving rats via catheters implanted on the morning of the experiment. Rats were anesthetized with diethyl ether (J.T. Baker Chemical Co., Philipsburg, NJ), the right external jugular vein was exposed and its cephalic aspect ligated. A segment of polyethylene tubing (I.D. 0.58 mm, O.D. 0.96 mm; PE-50; Clay Adams/Becton-Dickinson, Sparks, MD) full of 12.5% (v/v) heparinized (Schein Pharmaceutical Inc., Florham Park, NJ) saline was inserted through a nick in the distal right external jugular vein. The tubing was gently threaded caudally to the level of the right atrium where placement and patency of the catheter was verified by aspiration of dark venous blood. The catheter was then anchored at two points along its course through the external jugular vein, and the free end of the catheter was tunneled s.c. over the right shoulder and out a small opening over the dorsal aspect of the cervical spine. The incision was closed with two surgical staples. The catheter was purged with 200 µl of heparinized saline, trimmed to 2-4 cm external length and capped with the tip of a common pin. The entire procedure was completed in 7-14 minutes. After surgery,

individually caged rats were allowed to recover for 3-4 hours prior to use. At the time of intravenous injections, the patency of the catheter was verified via aspiration of blood. Conscious, freely moving rats with patent catheters were slowly injected with prolactin antiserum (200 µl/rat) or its normal rabbit serum vehicle (200 µl/rat) followed by 200 µl of 12.5% (v/v) heparinized saline.

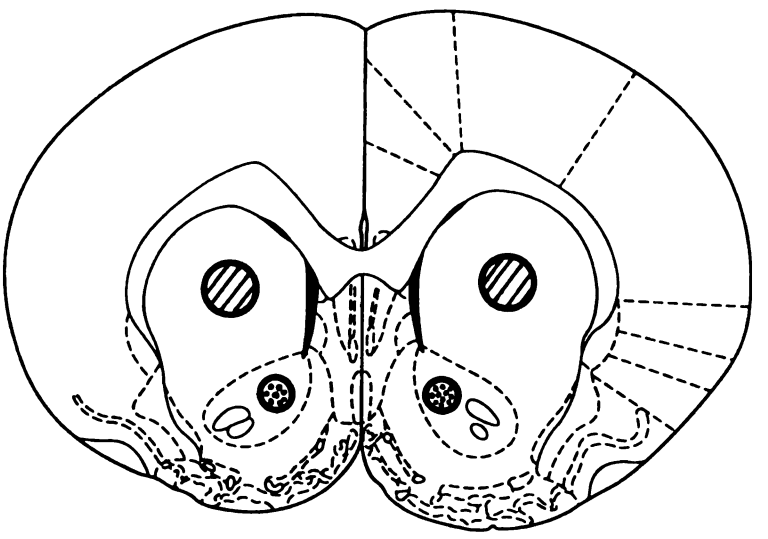
D. NEUROCHEMISTRY

1. Tissue preparation

Following appropriate treatments rats were decapitated and brains were quickly removed. Brainstems were transected at mid-cerebellar level and placed upright on their cut surfaces on aluminum foil directly over dry ice. Each brain was sliced into consecutive 600 µm frontal sections beginning at approximately interaural 11.20 mm and bregma 2.20 mm (Paxinos and Watson, 1986) and extending caudad in a -9 °C cryostat (Harris model CTD, International Equipment Co., Needham, MA) and thaw-mounted on a glass slide. Using a modification (Lookingland and Moore, 1984) of the method of Palkovits (Palkovits, 1973; Palkovits, 1974; Palkovits and Brownstein, 1983) the terminal fields of TIDA, NSDA and MLDA neurons were microdissected as follows. With the aid of a stereoscope and a round 21-gauge punch tool (fashioned from a 21-gauge stainless steel hypodermic needle; Beckton-Dickinson Co., Rutherford, NJ), the nucleus accumbens was bilaterally dissected from sections 1 and 2 (as illustrated in **figure 2.1**) and placed in 100 µl of tissue buffer (50 mM sodium phosphate; 30 mM citrate buffer; pH 2.5). Similarly, the striatum was bilaterally dissected from section 2 (**figure 2.1**) and placed in 100 µl of tissue buffer. The median eminence was dissected from sections 8 and



SECTION #1



SECTION #2

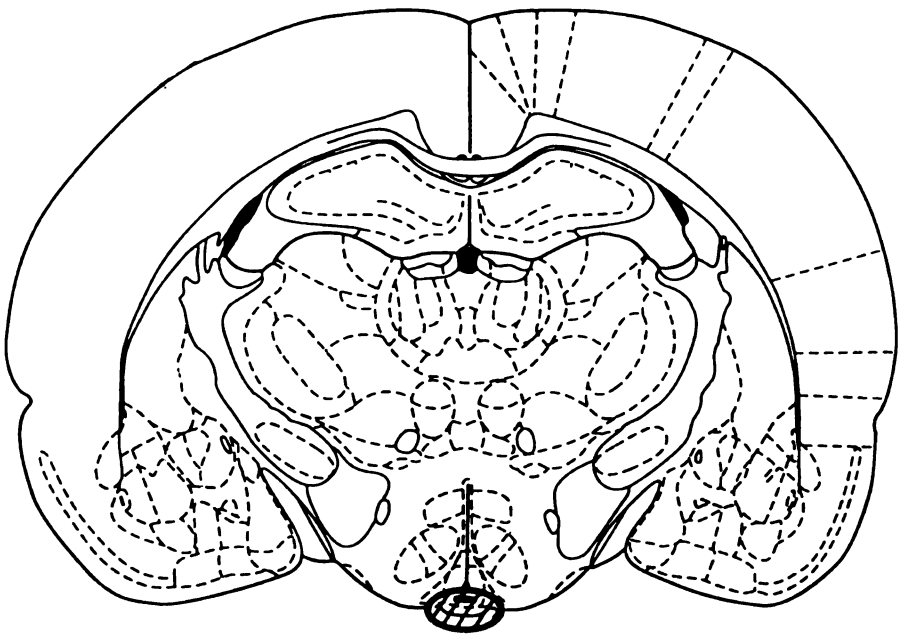
Figure 2.1 Anatomical locations of nucleus accumbens (stippled) and striatum (cross-hatched) samples collected for neurochemical analysis. With the aid of a stereoscope, the nucleus accumbens was bilaterally microdissected from the 1st (bregma 2.20 mm) and 2nd (bregma 1.60 mm) serially collected 600 μm frontal sections using a round 21-gauge punch tool. Similarly, the striatum was bilaterally microdissected from the 2nd section using a round 18-gauge punch tool. Figures modified from Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1997.

9 using an oval 18-gauge punch tool (as illustrated in **figure 2.2**) and placed in 50 µl of tissue buffer containing 15% methanol. All tissues were stored at –20 °C until assayed.

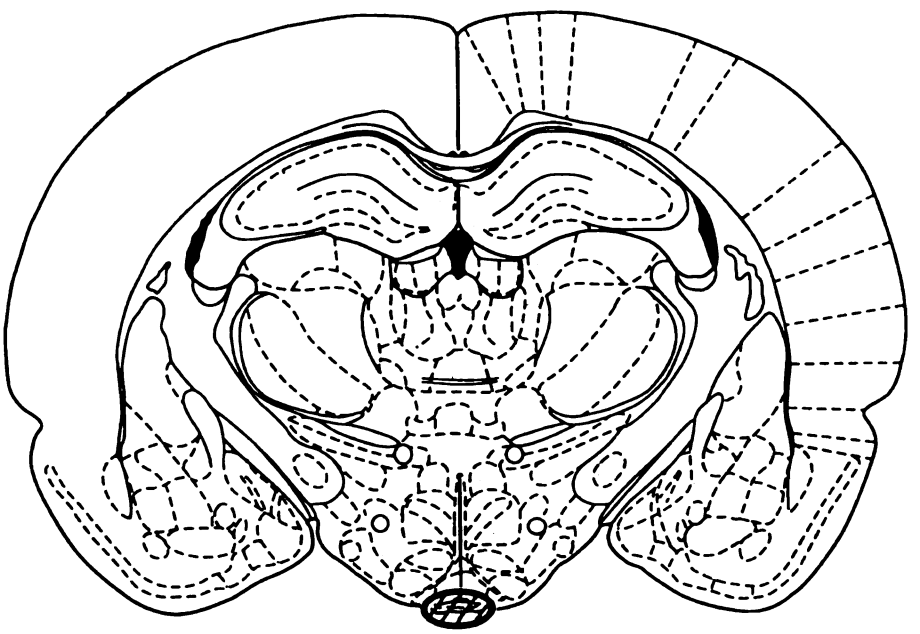
2. Assays

On the day of the assay, samples were thawed, sonicated for 3 s (Heat System-Ultrasonics, Plainview, NY USA) and centrifuged (microfuge model #152, Beckman Instruments, Inc., Palo Alto, CA) for either 30 s (samples from median eminence and nucleus accumbens) or 45 s (samples from striatum). Supernatants from median eminence and nucleus accumbens were transferred via Hamilton microsyringe to a second set of microtubes over ice; their volumes were brought to 65 µl/tube with tissue buffer (50 mM sodium phosphate; 30 mM citrate buffer; pH 2.5) containing 15% methanol. Supernatants from striatum were similarly transferred to a second set of microtubes, however the final volume of these samples was brought to 100 µl/tube. Volumes of eluent for the various brain regions were previously established based upon relative concentrations of catecholamines and the optimal range of assay detection.

The remaining tissue pellets were dissolved with 100 µl (median eminence and nucleus accumbens) or 200 µl (striatum) of 1.0 N sodium hydroxide for at least 24 h at room temperature and spectrophotometrically assayed for protein (Lowry et al., 1951) as follows. For median eminence samples, 100 µl of undiluted protein solution was added by Hamilton microsyringe to labeled glass cuvettes (12 x 75 mm) which served as the spectrophotometric reaction vessels. Fifty µl of each striatum protein sample was combined with 50 µl of 1.0 N sodium hydroxide in appropriate glass cuvettes to attain a four-fold dilution of the total protein content of the original microtube. Similarly, 50 µl of



SECTION #8



SECTION #9

Figure 2.2 Anatomical locations of median eminence (cross-hatched) samples collected for neurochemical analysis. With the aid of a stereoscope, the median eminence was microdissected from the 8th (bregma –2.56 mm) and 9th (bregma –3.30 mm) serially collected 600 μm frontal sections using an oval 18-gauge punch tool. Figures modified from Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1997.

each nucleus accumbens protein sample was combined with 50 µl of 1.0 N sodium hydroxide in appropriate glass cuvettes to attain a 2-fold dilution of the total protein content of the original microtube. Standards containing 12.5, 25 and 50 µg of bovine serum albumin (Sigma) dissolved in 100 µl 1.0 N sodium hydroxide were also transferred into appropriate glass cuvettes for the protein assay. For colorimetric reaction, 1 ml reagent A (100:1:1, 0.19 M sodium carbonate : 40 mM cupric sulfate : 70 mM potassium sodium tartrate) was added to each cuvette, vortexed and allowed 10 min incubation. Subsequently, each cuvette received 100 µl of reagent B (1:1, 2 N Folin & Ciocalteu's phenol reagent (Sigma): double distilled water), was vortexed and allowed 30 min incubation. Protein content of a sample was estimated by its absorbance on a Gilford spectrophotometer (model Stasar III, Gilford Instrument Laboratories, Inc., Oberlin, OH) tuned to 700 nm wavelength relative to standards run in the same reaction.

DOPAC and dopamine contents of standards and tissue samples were determined concurrently using ESA Coulochem Liquid Chromatographs (model 5100A; Bedford, MA) with electrochemical detection and Waters solvent delivery systems (model 510; Milford, MA) set at a flow rate of 0.9 ml/min as described previously (Chapin et al., 1986; Lindley et al., 1990b). Fifty µl of supernatant was injected onto a C18 reverse phase analytical column (5 µm spheres; 250 x 4.6 mm; Biophase ODS, Bioanalytical Systems, Inc., West Lafayete, IN) which was guarded by a precolumn cartridge filter (5 µm spheres; 30 x 4.6 mm). High-performance liquid chromatography mobile phase consisted of 50 mM sodium phosphate buffer (pH 2.65 – 2.70), 30 mM citric acid, 0.1 mM disodium ethylenediamine tetraacetic acid (EDTA; Sigma), 0.30% sodium octyl sulfate and 20-25% methanol. Depending on the characteristics of the column,

ingredients of the mobile phase were slightly adjusted to maintain separation of compounds of interest and minimize total retention times for efficiency (Chapin et al., 1986).

Amine content in the striatum was determined using an electrochemical detector (LC4A, Bioanalytical Systems, Inc.) equipped with a TL-5 glassy carbon electrode set at a potential of +0.75 V relative to a silver/silver chloride reference electrode. Since the median eminence and nucleus accumbens samples contain lesser amounts of DOPAC and dopamine than the striatum, supernatants from these regions were analyzed on more sensitive electrochemical detection systems which consisted of a single coulometric electrode (Coulochem model #5100A; ESA, Bedford, MA) in series with dual electrode analytical cells (models #5021, ESA). The potential of the conditioning cell electrode for these systems was set at +0.40 V; analytical cell electrodes were set at +0.12 V and -0.40 V, relative to an internal silver reference electrode. The current signal from the second electrode of the analytical cell was recorded by a Hewlett-Packard integrator (model #3395; Hewlett-Packard, Avondale, PA). Use of coulometric electrodes increases the sensitivity of the measurement of compounds of interest, and with the analytical electrodes set up in an oxidation-reduction mode, only compounds oxidized at the first electrode and reduced at the second electrode are detected. For example, the solvent front, composed predominantly of ascorbic acid, is minimized in this way as ascorbic acid does not back-reduce and is therefore not detected. The net effect of the oxidation-reduction mode of detection is increased selectivity and reduced noise signal (Lindley et al., 1990b).

The contents of DOPAC and dopamine in each sample were determined by comparing peak heights reported by the integrator with those of the standards run on the same day. The lower limit of sensitivity of this assay for DOPAC and dopamine was approximately 2 - 5 pg per sample.

E. PROLACTIN ASSAYS

Following appropriate treatments rats were decapitated and trunk blood was collected in heparinized glass vacutainer tubes (143 USP units/tube; Becton-Dickinson Co., Franklin Lakes, NJ) maintained on ice. Whole blood was centrifuged (model DPR-6000, International Equipment Co., Needham, MA) for 20 min at 2000 rpm and plasma collected and stored at -20°C in glass vials containing 100 µl saturated sodium citrate solution until assayed for prolactin.

1. Radioimmunoassay for prolactin

Serum prolactin concentrations were determined by double-antibody radioimmunoassay using the procedure developed by Drs. Parlow and Raiti of the National Hormone and Pituitary Program (NHPP), National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK; Rockville, MD). This procedure was optimized and performed as follows.

Prior to assay, a range (0.03 - 2.00 ng/50µl) of standards were made using highly purified rat prolactin (NIDDK rat PRL-RP-3; potency, 30 IU/mg as determined in pigeon local crop assay by Nicoll) diluted in 0.1 M phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA; Sigma, RIA grade, fraction V), aliquotted 50 µl per

microtube and stored at -20°C for no more than 3 months prior to use. Anti-rat prolactin S-9 antisera (NIDDK) was reconstituted, diluted to 1:35,000 using 0.1 M PBS containing 0.05 M EDTA and 1:400 normal rat serum (Sigma), aliquotted 100 ml/bottle and stored at -20°C until use. The solution of 0.1 M PBS/0.05 M EDTA/1:400 normal rat serum was stored at -20°C in 1 ml aliquots. One hundred µg of rat prolactin for iodination (rat PRL-I-6; NIDDK) was dissolved in 125 µl of 0.01 M $NaHCO_3$ (pH 8.6), aliquotted 5 µl/sterile siliconized 1.5 ml Eppendorf tube, and stored at -80°C for no more than 1 week before use.

On assay day 1, a set of rat prolactin standards, a tube of 0.1 M PBS/0.05 M EDTA/1:400 NRS, a tube of rat serum pool, rat prolactin primary antiserum and samples were thawed. All solutions were gently vortexed prior to use. Buffer (0.1 M PBS, 2% BSA, pH 7.4) was added to each disposable glass (12 x 75 mm) cuvette such that after subsequent addition of standard, sample or serum pool volume, the final volume was 500 µl. Cuvettes for the measurement of non-specific background and primary antibody controls received 500 µl of buffer. Appropriate cuvettes were hand-pipetted 50 µl of standards, three different volumes of unknown samples and 10, 20 and 50 µl of pooled serum. The three dilutions for samples were established in previous experiments from the 20 - 80% (linear) binding range for the expected concentration of prolactin. For example, if prolactinemia in the range of 2 – 250 ng/ml was expected, 10, 20 and 50 µl volumes of sample were utilized. If prolactinemia in the range of 0.1 – 70 ng/ml was expected, 50, 100 and 200 µl volumes of sample were utilized. Repeating pipettor systems (e.g. Digiflex automatic pipette) were not used in these assays because personal observations revealed that: (1) rapid aspiration of solutions produced air bubbles in the

syringe, and (2) clots within plasma samples often plugged the syringe, contributing to error and variability. All cuvettes were run in duplicate. Two hundred µl of 1:35,000 anti-rat prolactin S-9 antiserum was added to antibody control, standard, serum control and sample cuvettes; alternatively, 200 µl of 0.1 M PBS/0.05 M EDTA/1:400 NRS was added to non-specific background control cuvettes. Cuvettes were gently vortexed, covered and incubated at 4°C for 18 - 24 h.

On assay day 2, prolactin was iodinated by the chloramine-T method (Niswender et al., 1968) and prolactin tracer added to cuvettes as follows. The siliconized tube of rat prolactin for iodination (4 µg rat PRL-I-6 per 5 µl aliquot) was thawed and utilized as the reaction vessel. Accordingly, 20 µl of 0.5 M PBS (pH 7.4) was added and gently vortexed. Ten µl of $Na^{125}I$ (1 mCi; Amersham Corporation, Arlington Heights, IL) was added to the reaction vessel, gently vortexed and allowed to incubate for 15 s. To commence the oxidation of ^{125}I to prolactin, 10 µl chloramine-T (Sigma) solution (1.5 mg chloramine-T/ml 0.05 M PBS, pH 7.4) was added to the reaction vessel, gently vortexed and incubated for no more than 30 s. Dr. Parlow warned that only 4 - 5 µg of chloramine-T should be utilized for every µg prolactin to be iodinated as oxidative damage to prolactin could interfere with assay performance. A preliminary experiment testing duration of chloramine-T reaction (at 4 µg chloramine-T/µg prolactin) showed evidence of prolactin damage (shoulder on curve probably represents prolactin fragments) after 45 s incubation, and established 30 s incubation as optimal (**figure 2.3**). To quench the oxidative reaction, 50µl of sodium metabisulfite (Sigma) solution (2.5 mg sodium meta-bisulfite / ml 0.05 M PBS, pH 7.4) was added to the reaction vessel, gently vortexed and

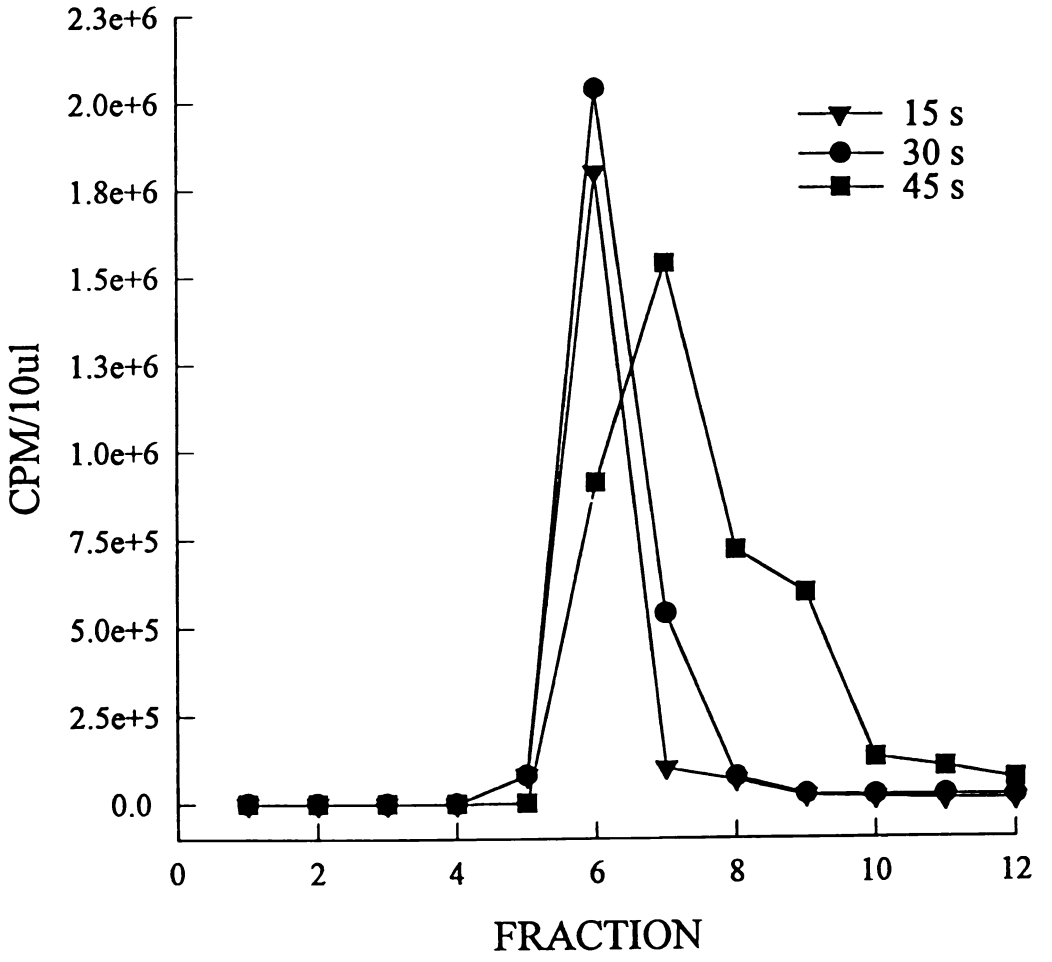


Figure 2.3 Effects of duration of chloramine-T oxidative reaction on iodinated prolactin curve morphology. In a siliconized tube, 4 μg rat prolactin for iodination in 5 μl $NaHCO_3$ was combined with 20 μl of PBS. Ten μl of $Na^{125}I$ (1 mCi) was added to the reaction vessel, gently vortexed and allowed to incubate for 15 s. Ten μl chloramine-T solution (1.5 mg/ml PBS) was added to the reaction vessel, gently vortexed and incubated for 15, 30 or 45 s. To quench the oxidative reaction, 50 μl of sodium metabisulfite solution (2.5 mg/ml PBS) was added to the reaction vessel, gently vortexed and allowed to incubate for 15 s. The reaction mixture was fractionated on a PD-10 Sephadex G-25M column and 10 μl of each fraction was quantified in an automated gamma counter.

allowed to incubate for 15 s.

The reaction mixture was fractionated on a PD-10 Sephadex G-25M column (Pharmacia Biotech, Uppsala, Sweden) previously rinsed with 5 ml of 0.05 M PBS (pH 7.4). Phosphate buffered saline was added dropwise to keep the column filter moist and facilitate the elution of the reaction mixture. A preliminary experiment utilized the Lowry protein assay to demonstrate that rat prolactin (rat PRL-I-6) elutes from the PD-10 Sephadex G-25M column in the 6th or 7th serially collected fraction (10 drops or 500 µl/fraction) (**figure 2.4**). To be conservative, the first 12 fractions were collected into test tubes containing 1 ml of 0.1 M PBS and 2% BSA (pH 7.4).

The collected fractions were vortexed and 10 µl from each test tube was counted for 1 min in an Micromedic plus series automatic gamma counter (model 4/600, ICN Biomedicals, Inc., Costa Mesa, CA) coupled to an IBM PC running bundled AGC operating software (version 2.07). The fraction containing maximal cpm (usually the 7th fraction) was diluted with 0.1 M PBS/2% BSA (pH 7.4) to 15,000 cpm/100 µl solution. After appropriate radioactivity of the iodinated prolactin tracer solution was established, each cuvette received 100 µl of tracer, was vortexed, covered and allowed to incubate for 18 - 24 h at 4°C.

On day 3, goat anti-rabbit gamma globulin antiserum (Accurate Chemical & Scientific Corporation, Westbury, NY) was reconstituted and diluted 1:200 with 0.1 M PBS (pH 7.4). All cuvettes except total count controls received 200 µl of secondary antiserum, were gently vortexed, covered and incubated for 18 - 24 h at 4°C.

On day 4, antibody-bound radiolabeled prolactin was precipitated and quantified. All cuvettes except total count controls received 2 ml of cold 0.1 M PBS (pH 7.4)

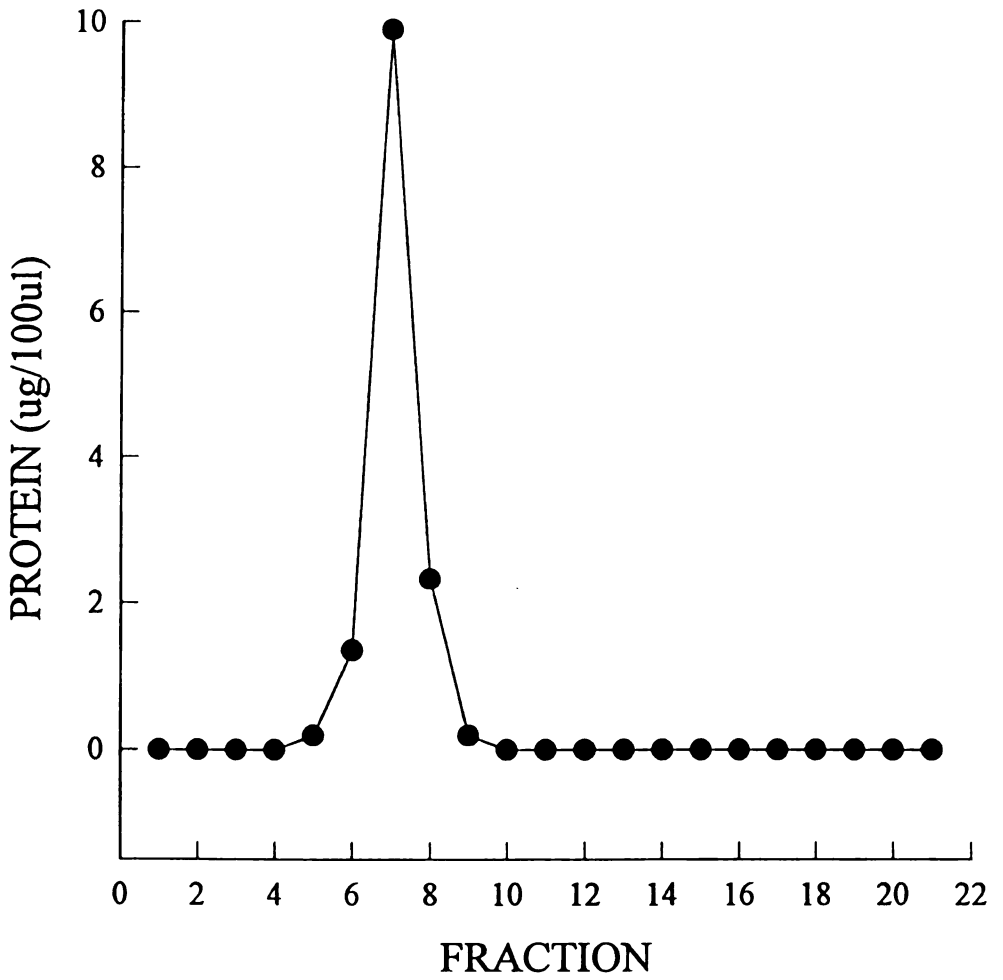


Figure 2.4 Characterization of PD-10 Sephadex G-25M column for fractionalization of rat prolactin for iodination via Lowry protein assay. A column was rinsed with 5 ml PBS prior to use. The solution containing 100 μg rat prolactin dissolved in 10 ml PBS was added to the column and separated into 500 μl fractions. The collected fractions were vortexed and 100 μl of each fraction was analyzed for protein content via Lowry protein assay relative to standards run on the same day.

containing 5% polyethylene glycol (Sigma; 8,000 mw) and were centrifuged (3,000 rpm; Damon/IEC model PR-6000; Needham Heights, MA) for 20 min at 4°C. After centrifugation, supernatants were decanted and cuvettes were air-dried. Dry cuvettes were loaded into racks and quantified in an Micromedic plus series automatic gamma counter coupled to an IBM PC running bundled AGC operating software.

The procedure as described routinely yielded 40 - 50% binding and an R value of 0.99. Using a 100 µl aliquot of plasma, the lower limit of sensitivity was 0.1 ng/cuvette, and the intra- and interassay coefficients of variance were 4.6% and 6.7%, respectively. The primary antiserum for rat prolactin showed no significant cross-reactivity with rat growth hormone, thyroid-stimulating hormone, follicle-stimulating hormone, or luteinizing hormone as reported by the supplier.

2. Bioassay for prolactin

Antiserum to prolactin interferes with plasma prolactin measurement by double antibody radioimmunoassay (Hattori et al., 1994). Consequently, plasma prolactin was measured indirectly by rat Nb2 node lymphoma cell bioassay in experiments in which prolactin antiserum was administered. The rat Nb2 lymphoma cell bioassay for prolactin is based on the observation that rat Nb2 node cell lines replicate (approximately 16-fold over 72 h) in a dose-dependent manner in response to prolactin (Tanaka et al., 1980; Gout et al., 1980; Noble et al., 1980). The assay is sensitive (lower limit 1.22 ng/ml), specific (clonal expansion is blocked by prolactin antiserum, and unaffected by rat growth hormone; Tanaka et al., 1980) and reproducible, yielding results comparable to prolactin radioimmunoassay (Lawson et al., 1982).

Frozen plasma samples were packed in a cooler of dry ice and shipped to Dr. David M. Lawson (Wayne State University School of Medicine, Detroit, MI) who performed conventional rat Nb2 node lymphoma cell bioassays for prolactin as follows. The Nb2 node cell line was originally derived from Drs. Noble, Beer and Gout (University of British Columbia, Vancouver, B.C., Canada) and stored in liquid nitrogen. Prior to the assay, viable cells were recovered by thawing in a 37°C bath of filter-sterilized Fischer's medium containing 10% fetal calf serum, 10% horse serum, 1 x 10^{-4} M 2-mercaptoethanol, 50 IU/ml penicillin and 50 µg/ml streptomycin (stock medium). The cells were then centrifuged (300 x g, 10 min), the pellet gently resuspended in stock medium and dispensed into tissue culture flasks which were supplied with a 95% air - 5% CO_2 gas mixture. The medium was changed after 24 h and the flasks were incubated at 37°C for 72 h. Cell numbers were monitored and when they reached 1x 10^6 cells/ml, they were diluted 10-fold with stock medium. Cells were used for assay when the doubling time was in the range of 18 - 20 h. Twenty four h before assay the cells were repeatedly centrifuged and resuspended in stock media containing 10%, 1% and 0% fetal calf serum. After washing the cells of fetal calf serum, the cells were diluted to 1 x 10^5 cells/ml aliquot in Fischer's medium and one aliquot was added to each well of a 24-well tissue culture plate.

Standard rat prolactin (NIDDK-RP-3; 11 IU/mg) was diluted with Fischer's medium to give a final concentration of 0.01 - 40.00 ng/50 µl. Each sample was diluted with Fischer's medium to three concentrations and brought to a final volume of 50 µl. Fifty µl of standards and samples were added to duplicate wells of the tissue culture plates. The plates were incubated in a humidified incubator at 37°C for 72 h with

constant exposure to 95% air - 5% CO_2 gas mixture. The entire well content was removed, diluted 10-fold with particle-free electrolyte solution (Hematall; Fisher Scientific, Itasca, IL) and counted in a model B Coulter Counter (Fisher Scientific) using a 70 μm aperture and a 100 μl mercury manometer. Cell counts from the Coulter Counter were entered into a Micromedic MAAC microprocessor and analyzed using a slight modification of a point to point linear RIA program (Lawson et al., 1982).

F. IMMUNOHISTOCHEMISTRY

1. Tissue preparation

After appropriate treatments, rats were anesthetized with Equithesin (4 ml/kg, i.p.) and transcardially perfused with cold 0.9% saline (~20 ml/rat) followed by 0.1 M PBS (pH 7.4) containing 4% paraformaldehyde (Sigma; 150 - 180 ml/rat) delivered via peristaltic pump (rabbit model; Rainin Instrument Co., Woburn, MA) at a flow rate of 10 ml/min. Brains were removed and post-fixed in 20 ml of 0.1 M PBS containing 4% paraformaldehyde (pH 7.4) for 18 - 24 h at 4°C. Brains were cryoprotected in 20 ml aliquots of 0.1 M PBS (pH 7.4) containing 20% sucrose changed daily until brains equilibrate and sink. Brains were sectioned at -15°C using an IEC Minotome cryostat (International Equipment Co., Needham Heights, MA) into 30 μm frontal sections through the entire rostrocaudal extent of the arcuate nucleus, beginning approximately 2.0 mm posterior to bregma (Paxinos and Watson, 1986). Sections were collected as 8 sets of serial sections in a multiwell tray of cryoprotectant (30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, 30% (v/v) ethylene glycol in 0.1 M PBS, pH 7.2; Watson et al., 1986) and stored at -20°C until required for immunohistochemistry.

Tissue collection and storage in this fashion facilitates tissue handling and minimizes variation in experiments. Considering that each well contains sections no closer than 240 μm, correction factors for error due to duplicate quantification of the same cells in adjacent sections are unnecessary. This method also provides two replicates of rostral, middle and caudal regions of the arcuate nucleus (**figure 2.5**) per well of tissue.

2. FRA and tyrosine hydroxylase detection

Two non-adjacent wells of tissue per brain (i.e. 4 replicate sections per region) were thawed and washed of cryoprotectant in 4 rinses (15 min/rinse) of 0.05 M Tris (GibcoBRL, Grand Island, NY)-buffered saline, pH 7.6 (TBS). Unless otherwise stated, tissue was continually gyrated (100 rpm) during latent periods between solution changes on a gyrotory shaker table (model G2; New Brunswick Scientific Co., Edison, NJ) at room temperature. Tissues were treated with 3% hydrogen peroxide (Sigma) in TBS for 10 min, and rinsed (4 x 10 min, TBS) to minimize non-specific background due to endogenous peroxidases. Tissues were treated with 1% sodium borohydride (w/v; Sigma) in TBS for 20 min, and rinsed (6 x 10 min, TBS) to neutralize non-specific binding due to reactive aldehydes from fixative solutions. Tissues were treated with 3% normal donkey serum (Sigma) in TBS containing 0.3% (v/v) of the detergent Triton-X100 (TX; Research Products International Corp., Elk Grove Village, IL) for 30 min to block non-specific binding with secondary antiserum. Tissue was treated with 1% (v/v) avidin solution (Avidin-Biotin Blocking kit; Vector Laboratories, Burlingame, CA) in

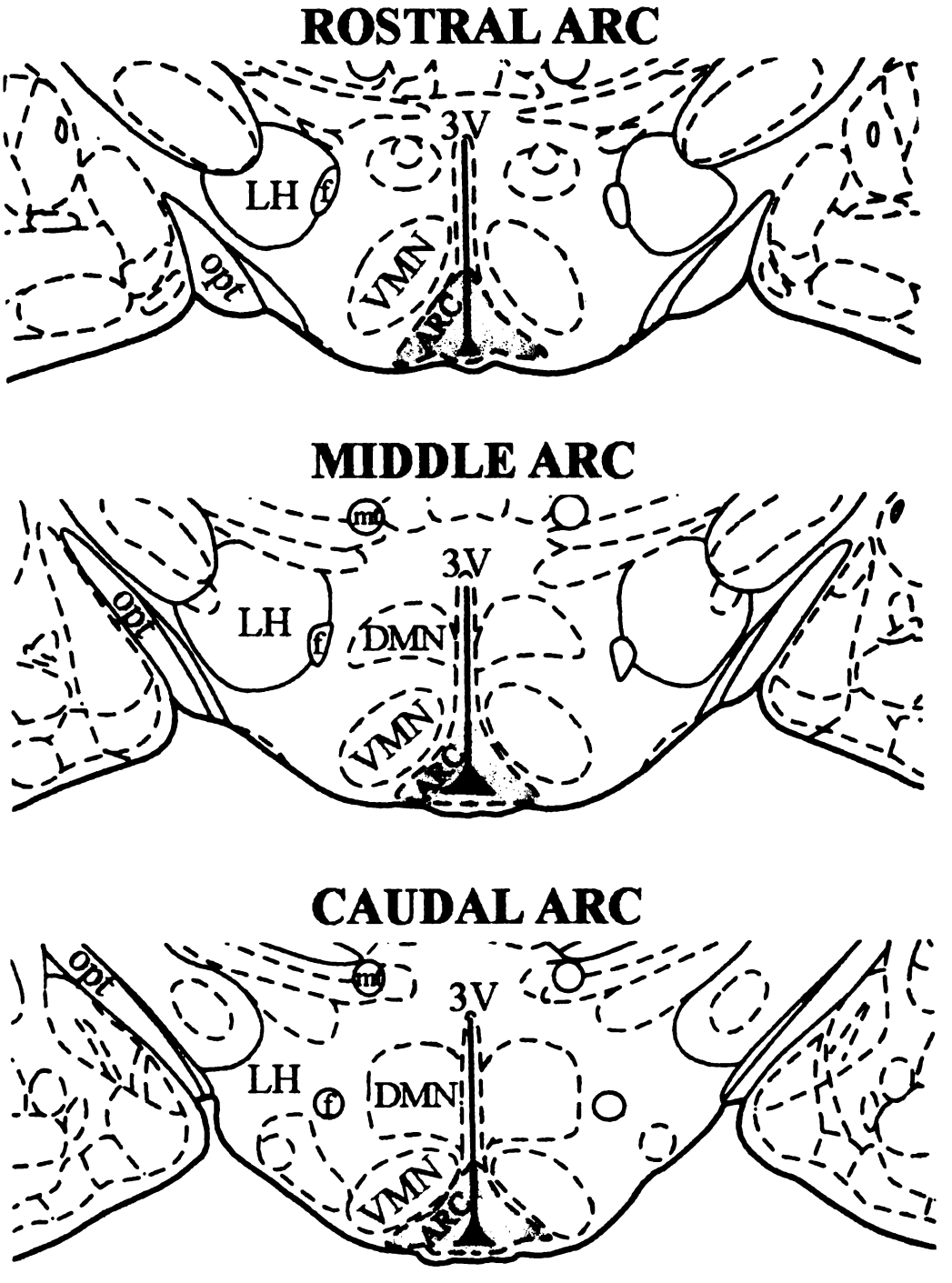


Figure 2.5 Representative rostrocaudal regions of the ARC highlighted in ventral hypothalamic frontal sections. Distances posterior to bregma for these regions were 2.3 - 2.8 mm for rostral ARC, 2.8 - 3.3 for middle ARC and 3.3 – 3.8 mm for caudal ARC (Paxinos and Watson, 1997). 3V, third ventricle; ARC, arcuate nucleus; DMN, dorsomedial hypothalamic nucleus; f, fornix; LH, lateral hypothalamus; opt, optic tract; VMN, ventromedial hypothalamic nucleus.

TBS-TX for 20 min, and rinsed (3 x 10 min, TBS-TX) to prevent non-specific binding to endogenous avidin. Tissues were treated with 1% (v/v) biotin solution (Avidin-Biotin Blocking kit; Vector Laboratories) in TBS-TX for 20 min, and rinsed (3 x 10 min, TBS-TX) to decrease non-specific binding due to endogenous biotin.

Anti-FRA antiserum (OA-11-824, Genosys Biotechnologies, The Woodlands, TX) was generated in sheep against a synthetic peptide derived from a conserved region of the c-fos gene common to all FRA genes (amino acid sequence: M-F-S-G-F-N-A-D-Y-E-A-S-S-S-R-C), including FOS, FRA1, FRA2, and FOSB (Hesketh, 1995). FRA antiserum specificity was determined by the manufacturer and another research group (Curran et al., 1985). Preliminary dual immunohistochemical titration studies determined that the optimal dilution for FRA antiserum was 1:5,000. Accordingly, anti-FRA antiserum was diluted in TBS-TX containing 1.5% normal donkey serum, added to tissue and incubated for 48 h on a gyrotory shaker table at 4°C.

After 4 rinses of TBS-TX (15 min/rinse), tissue-bound FRA antiserum was detected with biotinylated donkey anti-sheep IgG antiserum (Accurate Chemical & Scientific Corp.) diluted 1:500 in TBS-TX and incubated for 1.5 h. Excess secondary antiserum was removed with 6 x 10 min rinses in TBS-TX and remaining signal from the antibody complex was amplified by the avidin-biotin complex system (Vector Laboratories; Hsu et al., 1981). Vector ABC Elite kit reagents A (avidin) and B (biotinylated horse radish peroxidase) were diluted 1:200 (v/v) in TBS-TX and allowed to stand for 30 min before incubation with tissue for 1.5 h. Unbound avidin-biotin complex was washed from tissue via 3 TBS-TX rinses (10 min/rinse), followed by 3 rinses of similar duration in sodium acetate (0.175 M CH_3COONa, pH 8.2; JT Baker).

The chromagen utilized for FRA localization was nickel-intensified 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) freshly made using the Hsu recipe (Hsu et al., 1982). Tissue was incubated in 0.175 M sodium acetate containing 0.05% (w/v) DAB, 0.01% hydrogen peroxide, and 2.5% nickel sulfate (w/v; Sigma) for 3 - 5 min as determined by rate of color development. After 3 rinses of sodium acetate and 3 rinses of TBS-TX (10 min/rinse), tissues were treated with 3% normal horse serum (Sigma) in TBS-TX for 30 min to block non-specific binding with secondary antiserum.

Monoclonal (IgG_1 subclass) anti-tyrosine hydroxylase antiserum (#22941, Incstar Corp., Stillwater, MN) was generated in mouse against a fragment of purified tyrosine hydroxylase isolated from rat PC12 cells. The antiserum is believed to have wide species cross-reactivity because it recognizes an epitope derived from the midportion of the tyrosine hydroxylase molecule where extensive species homology exists. The manufacturer reports that this tyrosine hydroxylase antiserum did not cross react with dihydropterdine reductase, dopamine-β-hydroxylase, phenylethanolamine-N-methyl-transferase, phenylalanine hydroxylase or tryptophan hydroxylase as determined by western analysis. Preliminary dual immunohistochemical titration studies determined that the optimal dilution for tyrosine hydroxylase antiserum was 1:1,000. Accordingly, anti-tyrosine hydroxylase antiserum was diluted 1:1,000 in TBS-TX containing 1.5% normal horse serum, added to tissue and incubated for 48 h on a gyrotory shaker table at 4°C.

After 4 rinses of TBS-TX (15 min/rinse), tissue-bound tyrosine hydroxylase antiserum was detected with biotinylated horse anti-mouse IgG antiserum (BA-2000; Vector Laboratories) diluted 1:200 in TBS-TX and incubated for 1 h. Excess secondary antiserum was removed with 6 x 10 min rinses in TBS-TX. The chromagen utilized for

tyrosine hydroxylase localization was rhodamine-tagged avidin D (A-2002; Vector Laboratories). Tissues were incubated rhodamine-tagged avidin diluted 1:500 in TBS-TX for 1 h in darkness and rinsed in TBS (6 x 10 min/rinse). This double immunohistochemistry procedure routinely allowed visualization of tyrosine hydroxylase-immunoreactive (red-orange fluorescent) neurons which contained FRA-immunoreactive (black) nuclei (**figure 2.6**).

3. FRA and neurotensin detection

Tissue was thawed, rinsed of cryoprotectant, and non-specific background was minimized via treatments with 3% hydrogen peroxide, 1% sodium borohydride, 3% normal donkey serum, 1% (v/v) avidin solution, and 1% (v/v) biotin solution, as described for FRA and tyrosine hydroxylase detection. Anti-FRA antiserum (OA-11-824, Genosys Biotechnologies) was diluted in TBS-TX containing 1.5% normal donkey serum, added to tissue and incubated for 48 h on a gyrotory shaker table at 4°C. Tissue-bound FRA antiserum was detected with biotinylated donkey anti-sheep IgG antiserum (Accurate Chemical & Scientific Corp.) diluted 1:500 in TBS-TX and incubated for 1.5 h, and remaining signal from the antibody complex was amplified by Elite avidin-biotin complex kit (Vector Laboratories) diluted 1:200 (v/v) in TBS-TX and incubated with tissue for 1.5 h. The chromagen utilized for FRA localization was nickel-intensified DAB freshly made using the Hsu recipe (Hsu et al., 1982) and incubated with tissue 3 - 5 min as determined by rate of color development. After 3 rinses of sodium acetate and 3 rinses of TBS-TX, tissues were treated with 3% normal goat serum (Sigma) in TBS-TX

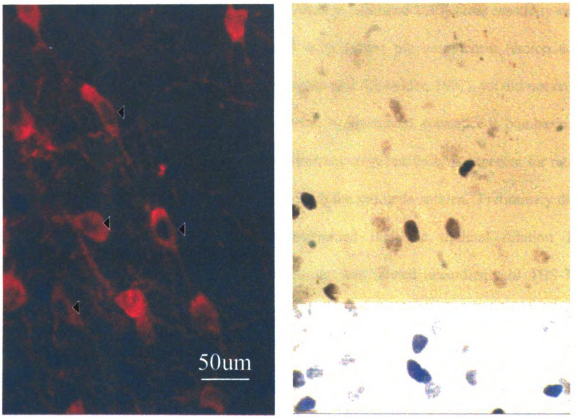


Figure 2.6 Digitized photomicrographs of tissue immunohistochemically processed for the detection of TH and FRA. Fluorescent TH-IR neurons under dark field optics (left panel) and the same image under bright field optics (right panel) showing blackened FRA-IR nuclei colocalized to some, but not all, of these neurons. Arrowheads represent TH-IR neurons with FRA-IR nuclei; i.e. double-labeled neurons.

for 30 min to block non-specific binding with secondary antiserum.

Polyclonal anti-neurotensin antiserum (#IHC 7351, Lot #971456, Peninsula Laboratories, Belmont, CA) was generated in rabbit against synthetic neurotensin (amino acid sequence: E-L-Y-E-N-K-P-R-R-P-Y-I-L; Carraway and Leeman, 1975). The manufacturer reports that this neurotensin antiserum exhibited 100% cross reactivity with synthetic neurotensin, 58% cross reactivity with guinea pig neurotensin (amino acid sequence: E-L-Y-E-N-K-*S*-R-R-P-Y-I-L; Rostene and Alexander, 1997), yet did not cross react with neurotensin 8-13, porcine neuromedin N, kinetensin, substance P, bombesin or eledoisin by western analysis. One hundred percent cross reactivity is expected for rat as this species has identical amino acid identity with the synthetic antigen. Preliminary dual immunohistochemical titration studies determined that the optimal dilution for neurotensin antiserum was 1:8,000. Antiserum was diluted accordingly in TBS-TX containing 1.5% normal goat serum, added to tissue and incubated for 48 h on a gyrotory shaker table at 4°C.

After 6 rinses of TBS-TX, tissue-bound neurotensin antiserum was detected with biotin-SP-conjugated AffiniPure goat anti-rabbit IgG, $F(ab')_2$ fragment specific antiserum (#111-065-006; Lot #38262; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in TBS-TX and incubated for 2 h. Excess secondary antiserum was removed with 6 x10 min rinses in TBS-TX, and remaining signal from the antibody complex was amplified by the avidin-biotin complex system. Vector ABC Elite kit reagents were diluted 1:200 (v/v) in TBS-TX and incubated with tissue for 2 h. Unbound avidin-biotin complex was rinsed from tissue via 3 TBS-TX rinses (10 min/rinse), followed by 3 rinses of similar duration in TBS-TX (pH 7.2).

The chromagen utilized for neurotensin localization was DAB *without nickel* freshly made according to the Hsu recipe (Hsu et al., 1982). Tissue was incubated in TBS-TX (pH 7.2) containing 0.05% (w/v) DAB, and 0.01% hydrogen peroxide for 7 - 10 min as determined by rate of color development. Tissues were rinsed (10 min/rinse) 3 times with TBS-TX (pH 7.2) and 3 times with TBS-TX prior to storage or mounting. This double immunohistochemistry procedure routinely allowed visualization of neurotensin-immunoreactive (brown) neurons which contained FRA-immunoreactive (black) nuclei (**figure 2.7**).

4. Neurotensin and tyrosine hydroxylase detection

Tissue was thawed, rinsed of cryoprotectant, and non-specific background was minimized via treatments with 3% hydrogen peroxide, 1% sodium borohydride, 3% normal goat serum, 1% (v/v) avidin solution, and 1% (v/v) biotin solution, as described above. Anti-neurotensin antiserum was diluted 1:8,000 in TBS-TX containing 1.5% normal goat serum, added to tissue and incubated for 48 h on a gyrotory shaker table at 4°C. Tissue-bound neurotensin antiserum was detected with biotin-SP-conjugated AffiniPure goat anti-rabbit IgG, $F(ab')_2$ fragment specific antiserum (Jackson ImmunoResearch Laboratories) diluted 1:500 in TBS-TX and incubated for 2 h, and remaining signal from the antibody complex was amplified by Elite avidin-biotin complex kit (Vector Laboratories) diluted 1:200 (v/v) in TBS-TX and incubated with tissue for 2 h. The chromagen utilized for neurotensin localization was nickel-intensified DAB freshly made and incubated with tissue 7 - 10 min as determined by rate of color development. After rinsing, tissues were treated with 3% normal horse serum in TBS-TX

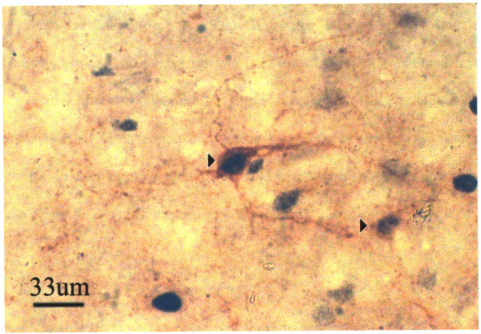


Figure 2.7 Digitized bright field photomicrograph of tissue immunohistochemically processed for the detection of NT and FRA. Clear or blackened FRA-IR nuclei were localized within brown NT-IR perikarya. Arrowheads represent NT-IR neurons with FRA-IR nuclei; i.e. double-labeled neurons.

for 30 min to block non-specific binding with secondary antiserum.

Anti-tyrosine hydroxylase antiserum (Incstar Corp.) was diluted 1:1,000 in TBS-TX containing 1.5% normal horse serum, added to tissue and incubated for 48 h on a gyrotory shaker table at 4°C. Tissue-bound tyrosine hydroxylase antiserum was detected with biotinylated horse anti-mouse IgG antiserum (Vector Laboratories) diluted 1:200 in TBS-TX and incubated for 1 h. The chromagen utilized for tyrosine hydroxylase localization was rhodamine-tagged avidin D (Vector Laboratories) diluted 1:500 in TBS-TX and incubated with tissues for 1 h in darkness. This double immunohistochemistry procedure allowed visualization of tyrosine hydroxylase- (red-orange fluorescent) and neurotensin- (black) immunoreactive perikarya in the same section by alternating between dark and bright field optics, respectively (**figure 2.8**).

5. Slide preparation and visualization

Following immunohistochemistry, sections were stored in TBS at 4°C in darkness until mounted. Glass slides were washed, rinsed 5 times with distilled water and air-dried. Clean slides were twice dipped in warm (60°C) subbing solution containing 0.188% gelatin (300 bloom swine; Sigma) 0.0188% chromium potassium sulfate (Sigma) in double-distilled water. Sections were floated in TBS onto subbed slides and air-dried. Dry slides were loaded into racks and dehydrated via graded series of filtered ethanol baths (70%, 90% and 100%; 10 min/rinse). Tissues were delipidated in a series of xylene baths (2 x 15 min/rinse) and slides coverslipped using DPX mountant (Fluka, St. Louis, MO).

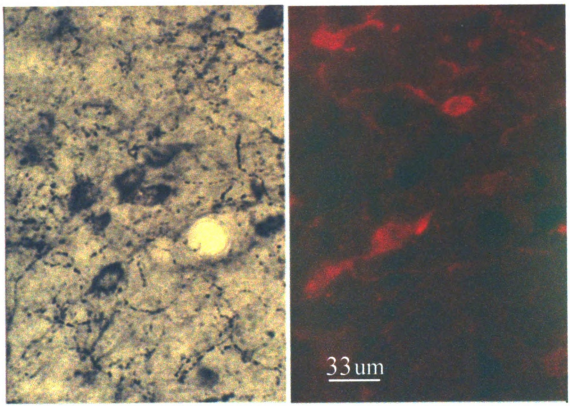


Figure 2.8 Digitized photomicrographs of tissue immunohistochemically processed for the detection of NT and TH. Blackened NT-IR perikarya visualized under bright field optics (left panel) and the same image under dark field optics (right panel) showing localization of fluorescent TH-IR.

Tissues processed by immunohistochemistry using DAB or DAB-nickel as the chromagen were viewed by standard bright field microscopy on a Leitz Laborlux microscope (model S, Wetzlar, Germany). When rhodamine was the chromagen, dark field fluoromicroscopy was employed on the same microscope (equipped with mercury lamp and model 103 housing, Leitz). Bright field photomicrographs were captured on Motophoto (color) 400 film using an interfaced photosystem (model Wild MPS28/32, Leitz) with automated exposure. Since dark field images were routinely over-exposed using the automated photosystem, these photomicrographs were manually exposed for 1-15 s. Images were digitized using a scanner (model 5100C, Hewlett Packard) and PrecisionScan (version 2.0, Hewlett Packard) software/driver, and labeled using PhotoShop software (version 5.0, Adobe, San Jose, CA). Magnifications are indicated in the legends of the appropriate figures.

G. QUANTIFICATION & STATISTICAL ANALYSES

Group averages from prolactin radioimmunoassays were based upon individual animal averages derived from 2 replicates at each of 3 dilutions, and only values which were extrapolated from the linear portion of the standard curve (20 – 80% binding of radiolabeled prolactin) were included.

In all immunohistochemistry experiments, 2 - 6 sections per region (rostral, middle or caudal ARC **figure 2.5**) per animal were bilaterally quantified at 400x magnification for total numbers of single- and double-labeled cells in each ARC subdivision: dorsomedial-magnocellular, dorsomedial-parvocellular and ventrolateral (**figure 1.4**). Consequently, the contribution of one animal was based on no fewer than 6

sections, and as many as 18 sections through the arcuate nucleus. Individual animal means were generated and compiled to produce a group average. Since raw counts were not normally distributed, they were systematically transformed prior to statistical analyses using a square root function.

Statistical analyses of the data were conducted using SigmaStat software (version 2.0, Jandel Scientific, San Rafael, CA). Statistical analyses of two groups with normal distribution and equal variance were conducted using Student's t-test. If normality or equal variance failed, the Mann-Whitney rank sum test was utilized. Statistical analyses of greater than two groups with normal distributions and equal variances were conducted using one way analysis of variance followed by Dunnett's test for multiple comparisons versus a control group. If normality or equal variance tests failed, statistical analyses were conducted using Kruskal-Wallis one way analysis of ranks followed by Dunn's test for multiple comparisons versus a control group (Steel and Torrie, 1960). Differences were considered significant if the probability of error was less than 5% and the desired power of tests was set as 0.80. Group averages ± one standard error of the mean were utilized for representation in all graphs and tables.

3. REGULATION OF TIDA NEURONS BY PROLACTIN

A. INTRODUCTION

The mammalian brain contains several different dopaminergic neuronal systems. The largest and most studied is the mesotelencephalic system, comprised of NSDA and MLDA neurons. NSDA neurons have perikarya in the substantia nigra pars compacta and axons that terminate in the striatum. These neurons regulate sensorimotor integration and motor movement. MLDA neurons have perikarya in the ventral tegmental area and axons that terminate in limbic structures including the nucleus accumbens, olfactory tubercles, septum, amygdala, and various cortical regions. These neurons regulate motivational behavior. TIDA neurons have perikarya in the arcuate nucleus and axons that terminate in the median eminence. Dopamine released in the median eminence from TIDA neurons travels in the hypophysial portal blood to the anterior pituitary where it tonically inhibits prolactin secretion. Much less is known about the TIDA neuronal system in comparison to the "classical" mesotelencephalic system, hence the primary focus of this chapter will be upon the regulation of TIDA neurons by prolactin, incorporating the NSDA and MLDA neuronal systems for comparison.

TIDA neurons lack dopamine autoreceptors characteristic of NSDA and MLDA systems (Demarest and Moore, 1979), and instead, are regulated in part by the feedback effects of prolactin (for review see Moore and Lookingland, 1995). Feedback activation of TIDA neurons by prolactin is comprised of interrelated tonic and inductive components (Demarest et al., 1984; 1986). The tonic component determines the basal rate of prolactin secretion by regulating short-term changes in TIDA neuronal activity in response to acute changes in circulating prolactin concentrations. This component is

unique to females and sets the basal level of activity of TIDA neurons with a latency of 2-4 h. The inductive component involves a change in the capacity of TIDA neurons to respond to more prolonged periods of hyperprolactinemia (Moore et al., 1987a) and is observed in both males and females. The inductive component stimulates TIDA neuronal activity to levels 2-3 times basal (Demarest et al., 1985b; Selmanoff, 1985) 12 h following systemic (Hokfelt and Fuxe, 1972; Selmanoff, 1981; Demarest et al., 1984; 1986) or central (Annunziato and Moore, 1978) administration of exogenous prolactin, or after experimental manipulations which result in endogenous hyperprolactinemia (Moore et al., 1985).

Much of what is known regarding prolactin feedback regulation of TIDA neurons is based upon pharmacological studies employing non-selective dopamine receptor agonists (e.g. bromocriptine; Demarest et al., 1985b) and antagonists (e.g. haloperidol; Demarest and Moore, 1979) to alter endogenous prolactin secretion. While it is generally accepted that these ligands bind to D_2 receptors on lactotrophs and thereby act indirectly via changes in circulating prolactin to regulate the activity of TIDA neurons (Moore and Lookingland, 1995), these neurons are also acutely regulated by a D_2 receptor-mediated mechanism which acts independently of prolactin (Eaton et al., 1993b; Durham et al., 1997; 1998). Moreover, many early neurochemical estimates of TIDA neuronal activity employed drugs (e.g. α-methyltyrosine, NSD 1015) that by design inhibit dopamine biosynthetic enzymes (Moore, 1987b). Since newly synthesized dopamine is preferentially released from TIDA neurons (Moore and Lookingland, 1995), these inhibitors reduce dopamine release, and thereby remove dopamine inhibition of prolactin secretion (Reymond and Porter, 1982). Because these drugs were administered to all

experimental groups (including controls), a period of hyperprolactinemia preceding and during the time of sampling may have confounded the results, especially those concerning the early time course effects of prolactin on TIDA neurons.

The overall aims of the experiments described in this study were to characterize the temporal aspects of the delayed inductive activation of TIDA neurons by endogenous hyperprolactinemia and confirm that prolactin mediates the stimulatory effects of haloperidol on these neurons. The underlying hypothesis to be tested is that delayed activation of TIDA neurons by prolactin is dependent upon a period of sustained hyperprolactinemia for both the initiation and maintenance of this response. If the delayed inductive activation of TIDA neurons by prolactin is dependent upon a period of sustained hyperprolactinemia for both initiation and maintenance of the response, then exposure to 3 or 6 h periods of hyperprolactinemia followed by 9 or 6 h periods, respectively, of hypoprolactinemia will prevent the delayed inductive activation of TIDA neurons. Dopaminergic neuronal activity will be estimated in these studies by measuring the concentrations of the dopamine metabolite DOPAC in terminal regions of these neurons which requires no pharmacologic intervention. Specific prolactin antiserum (PRL-AB) will be utilized to immunoneutralize circulating prolactin thereby avoiding the use of dopaminergic agonists.

B. RESULTS

Haloperidol is a D_2 dopamine receptor antagonist that stimulates prolactin secretion via blockade of the tonic inhibitory action of dopamine on pituitary lactotrophs. As shown in **figure 3.1**, systemic administration of haloperidol (1 mg/kg; sc) increased plasma prolactin concentrations in male rats to peak levels by 1 h, and maintained a state of hyperprolactinemia for at least 12 h. Haloperidol also increased median eminence DOPAC concentrations by 6 and 12 h post-injection, but had no effect on dopamine concentrations in this region. Systemic administration of haloperidol induced rapid (by 1 h) and prolonged (through 12 h) increases in DOPAC concentrations in both the striatum (**figure 3.2**) and nucleus accumbens (**figure 3.3**) and acute decreases in dopamine concentrations in both the striatum (for 1-3 h; **figure 3.2**) and nucleus accumbens (for 1-6 h; **figure 3.3**).

The time course effects of PRL-AB on concentrations of plasma prolactin in male rats pretreated with haloperidol were investigated to determine the capacity of PRL-AB to immunoneutralize endogenous hyperprolactinemia (**figure 3.4**, upper panel). DOPAC and dopamine concentrations in the median eminence of these rats were measured concurrently (**figure 3.4**, lower panel). As expected, plasma prolactin concentrations in normal rabbit serum-treated rats were elevated 1 h after coadministration of haloperidol. In haloperidol-treated rats, a single injection of undiluted PRL-AB (200 µl/rat; iv) caused a prompt (by 1 h) decrease in plasma prolactin concentrations to non-detectable levels which persisted until at least 3 h after administration. By 6 h however, male rats coadministered haloperidol and PRL-AB had elevated plasma prolactin concentrations compared with zero time vehicle-treated controls. PRL-AB had no effect on median

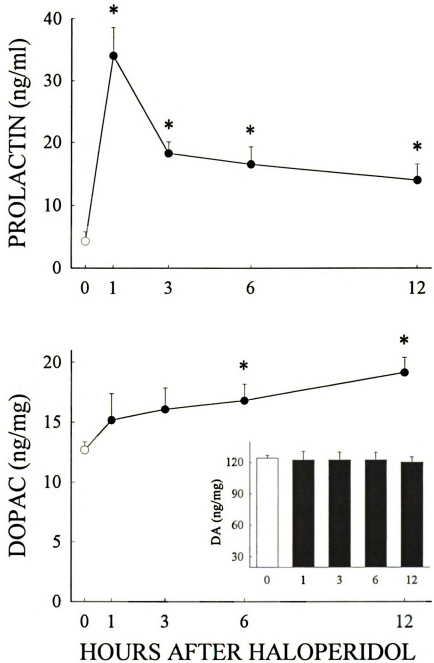


Figure 3.1 Time course effects of haloperidol on concentrations of prolactin in plasma (UPPER PANEL), and DOPAC and DA in the median eminence (LOWER PANEL) of male rats. Rats were injected with haloperidol (1.0 mg/kg; sc; solid symbols and columns) and decapitated either 1, 3, 6 or 12 h later. Zero time controls were injected with 0.3% tartaric acid (1.0 ml/kg; sc; open symbols and column) and decapitated 12 h later. Symbols represent means and vertical lines 1 SEM of 7-8 determinations of concentrations of prolactin in plasma (ng/ml) and DOPAC (ng/mg protein) in the median eminence. Columns represent the means and vertical lines 1 SEM of 7-8 determinations of DA concentrations (ng/mg protein) in the median eminence. *, Values that are significantly different ($p<0.05$) from zero time controls.

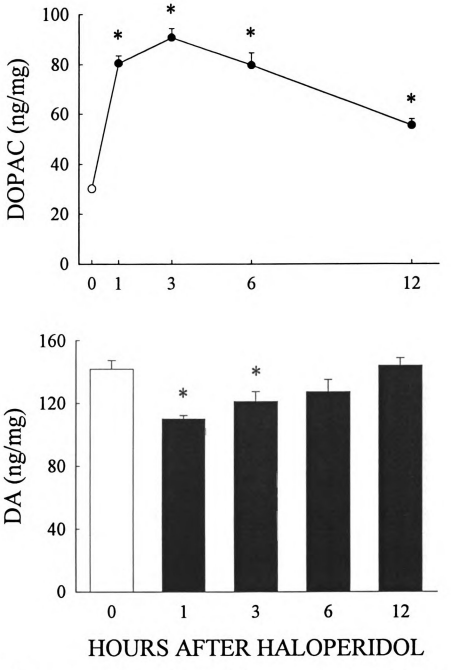


Figure 3.2 Time course effects of haloperidol on concentrations of DOPAC (UPPER PANEL), and DA (LOWER PANEL) in the striatum of male rats. Rats were injected with haloperidol (1.0 mg/kg; sc; solid symbols and columns) and decapitated either 1, 3, 6 or 12 h later. Zero time controls were injected with 0.3% tartaric acid (1.0 ml/kg; sc; open symbol and column) and decapitated 12 h later. Symbols represent means and vertical lines 1 SEM of 7-8 determinations of concentrations (ng/mg protein) of DOPAC and DA in the striatum. *, Values that are significantly different ($p<0.05$) from zero time controls.

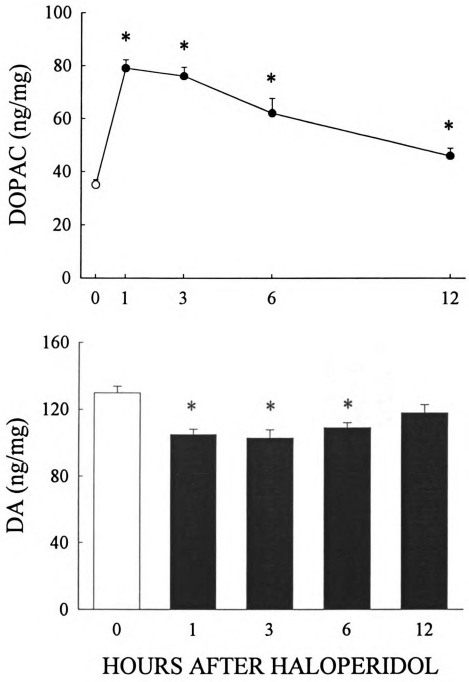


Figure 3.3 Time course effects of haloperidol on concentrations of DOPAC (UPPER PANEL), and DA (LOWER PANEL) in the nucleus accumbens of male rats. Rats were injected with haloperidol (1.0 mg/kg; sc; solid symbols and columns) and decapitated either 1, 3, 6 or 12 h later. Zero time controls were injected with 0.3% tartaric acid (1.0 ml/kg; sc; open symbol and column) and decapitated 12 h later. Symbols represent means and vertical lines 1 SEM of 7-8 determinations of concentrations (ng/mg protein) of DOPAC and DA in the nucleus accumbens. *, values that are significantly different ($p<0.05$) from zero time controls.

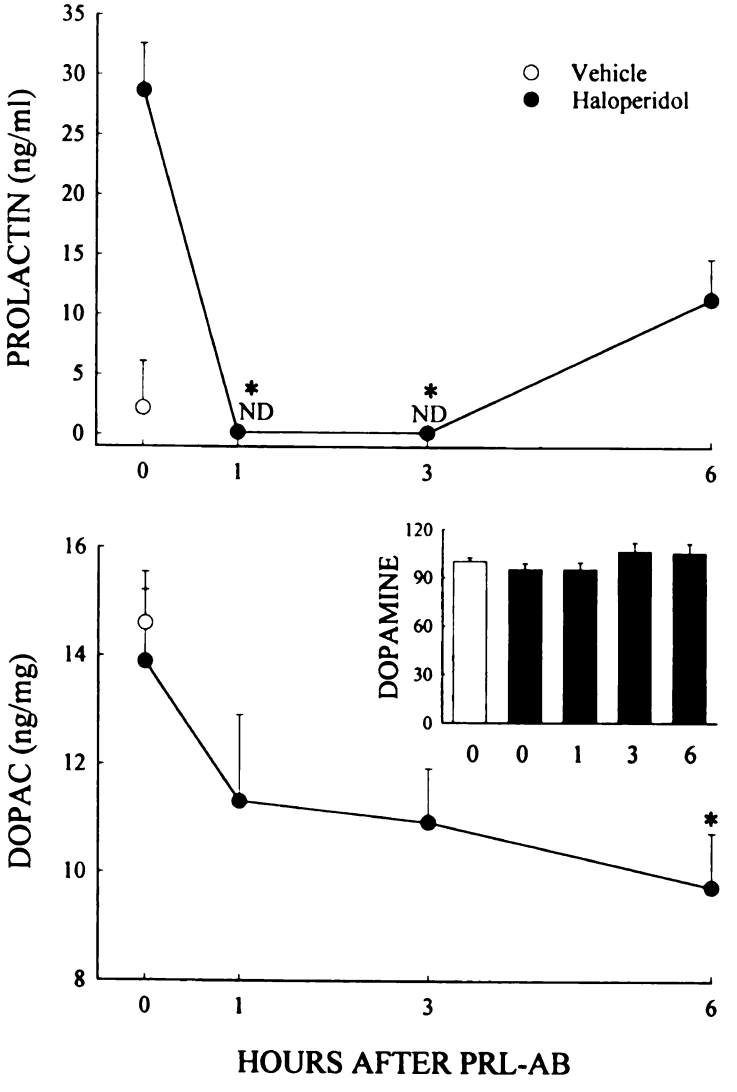


Figure 3.4 Time course effects of PRL-AB on concentrations of plasma prolactin (UPPER PANEL), and DOPAC and DA in the median eminence (LOWER PANEL) of male rats. Rats were injected with haloperidol (1.0 mg/kg; sc; solid symbols and columns) and PRL-AB (200µl/rat; iv) and decapitated either 1, 3, or 6 h later. Zero time controls were obtained from rats injected with haloperidol and normal rabbit serum (NRS; 200µl/rat; iv) 1 h prior to decapitation. Vehicle controls (open symbol and column) were injected with 0.3% tartaric acid (1 ml/kg; sc) and NRS 1 h prior to decapitation. Symbols and columns represent means and vertical lines 1 SEM of 7-8 determinations of concentrations of prolactin in plasma (ng/ml; UPPER PANEL), DOPAC (ng/mg protein; LOWER PANEL) and DA (ng/mg protein; LOWER PANEL INSET) in the median eminence. *, Values that are significantly different ($p<0.05$) from zero time controls.

eminence DOPAC concentrations in male rats through 3 h, but was decreased at 6 h. PRL-AB had no effect on DA concentrations in this region at any time after its administration. These studies established that a single injection of PRL-AB (200 µl/rat; iv) immunoneutralized haloperidol-induced hyperprolactinemia for at least 3 h.

Recall from **figures 3.1-3.3,** haloperidol increased concentrations of DOPAC 12 h after injection, but had no effect on dopamine concentrations in the median eminence, striatum and nucleus accumbens. PRL-AB blocked the delayed (12 h) stimulatory effect of haloperidol on DOPAC concentrations in the median eminence and had no effect on dopamine concentrations in this region (**figure 3.5**). In contrast, PRL-AB failed to block the stimulatory effects of haloperidol on DOPAC concentrations in the striatum and nucleus accumbens, and dopamine concentrations in these regions were no different from controls (**figure 3.6**).

The results presented in **figure 3.5** are consistent with the view that prolactin mediates the stimulatory effects of haloperidol on TIDA neurons (for review see Moore and Lookingland, 1995), and suggest that delayed activation of these neurons by prolactin is dependent upon a period of sustained hyperprolactinemia for both the initiation and maintenance of this response. To determine if this is the case, haloperidol-treated rats received injections of either PRL-AB or NRS every 3 h such that animals were exposed to periods of hyperprolactinemia of 3, 6 or 12 h duration, followed by 9, 6, and 0 h, respectively, of immunoneutralized circulating prolactin (**figure 3.7**, upper panel). Responses in these animals were examined 12 h after haloperidol administration. As shown in **figure 3.8** (left panel), 12 h of sustained haloperidol-induced hyperprolactinemia increased DOPAC concentrations in the median eminence, whereas

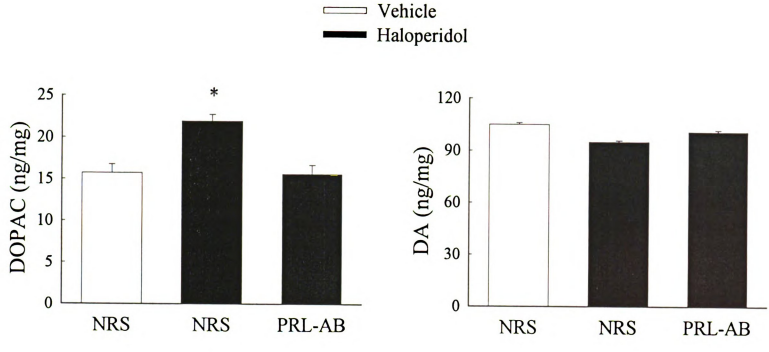


Figure 3.5 Effects of PRL-AB on concentrations of DOPAC (LEFT PANEL) and DA (RIGHT PANEL) in the median eminence of haloperidol-treated male rats. Rats were injected with haloperidol (1.0 mg/kg; sc; solid columns) 12 h before decapitation, and with PRL-AB (200µl/rat; iv; q 3 h) 6 h before decapitation. Controls were injected with haloperidol 12 h before decapitation, and with NRS (200µl/rat; iv; q 3 h) 6 h before decapitation. Vehicle controls (open columns) were injected with 0.3% tartaric acid (1.0 ml/kg; sc; open columns) 12 h before decapitation, and with NRS 6 h before decapitation. Columns represent means and vertical lines 1 SEM of 7-9 determinations of concentrations (ng/mg protein) of DOPAC and DA in the median eminence. *, values that are significantly different ($p<0.05$) from vehicle-treated controls.

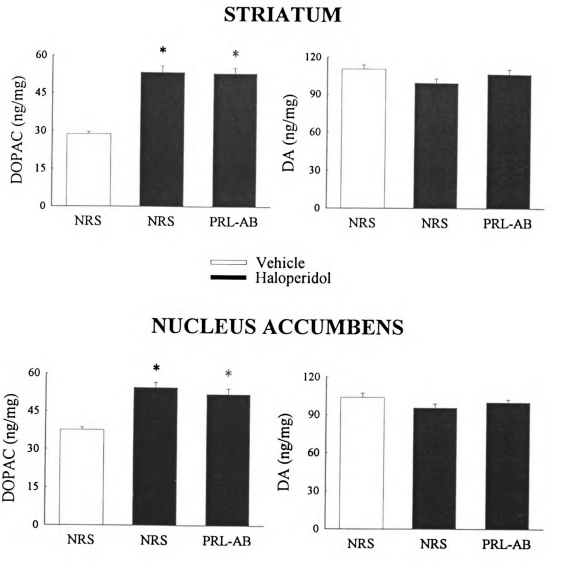


Figure 3.6 Effects of PRL-AB on concentrations of DOPAC (LEFT PANELS) and DA (RIGHT PANELS) in the striatum (UPPER PANELS) and nucleus accumbens (LOWER PANELS) of haloperidol-treated male rats. Rats were injected with haloperidol (1.0 mg/kg; sc; solid columns) 12 h before decapitation, and with PRL-AB (200µl/rat; iv; q 3 h) 6 h before decapitation. Controls were injected with haloperidol 12 h before decapitation, and with NRS (200µl/rat; iv; q 3 h) 6 h before decapitation. Vehicle controls (open columns) were injected with 0.3% tartaric acid (1.0 ml/kg; sc; open columns) 12 h before decapitation, and with NRS 6 h before decapitation. Columns represent means and vertical lines 1 SEM of 7-9 determinations of concentrations (ng/mg protein) of DOPAC and DA in the striatum and nucleus accumbens. *, values that are significantly different ($p<0.05$) from vehicle-treated controls.

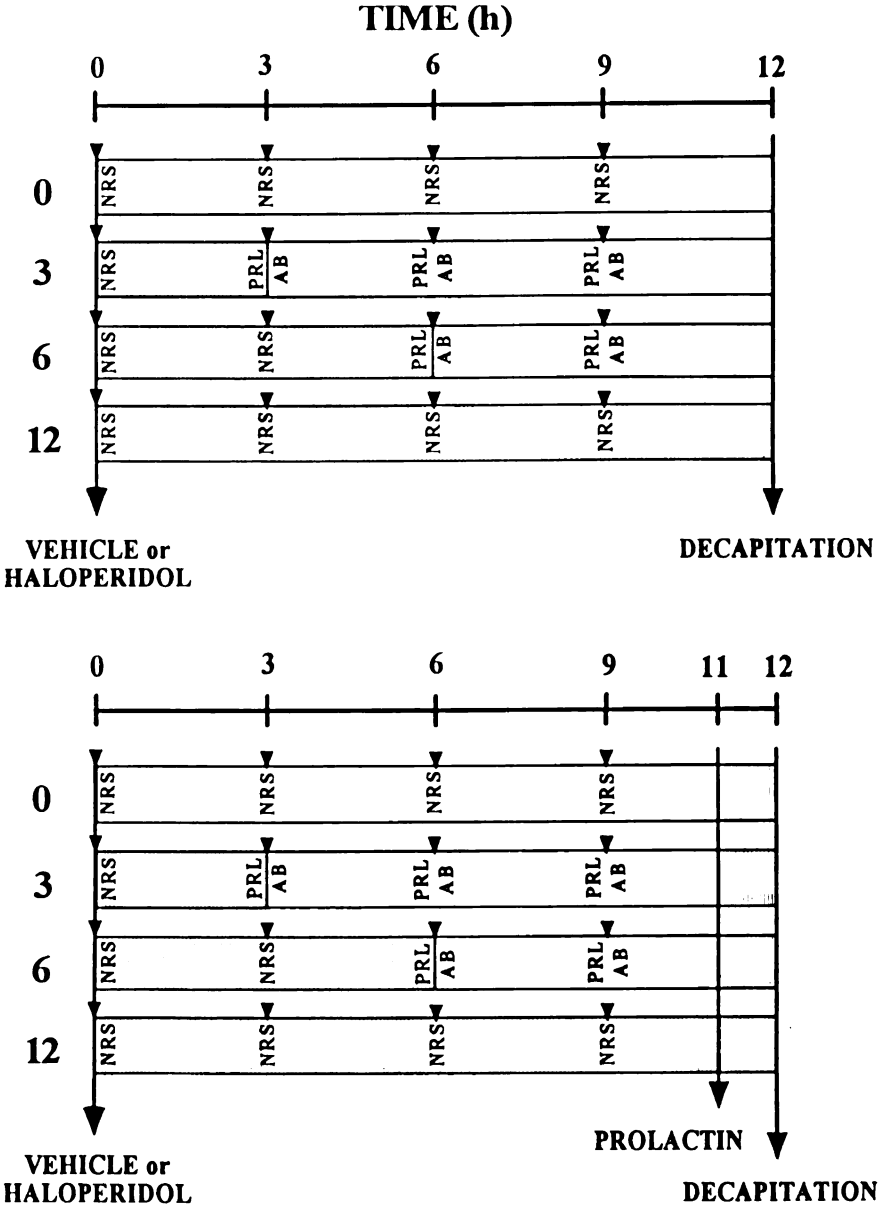


Figure 3.7 Treatment paradigm for comparison of the effects of haloperidol on median eminence DOPAC concentrations in PRL-AB- (UPPER PANEL) and PRL-AB + prolactin-treated (LOWER PANEL) male rats. Horizontal shaded bars represent periods of exposure to hyperprolactinemia before decapitation at 12 h. **UPPER PANEL:** Controls were injected with 0.3% tartaric acid (1 ml/kg; sc; 0 h) and NRS (200µl/rat; iv; 0 h; q 3 h; arrow heads) before decapitation at 12 h; there was no hyperprolactinemia. Haloperidol (1 mg/kg; sc; 0 h)-treated rats were injected with either NRS or PRL-AB (200µl/rat; iv; 0 h; q 3 h; arrow heads) such that groups of rats were exposed to initial periods of hyperprolactinemia lasting 3, 6 or 12 h in duration. **LOWER PANEL:** Controls were injected with 0.3% tartaric acid (1 ml/kg; sc; 0 h), NRS (200µl/rat; iv; 0 h, q 3 h; arrow heads) and prolactin (10µg/rat; icv; 11 h) before decapitation at 12 h such that the initial duration of exposure to hyperprolactinemia was zero, despite hyperprolactinemia at the time of measurement. Haloperidol (1 mg/kg; sc; 0 h)-treated rats were injected with either NRS or PRL-AB (200µl/rat; iv; 0 h; q 3 h; arrow heads) and prolactin (10µg/rat; icv; 11 h) before decapitation at 12 h such that groups of rats were exposed to initial periods of hyperprolactinemia lasting 3, 6 or 12 h in duration and hyperprolactinemia at the time of measurement.

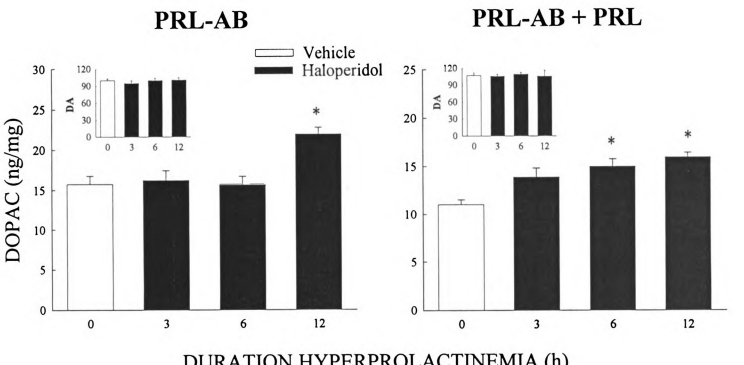


Figure 3.8 Comparison of the effects of haloperidol on median eminence DOPAC concentrations in PRL-AB- and PRL-AB + prolactin-treated male rats. All rats were injected with either haloperidol (1.0 mg/kg; sc; solid columns) or 0.3% tartaric acid vehicle (1.0 ml/kg; sc; open columns) and decapitated 12 h later. **LEFT PANEL:** Haloperidol-treated rats were injected with either NRS (200µl/rat; iv) or PRL-AB (200µl/rat; iv) every 3 h beginning at the time of haloperidol administration such that groups of rats were exposed to initial periods of hyperprolactinemia lasting 3, 6 or 12 h in duration. Controls were injected with NRS every 3 h beginning at the time of 0.3% tartaric acid administration until decapitation at 12 h. **RIGHT PANEL:** Haloperidol-treated rats administered NRS (200µl/rat; iv) or PRL-AB (200µl/rat; iv) as described for the left panel also received a single injection of prolactin (10 µg/rat; icv) 11 h after haloperidol administration and were decapitated 1 h later. Controls were injected with NRS every 3 h beginning at the time of 0.3% tartaric acid administration, prolactin 11 h after tartaric acid administration, and were decapitated at 12 h. Columns represent the means and vertical lines 1 SEM of 8-9 determinations of DOPAC concentrations (ng/mg protein) and DA concentrations (ng/mg protein; INSET PANELS) in the median eminence. *, values for haloperidol-treated rats that are significantly different ($p<0.05$) from vehicle-treated controls.

in rats exposed to only 3 or 6 h of haloperidol-induced hyperprolactinemia median eminence DOPAC concentrations were not significantly different from vehicle-treated controls. No duration of hyperprolactinemia effected concentrations of dopamine in the median eminence. This experiment was repeated in another set of animals except that rats exposed initially to 3 or 6 h of hyperprolactinemia (followed by 9 and 6 h of hypoprolactinemia, respectively) received an icv injection of prolactin 1 h prior to decapitation (**figure 3.7**, lower panel). As shown in **figure 3.8** (right panel), median eminence DOPAC concentrations were increased in response to prolactin in rats exposed to 6 or 12 h of haloperidol-induced hyperprolactinemia, but prolactin-treated rats exposed to 3 h of hyperprolactinemia were not significantly different from vehicle-treated controls. No duration of hyperprolactinemia effected concentrations of dopamine in the median eminence.

C. DISCUSSION

Passive immunoneutralization of circulating hormones represents a useful alternative to disruptive pharmacological and surgical procedures to study neuroendocrine feedback regulation of the central nervous system. In lieu of altering hormone secretion, injection of antiserum directly into the blood causes rapid and selective binding of the targeted hormone thereby rendering it inaccessible to its site of action. In the case of prolactin, passive immunoneutralization has been used successfully to ascribe its role in a variety of neurologic (Roky et al., 1995; Crisostoma et al., 1997), autonomic (Mills and Ward, 1985) and neuroendocrine functions (Milenkovic et al., 1990; Parker et al., 1991; Pape and Tramu, 1996), including feedback regulation of TIDA neurons (Gudelsky and Porter, 1980; Porter et al., 1990; Toney et al., 1992b; Wagner et al., 1993).

Interpretation of the results of immunoneutralization studies is dependent upon both selectivity of antiserum for its targeted hormone and capacity of antiserum to bind sufficient free hormone to eliminate its biological actions. PRL-AB used in these studies was generated in rabbits using NIDDK rat prolactin as the directed antigen. Since this preparation was obtained from anterior pituitary extracts the possibility existed that PRL-AB may also contain antibody directed against other (albeit minor) contaminant hormones. Radioimmunoasssay analyses of binding specificity for this antiserum revealed, however, that PRL-AB used in these experiments was selective for prolactin, having no affinity for luteinizing hormone or growth hormone (Hentschel et al., 2000a).

The capacity of PRL-AB to eliminate free bioactive prolactin from the circulation was evaluated by determining the time course effects of PRL-AB on plasma prolactin concentrations quantified using the Nb2 lymphoma bioassay (Canon et al., 1991).

Intravenous administration of PRL-AB decreased plasma prolactin to non-detectable levels for up to 3 h in haloperidol-pretreated male rats. The capacity of PRL-AB to bind prolactin in the face of activated secretion may be exceeded after 3 h, and hence repeated injections of PRL-AB were utilized to maintain more prolonged periods of hypoprolactinemia.

The primary function of TIDA neurons is to suppress the secretion of prolactin from the anterior pituitary. Indeed, experimental procedures which either; 1) disrupt synthesis and release of dopamine from TIDA neurons, 2) prevent access of dopamine to the anterior pituitary lactotrophs (i.e. pituitary stalk section; pituitary transplantation), or 3) block pituitary D_2 receptors, all increase prolactin secretion (Ben-Jonathan, 1985). In females, the inhibitory effect of dopamine on the synthesis and secretion of prolactin is opposed by estrogen, consequently circulating prolactin levels in females are higher than males. Higher basal prolactin in females, in turn, tonically stimulates TIDA neurons such that under normal conditions the activity of these neurons is 2-3 times that of males (Moore and Lookingland, 1995). In males, testosterone has no effect on prolactin secretion (Toney et al., 1991), and circulating prolactin levels are insufficient to tonically activate TIDA neurons (Demarest and Moore, 1981b).

The results of the present study confirm that TIDA neurons in male rats are unresponsive to acute PRL-AB-induced hypoprolactinemia. Median eminence DOPAC concentrations remain unchanged up to 3 h, yet are decreased at 6 h. These results are consistent with a previous study in which median eminence DOPAC concentrations were decreased 12 h, but not 4 h after PRL-AB injection in male rats (Hentschel et al., 2000a). These results differ from another study (Demarest and Moore, 1981b) in which

bromocriptine was used to depress endogenous prolactin secretion. In that study DOPA accumulation was not decreased in the median eminence of male rats until 48 h after the first injection of bromocriptine. The findings of Demarest and Moore may be reconciled with our results in the light of recent reports (Durham et al., 1997;1998). It is now known that in male rats TIDA neuronal activity is tonically inhibited by dynorphinergic neurons, and that these neurons are inhibited by D_2 receptor agonists such as bromocriptine. Accordingly, while bromocriptine suppressed endogenous prolactin secretion and removed this stimulus from TIDA neurons, this drug simultaneously disinhibited TIDA neuronal activity. Hence the effect of hypoprolactinemia could have been masked. The data suggest that in male rats prolonged periods of hypoprolactinemia are required to reduce basal activity of TIDA neurons. Furthermore, since median eminence DOPAC concentrations remained decreased at 6 h at which time hyperprolactinemia had overcome the capacity of PRL-AB to immunoneutralize circulating prolactin, the data also suggest that once TIDA neuronal activity has been depressed there is a latent period following acute changes in circulating prolactin concentrations that precedes activation of these neurons in male rats.

Prolactin induces changes in TIDA neurons which result in delayed activation of these neurons to levels 2-3 times basal (Demarest et al., 1985a; Selmanoff, 1985), and maintenance of this response during chronic stimulation (Moore et al., 1985). This inductive component of prolactin feedback regulation of TIDA neurons is dependent upon protein synthesis (Johnston et al., 1980) and is associated with increased expression of tyrosine hydroxylase mRNA in neuronal perikarya in the arcuate nucleus (Arbogast and Voogt, 1991; Selmanoff et al., 1991). These features suggest that prolactin induces

long-term changes in gene expression which result in an increased capacity of TIDA neurons to synthesize and release dopamine.

Temporal aspects of prolactin feedback related to the initiation and maintenance of activation of TIDA neurons were determined in the present study using the haloperidol-induced hyperprolactinemia experimental model in combination with systemic administration of PRL-AB. This approach permitted the examination of the effects of finite initial periods of hyperprolactinemia on TIDA, NSDA and MLDA neurons, and of the dependence on circulating prolactin in this process. The results confirm that haloperidol-induced hyperprolactinemia is associated with delayed (by 6-12 h) activation of TIDA neurons (Moore and Lookingland, 1995), rapid, prolonged activation of NSDA and MLDA neurons (Gudelsky and Moore, 1977), and that the stimulatory effects of haloperidol on these neurons are mediated by prolactin in TIDA neurons (Gudelsky and Porter, 1980), but not in NSDA or MLDA neurons.

The results are consistent with regulation of NSDA and MLDA, but not TIDA neurons by autoreceptors (Nowycky and Roth, 1978; Demarest and Moore, 1979). Accordingly, the acute decrease in dopamine concentrations in terminal regions of NSDA and MLDA, but not TIDA neurons may be due to autoreceptor-mediated disinhibition of these systems and the transient inability of synthesis to keep pace with release.

Moreover, these results reveal that more than 3 h of exposure to elevated prolactin is necessary to prime TIDA neurons to the stimulatory actions of prolactin, and that hyperprolactinemia need not continue beyond 6 h for priming to occur. Indeed, median eminence DOPAC concentrations in rats exposed to hyperprolactinemia for 6 h (followed by 6 h of PRL-AB-induced hypoprolactinemia) were significantly elevated in response to

central administration of prolactin as compared with vehicle-treated controls, whereas values in rats exposed to hyperprolactinemia for only 3 h (followed by 9 h of PRL-AB-induced hypoprolactinemia) were not. The observation that median eminence DOPAC concentrations in rats exposed to 6 h of hyperprolactinemia followed by 6 h of hypoprolactinemia were not significantly different from zero time controls suggests that priming is not sufficient to induce delayed activation of these neurons in the absence of circulating prolactin.

In summary, the results of the present study confirm: (1) that the activity of NSDA and MLDA neurons are not regulated by prolactin, (2) the temporal characteristics of the delayed (6-12 h) inductive activation of TIDA neurons by prolactin, (3) that prolactin mediates the activation of TIDA neurons by haloperidol, and (4) that prolonged periods of hypoprolactinemia are required to depress the basal activity of TIDA neurons in male rats. The results of the present study also reveal that delayed induced activation of TIDA neurons by prolactin is dependent upon a priming period of hyperprolactinemia of greater than 3 h duration for initiation and sustained hyperprolactinemia for maintenance of this response.

4. EFFECTS OF PROLACTIN ON EXPRESSION OF FRA IN TH-IR NEURONS IN SUBDIVISIONS OF THE ARCUATE NUCLEUS

A. INTRODUCTION

TIDA neurons located in the ARC project to the median eminence where dopamine released from these neurons is transported in the hypophysial portal blood to the anterior pituitary where it tonically inhibits prolactin secretion from lactotrophs. TIDA neurons lack dopamine autoreceptors characteristic of other central dopaminergic systems (Demarest and Moore, 1979) and are regulated by prolactin feedback. Increases or decreases in circulating prolactin concentrations produce corresponding changes in the synthesis and metabolism of dopamine in terminals of these neurons in the median eminence (for review see Moore and Lookingland, 1995).

In addition to the "tonic" stimulatory action of prolactin that sets the higher basal TIDA neuronal activity in females as compared with males (Moore and Lookingland, 1995), prolactin induces changes in TIDA neurons that culminate in both delayed activation of these neurons to levels 2-3 times basal (Demarest et al., 1985a; Selmanoff, 1985) and maintenance of this response during chronic stimulation (Moore et al., 1985). This "inductive" component of prolactin feedback regulation of TIDA neurons is dependent upon protein synthesis (Johnston et al., 1980) and associated with increased expression of TH mRNA in the ARC (Arbogast and Voogt, 1991; Selmanoff et al., 1991). These features suggest that prolactin induces long-term changes in gene expression that result in an increased capacity of TIDA neurons to synthesize and release dopamine; such changes are characteristic of late-response gene expression.

In TIDA neurons, various stimuli and neuroendocrine manipulations induce the rapid transient expression of immediate early genes including FRA (Hoffman et al., 1993a; 1994). Newly translated FRA proteins translocate to the nucleus and heterodimerize with constitutively expressed Jun-related transcription factors (Morgan and Curran, 1989; Hughes and Dragunow, 1995). These heterodimers bind AP-1 elements (Sheng and Greenberg, 1990) identified in the TH gene promoter (Kumer and Vrana, 1996) and modulate transactivation rate of various late response genes, including TH (Icard-Liepkalns et al., 1992; Guo et al., 1998). Accordingly, expression of FRA in TH-containing perikarya of the ARC has been shown to reflect the activity of TIDA neurons (Hoffman et al., 1994; Lerant et al., 1996; Lerant and Freeman, 1997; Cheung et al., 1997), and represents an early neurochemical marker of activity which permits the identification of populations of chemically similar neurons uniquely responsive to specific stimuli.

While TIDA neurons share TH-IR and residence within the ARC, there is anatomical, histochemical and biochemical evidence to divide the ARC into dorsomedial (DM) and ventrolateral (VL) subdivisions. Perikarya of the DM-ARC have more intense TH-IR staining than those of the VL-ARC. In addition, there is a discrepancy in TH- and AAAD-IR between the DM and VL subdivisions of the ARC. The DM-ARC contains both TH-IR and AAAD-IR, whereas the VL-ARC exhibits only TH-IR (Skagerberg et al., 1988; Misu et al., 1996). Hence, the DM-ARC contains the full complement of enzymes necessary to synthesize dopamine, whereas the VL-ARC lacks AAAD and produces only DOPA (Meister et al., 1988a; Komori et al., 1991). However, since vascular tissue may express AAAD, it is possible that DOPA released at the median eminence into the

hypophysial portal vessels may be converted to dopamine *en route* to the pituitary (Bertler et al., 1964; Constantinidis et al., 1969).

Since prolactin activates TIDA neurons after long latency, and changes in immediate early gene products like FRA, can be used as an index of activity in these neurons, we hypothesize that prolactin will cause changes in FRA expression in TIDA neurons. If FRA expression in TIDA neurons is regulated by prolactin, then dual TH/FRA immunohistochemistry will show differential expression of FRA-IR in TH-IR neurons in the ARC after prolactin treatment. To this end, initial experiments determined the effects of haloperidol-induced endogenous hyperprolactinemia on overall numbers of TH-IR neurons and the percentage of these neurons expressing FRA in the DM and VL subdivisions of the rostral, middle and caudal ARC (Cheung et al., 1997; Paxinos and Watson, 1997) of male rats. Follow-up experiments examined the duration of initial hyperprolactinemia required to elicit an effect, and compared the effects of haloperidol to those of exogenous prolactin administration in both male and diestrous female rats.

B. RESULTS

TH-IR neurons are not homogeneously distributed throughout the rostrocaudal extent of the ARC. Rather, there exists a rostrocaudal gradient in numbers of TH-IR neurons within ARC subdivisions (Cheung et al., 1997). In male rats, there are fewer TH-IR neurons in the DM subdivision as ARC regions extend caudally **(figure 4.1)**. In contrast, there are successively more TH-IR neurons in the VL subdivision as ARC regions extend caudally. Hence, numbers of TH-IR neurons in the DM relative to the VL ARC subdivision vary by rostrocaudal region. In male rats, the ratio of numbers of TH-IR neurons in the DM-ARC to those of the VL-ARC is greater in the rostral ARC (4:1) than the middle ARC (3:2) or caudal ARC (1:1). Despite the difference in numbers of TH-IR neurons in ARC subdivisions and rostrocaudal regions, the percentage of TH-IR neurons expressing FRA-IR nuclei remains uniformly low (3-5%) in all subdivisions and regions of untreated male rats **(figure 4.2)**.

Haloperidol is a D_2 dopamine receptor antagonist that stimulates prolactin secretion via disinhibition of pituitary lactotrophs. Systemic administration of haloperidol increased plasma prolactin concentrations to peak levels by 1 h, and maintained endogenous hyperprolactinemia for 12 h post-injection **(figure 4.3)**. Haloperidol caused a transient increase at 3 h in the percentage of TH-IR neurons expressing FRA-IR nuclei in the DM subdivision of all rostrocaudal ARC regions in male rats **(figure 4.4)**. Furthermore, there was a rostrocaudal gradient with respect to magnitude of response; effects were greater in the rostral ARC (8-fold increase) than the middle ARC (5-fold increase) or caudal ARC (3-fold increase). In the VL subdivision of all rostrocaudal ARC regions haloperidol caused a rapid (by 1.5 h) and prolonged (through 12 h) decrease in the

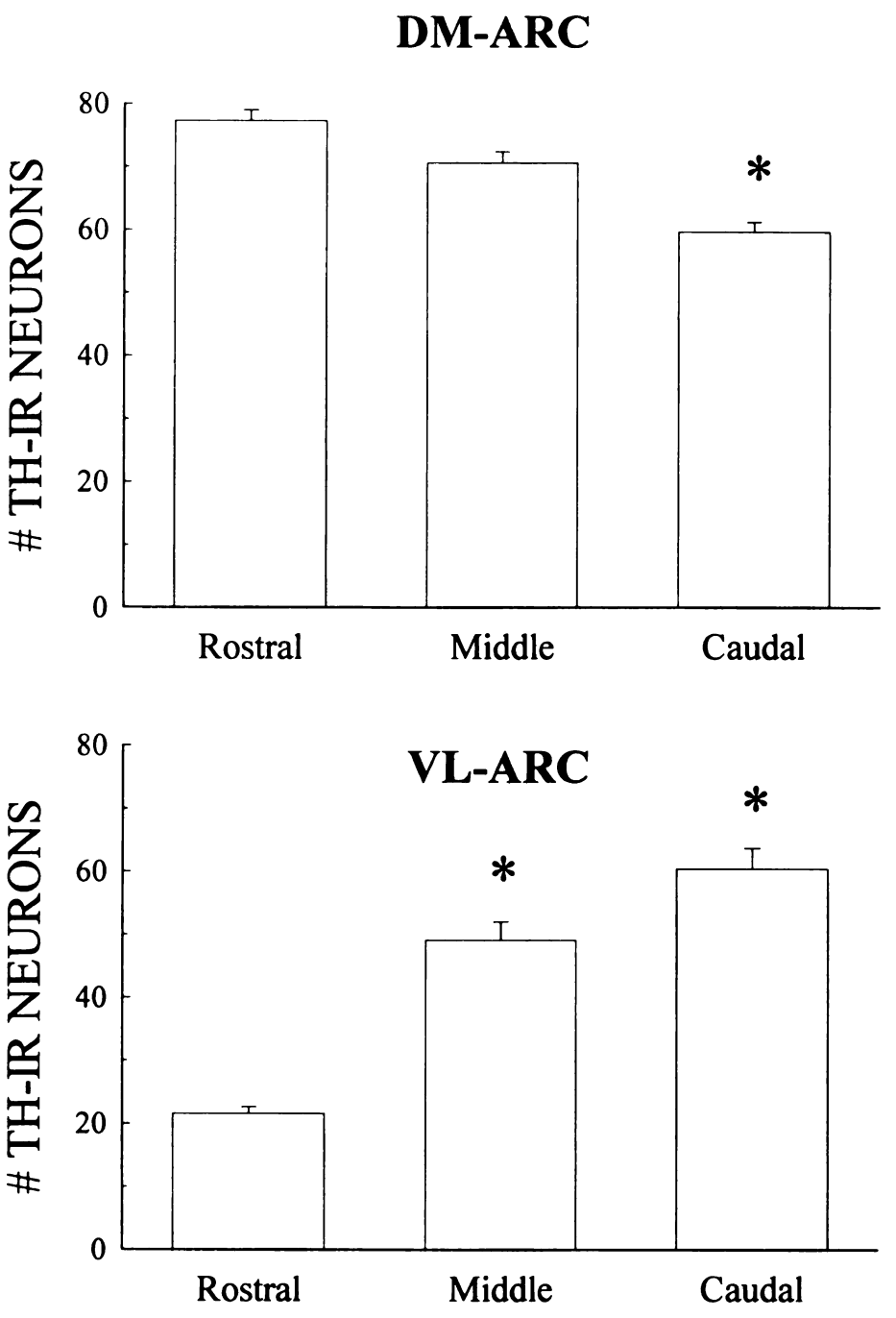


Figure 4.1 Comparison of the numbers of TH-IR neurons in the DM and VL subdivisions of rostral, middle and caudal ARC of male rats. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of TH-IR and FRA-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 6-8 animals. *, Values that are significantly different ($p < 0.05$) from the rostral ARC.

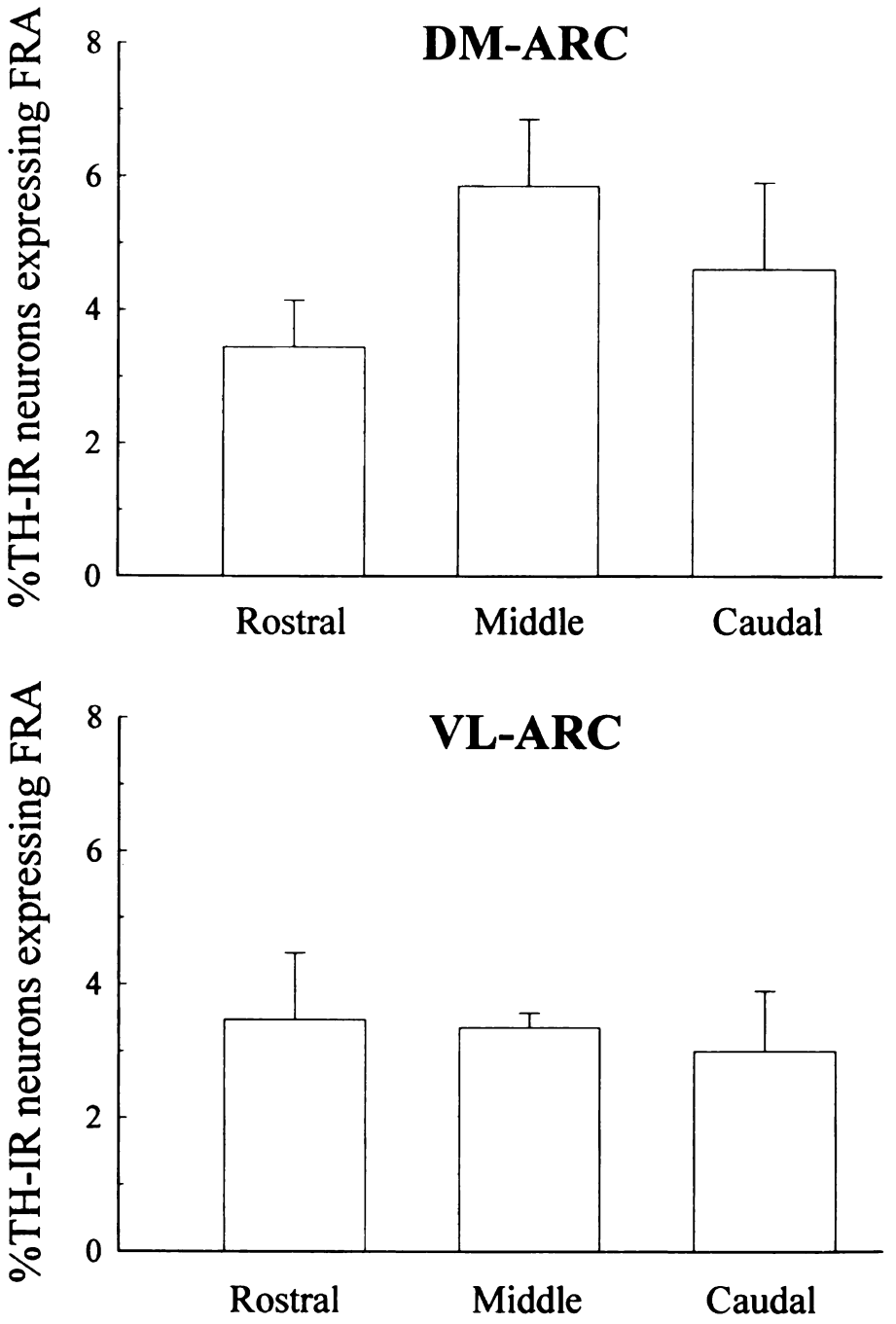


Figure 4.2 Comparison of %TH-IR neurons expressing FRA in the DM and VL subdivisions of rostral, middle and caudal ARC of male rats. Rats were perfused, brains sectioned and tissue immunohistochemically processed for the detection of TH-IR and FRA-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals.

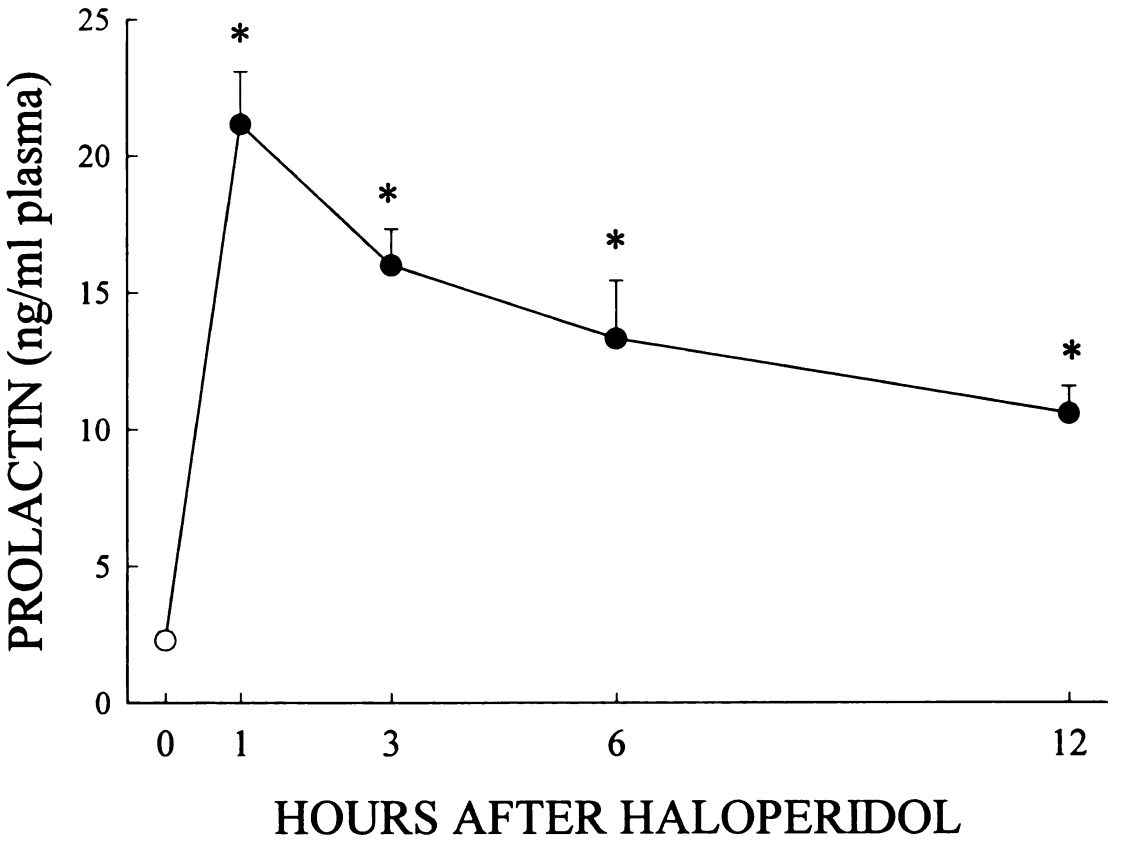


Figure 4.3 Time course effects of haloperidol on plasma prolactin concentrations in male rats. Rats were injected with haloperidol (1 mg/kg; sc) and decapitated 1, 3, 6 or 12 h later. Zero time controls were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to decapitation. Symbols represent the means and vertical lines 1 SEM of prolactin concentrations in plasma of 7-8 rats. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

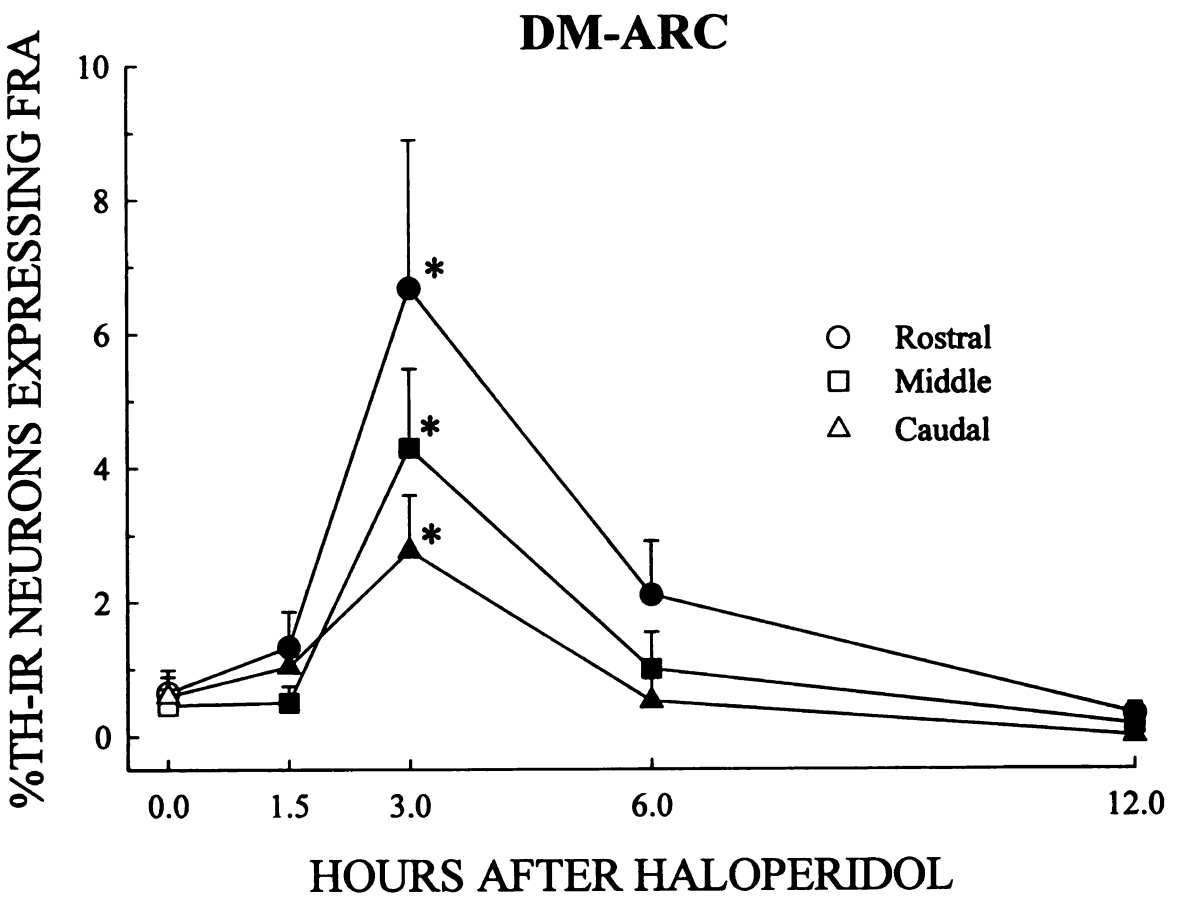


Figure 4.4 Time course effects of haloperidol on %TH-IR neurons expressing FRA in the DM subdivision of rostral, middle and caudal ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC (circles), -2.8 mm for middle ARC (squares) and -3.3 mm for caudal ARC (triangles) (Paxinos and Watson, 1997). Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

percentage of TH-IR neurons expressing FRA-IR nuclei **(figure 4.5)**. However, haloperidol had no effect on numbers of TH-IR neurons at any time point in any ARC subdivision or rostrocaudal region **(figure 4.6)**.

The effects of haloperidol could be mediated by direct action on central dopamine receptors, or indirectly via endogenous hyperprolactinemia. Accordingly, endogenous prolactin was immunoneutralized to determine if the effects of haloperidol on FRA expression in TIDA neurons were mediated by prolactin. Prolactin antiserum was administered at a dose known to block the stimulatory effects of estrogen on TIDA neuronal activity (Toney et al., 1992b) and suppress plasma prolactin concentrations for at least 3 h after haloperidol treatment **(figure 3.4)**. Co-administration of prolactin antiserum completely blocked the haloperidol-induced increase in percentage of TH-IR neurons expressing FRA at 3 h in the DM-ARC of all rostrocaudal regions **(figure 4.7)**. In contrast, prolactin antiserum failed to block the haloperidol-induced decrease in percentage of TH-IR neurons expressing FRA at 3 h in the VL-ARC of all rostrocaudal regions **(figure 4.8)**.

Hyperprolactinemia of different durations was investigated to further characterize the stimulus required to induce FRA expression in DM-ARC TH-IR neurons. To this end, rats were exposed to initial periods of haloperidol-induced hyperprolactinemia of 0, 1 or 3 h duration. Rats that had experienced 3 h of hyperprolactinemia (positive controls) demonstrated the expected increase in percentage of DM-ARC TH-IR neurons expressing FRA at 3 h **(figure 4.9)**. In contrast, FRA expression in rats co-administered prolactin antiserum and haloperidol (antibody and negative controls), or exposed to only 1 h of hyperprolactinemia, were no different from vehicle-treated controls. This study suggested

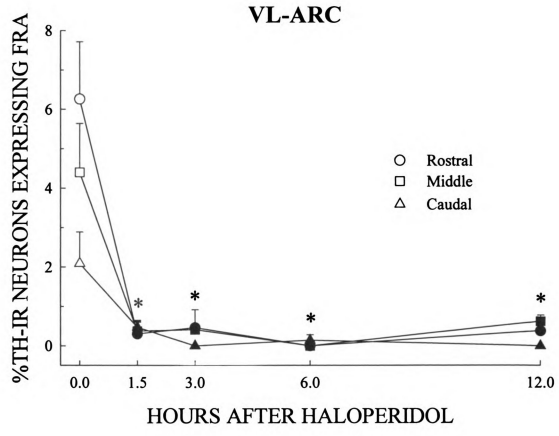


Figure 4.5 Time course effects of haloperidol on %TH-IR neurons expressing FRA in the VL subdivision of rostral, middle and caudal ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC (circles), -2.8 mm for middle ARC (squares) and -3.3 mm for caudal ARC (triangles) (Paxinos and Watson, 1997). Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

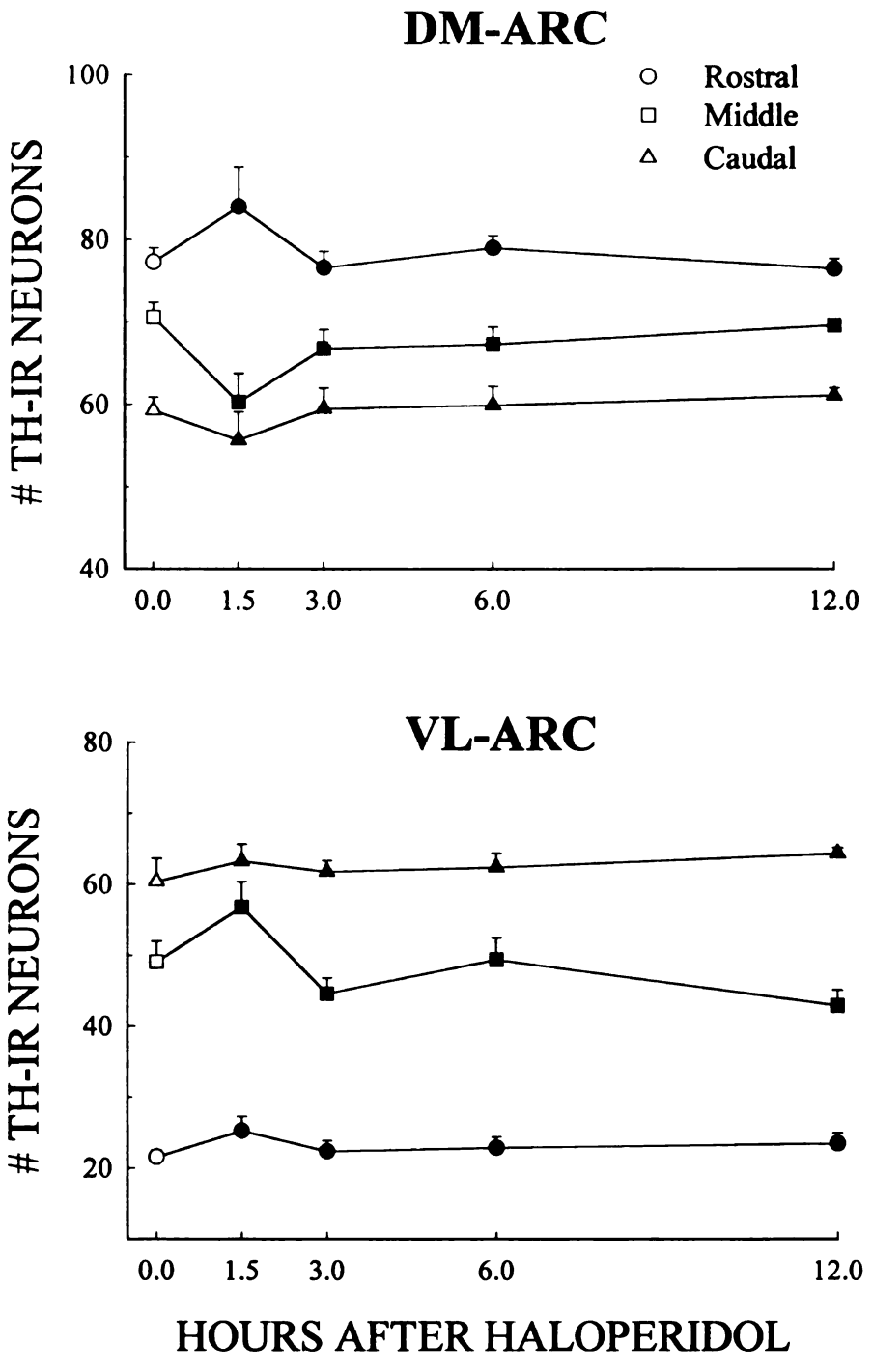


Figure 4.6 Time course comparison of the numbers of TH-IR neurons in the DM and VL subdivisions of rostral, middle and caudal ARC of vehicle- and haloperidol-treated male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC (circles), -2.8 mm for middle ARC (squares) and -3.3 mm for caudal ARC (triangles) (Paxinos and Watson, 1997). Symbols represent means and vertical lines 1 SEM from 6-8 animals.

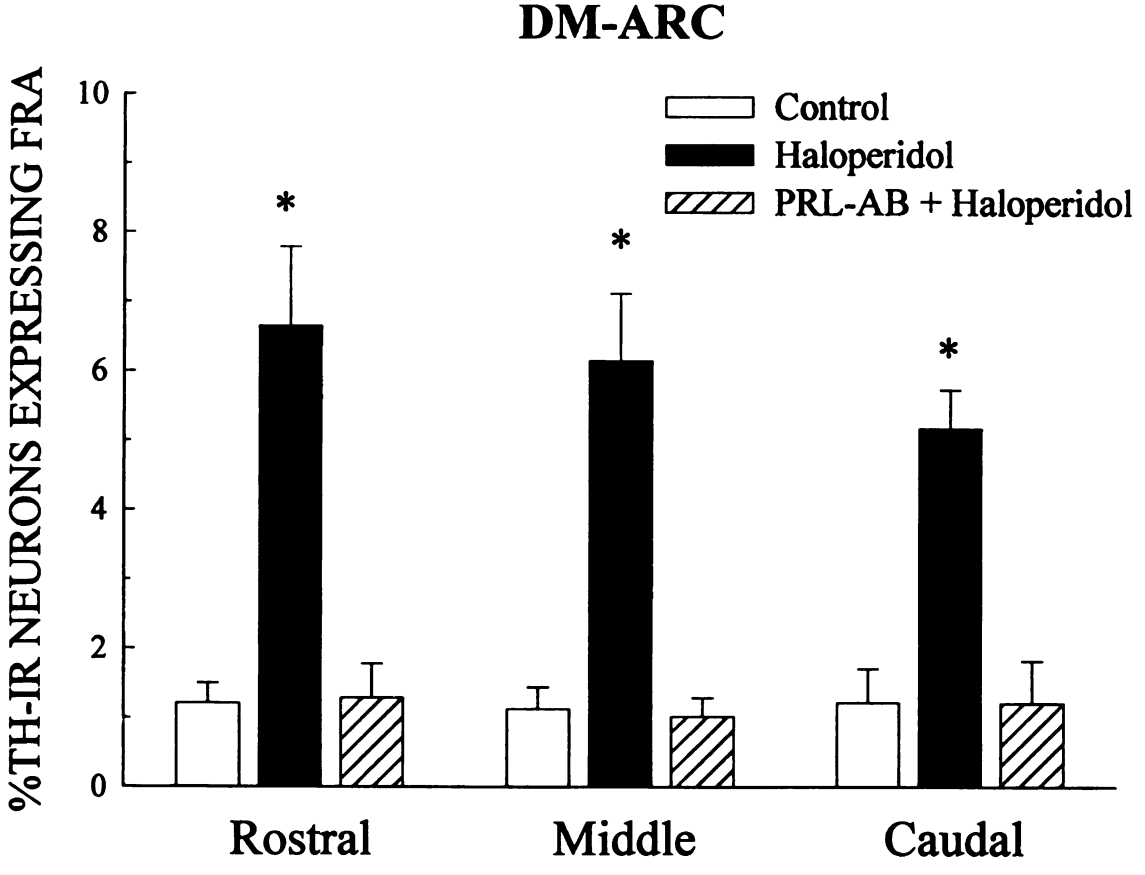


Figure 4.7 Effects of prolactin antiserum on %TH-IR neurons expressing FRA in the DM subdivision of rostral, middle and caudal ARC of haloperidol-treated male rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; sc) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; sc), and with either prolactin antiserum (hatched columns; 200 µl/rat; iv) or vehicle (normal rabbit serum, 200 µl/rat; iv) 3 h prior to decapitation. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from vehicle-treated controls.

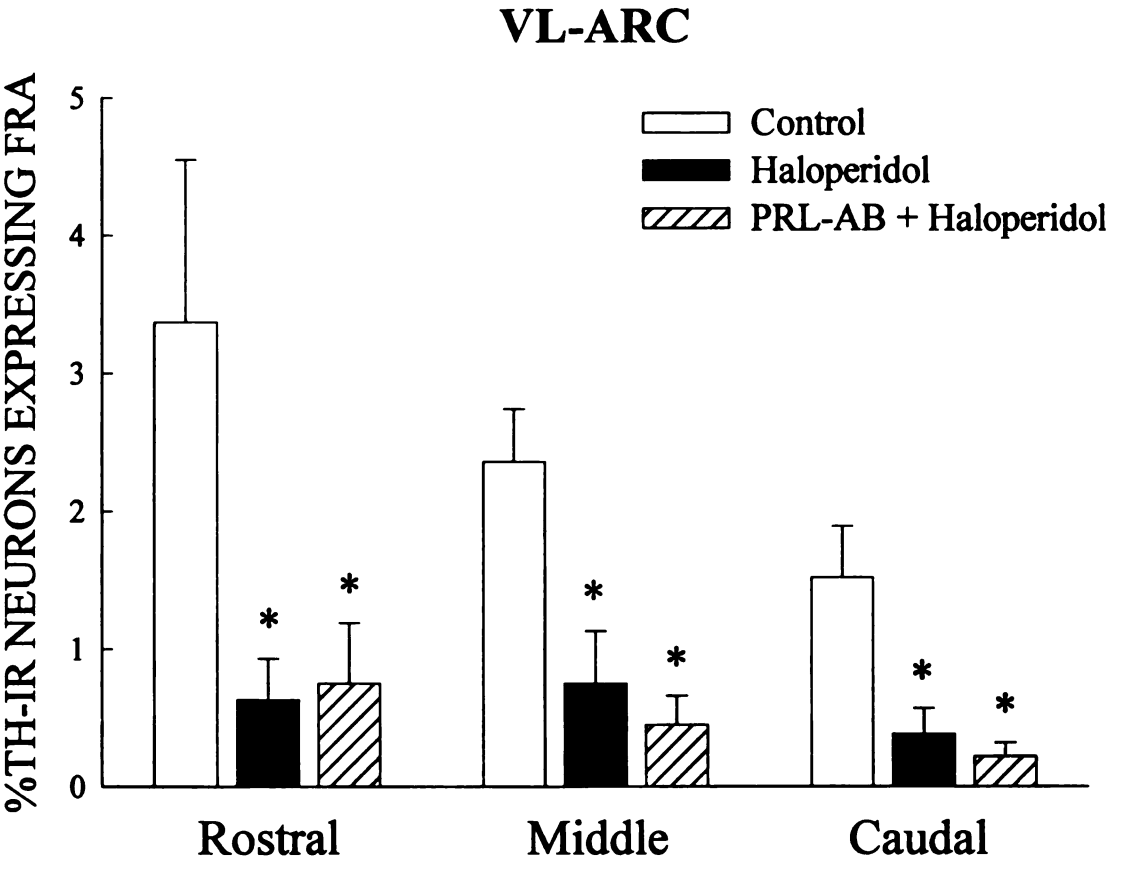


Figure 4.8 Effects of prolactin antiserum on %TH-IR neurons expressing FRA in the VL subdivision of rostral, middle and caudal ARC of haloperidol-treated male rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; sc) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; sc), and with either prolactin antiserum (hatched columns; 200 µl/rat; iv) or vehicle (normal rabbit serum, 200 µl/rat; iv) 3 h prior to decapitation. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from vehicle-treated controls.

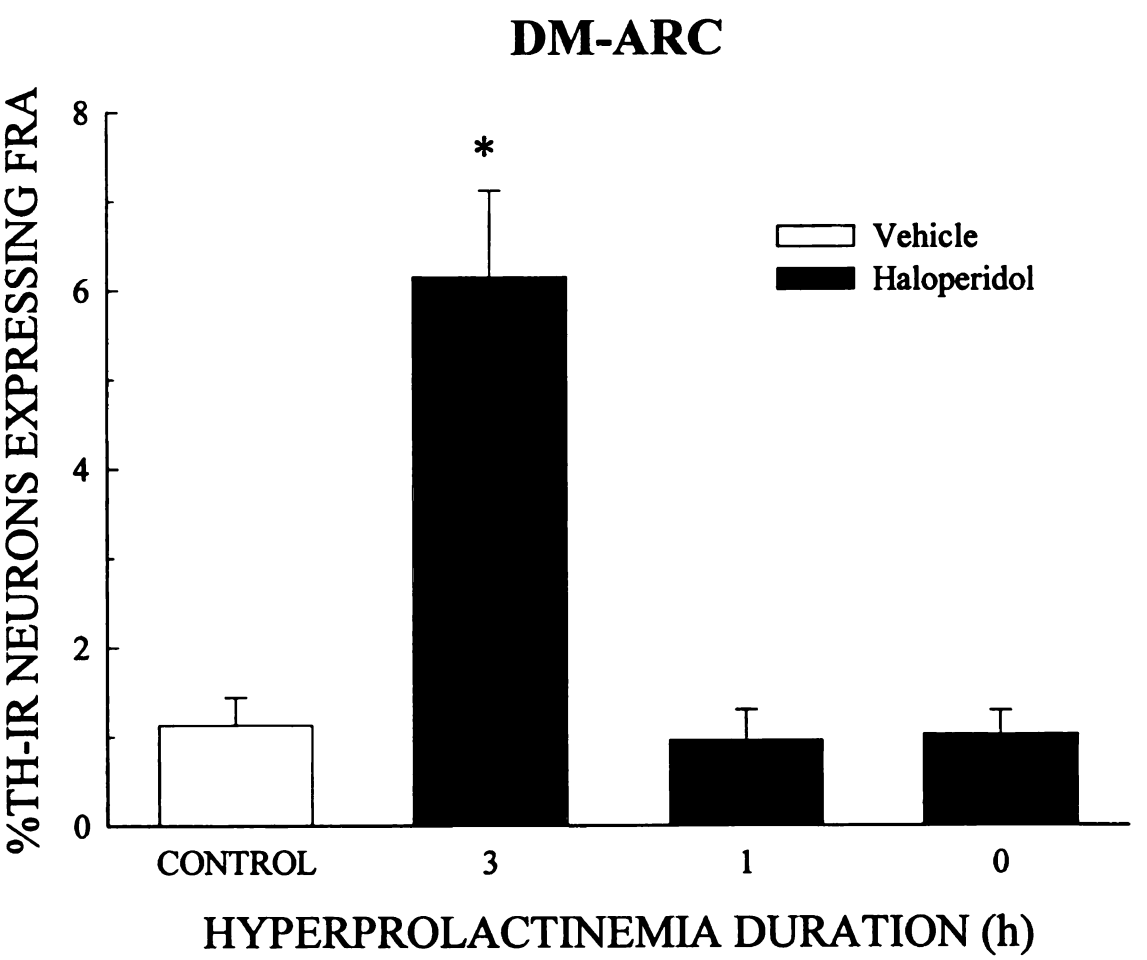


Figure 4.9 Effects of duration of hyperprolactinemia on %TH-IR neurons expressing FRA in the DM-ARC of haloperidol-treated male rats. Rats were injected with haloperidol (filled columns; 1 mg/kg; sc) 3 h prior to perfusion and with prolactin antiserum (200 µl/rat; iv) 2 h prior to perfusion. Vehicle controls (open column) were injected with 0.3% tartaric acid (1.0 ml/kg; sc) and with normal rabbit serum (200 µl/rat; iv) 3 h before perfusion. Positive and negative controls were injected with haloperidol 3 h prior to perfusion and with either normal rabbit serum or prolactin antiserum 3 h prior to perfusion, respectively. Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from vehicle-treated controls.

that in male rats, greater than 1 h of haloperidol-induced hyperprolactinemia is required for increasing FRA expression in DM-ARC TH-IR neurons at the time of peak response at 3 h.

Similar to the effects of haloperidol injection, central administration of prolactin to male rats increased the percentage of DM-ARC TH-IR neurons expressing FRA at 3 h in all rostrocaudal ARC regions **(figure 4.10).** This effect remained significant through 6 h in the rostral and middle ARC. The magnitude of response exhibited a rostrocaudal gradient; effects were greater in the rostral and middle ARC (5-fold increases) than the caudal ARC (4-fold increase). In the VL-ARC of male rats, prolactin caused a transient increase in the percentage of TH-IR neurons expressing FRA at 1.5 h in all rostrocaudal ARC regions which returned to control levels by 3 h **(figure 4.11)**. Despite the changes in immediate early gene expression, prolactin had no effect on numbers of TH-IR neurons at any time point in any ARC subdivision or rostrocaudal region of male rats **(figure 4.12)**.

By way of summary, **figure 4.13** compares representative photomicrographs of TH-IR neurons expressing FRA-IR nuclei in the rostral DM-ARC of male rats injected with vehicle, haloperidol or prolactin 3 h prior to perfusion. The percentage of TH-IR neurons expressing FRA was approximately 1% after vehicle treatment, 6% after haloperidol treatment, and 25% after prolactin treatment in male rats.

Complementary studies performed in diestrous female rats demonstrated a descending gradient in numbers of TH-IR neurons in the DM subdivision as the ARC extends caudally, whereas numbers of these neurons in the VL subdivision remain fairly constant throughout the rostrocaudal ARC **(figure 4.14)**. Hence, numbers of TH-IR neurons in the DM relative to the those of the VL ARC subdivision vary by rostrocaudal

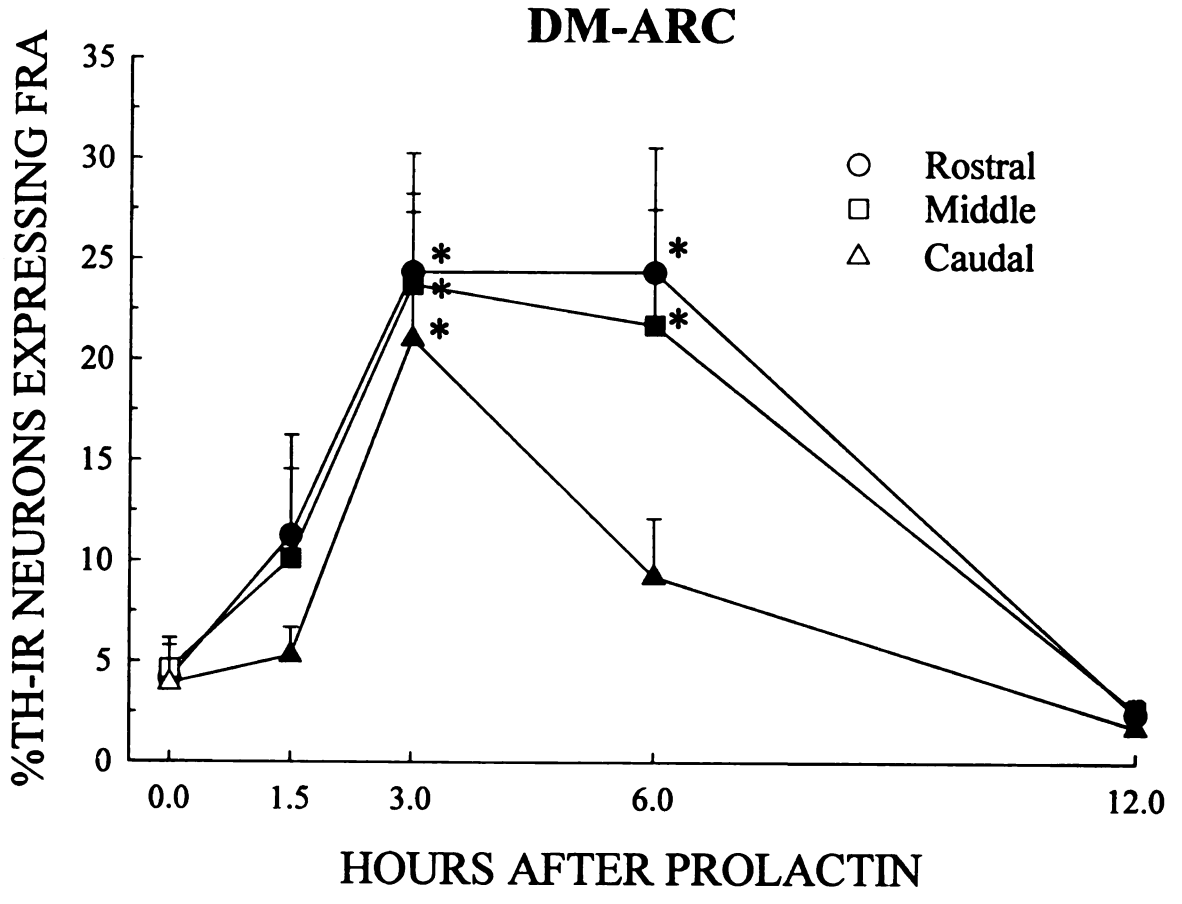


Figure 4.10 Time course effects of prolactin on %TH-IR neurons expressing FRA in the DM subdivision of rostral, middle and caudal ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with water vehicle (3 μl/rat; icv) 6 h prior to perfusion. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC (circles), -2.8 mm for middle ARC (squares) and -3.3 mm for caudal ARC (triangles) (Paxinos and Watson, 1997). Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

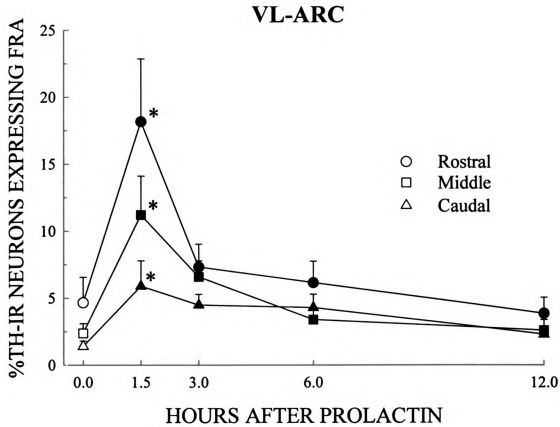


Figure 4.11 Time course effects of prolactin on %TH-IR neurons expressing FRA in the VL subdivision of rostral, middle and caudal ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with water vehicle (3 μl/rat; icv) 6 h prior to perfusion. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC (circles), -2.8 mm for middle ARC (squares) and -3.3 mm for caudal ARC (triangles) (Paxinos and Watson, 1997). Symbols represent means and vertical lines 1 SEM from 7-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

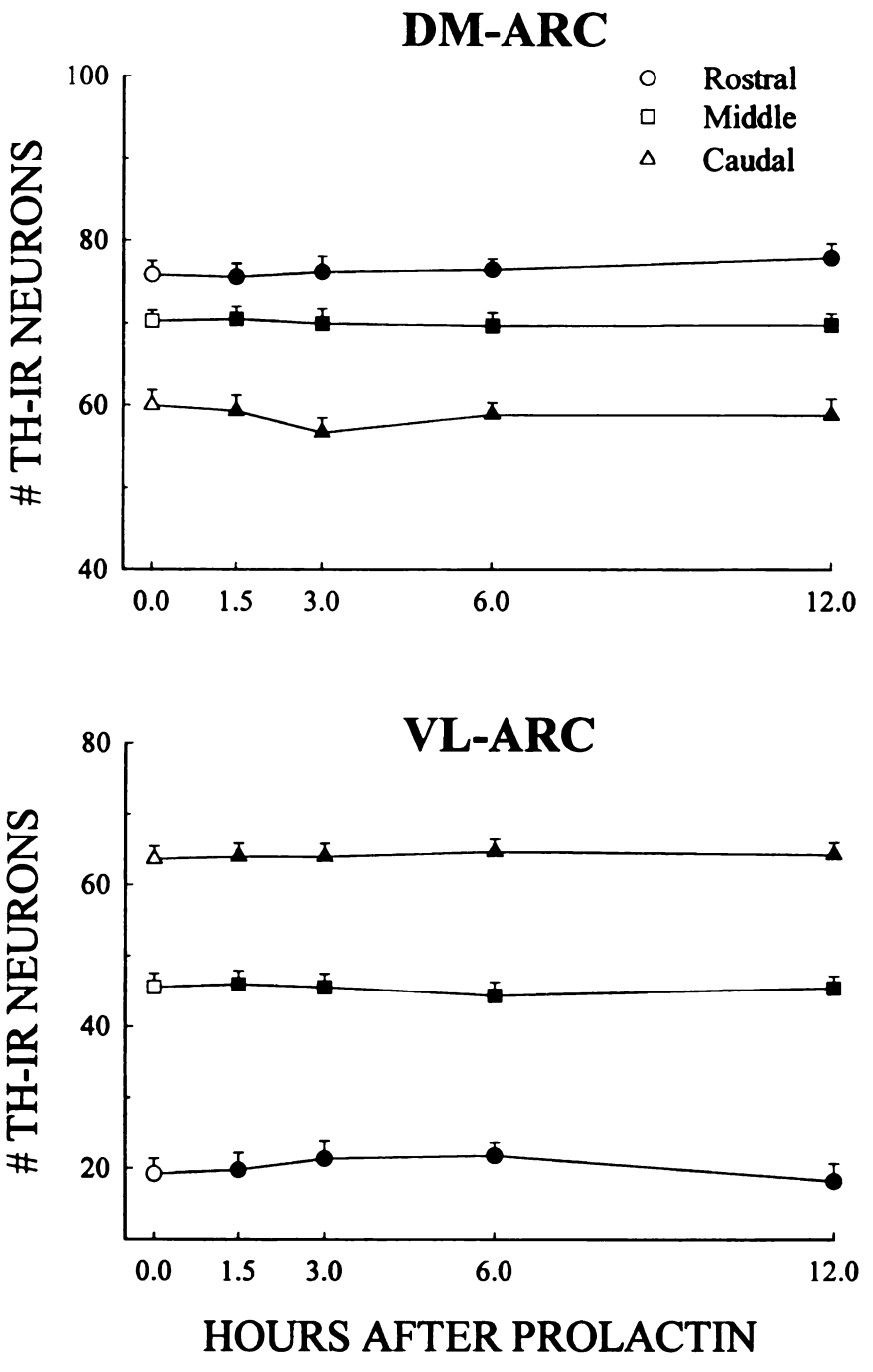


Figure 4.12 Time course comparison of the numbers of TH-IR neurons in the DM and VL subdivisions of rostral, middle and caudal ARC of vehicle- and prolactin-treated male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat, icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (distilled water; 3μl/rat, icv) 3 h prior to perfusion. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC (circles), -2.8 mm for middle ARC (squares) and -3.3 mm for caudal ARC (triangles) (Paxinos and Watson, 1997). Symbols represent means and vertical lines 1 SEM from 6-8 animals.

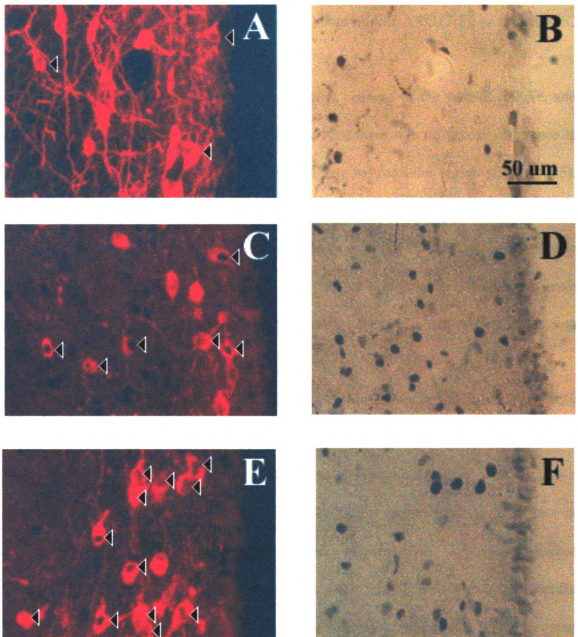


Figure 4.13 Comparison of digitized photomicrographs of TH-IR neurons expressing FRA in the rostral DM-ARC of male rats injected with vehicle, haloperidol or prolactin. Fluorescent TH-IR neurons under dark-field optics (panels A, C, E) and the same images under bright-field optics (panels B, D, F, respectively) showing blackened FRA-IR nuclei. Rats injected with either 0.3% tartaric acid (panels A and B; 1 ml/kg; sc), haloperidol (panels C and D; 1 mg/kg; sc) or prolactin (panels E and F; 10 μg/rat; icv) were perfused 3 h later. Arrowheads represent TH-IR neurons with FRA-IR nuclei.

region. In female rats, the ratio of numbers of TH-IR neurons in the DM-ARC to those of the VL-ARC is greater in the rostral ARC (11:1) than middle ARC (7:1), or caudal ARC (5:1).

In a comparison of the numbers of TH-IR neurons in the rostral, middle, and caudal ARC between male and diestrous female rats, there was no gender difference in numbers of TH-IR neurons in the DM-ARC at any rostrocaudal region **(figure 4.15)**. Yet, in the VL-ARC there were more TH-IR neurons in male rats than females at all rostrocaudal regions. Since numbers of TH-IR neurons in the VL-ARC remain relatively constant in females, yet increase caudally in males, this gender difference is most pronounced in the caudal ARC where the number of TH-IR neurons in males was approximately 5 times greater than that in females.

Neurochemical studies have demonstrated that in female rats circulating prolactin tonically stimulates TIDA neuronal activity to 2-3 times that of males (for review see Moore and Lookingland, 1995). Accordingly, while the percentage of TH-IR neurons expressing FRA-IR nuclei did not significantly differ across ARC subdivisions or rostrocaudal regions within basally active females **(figure 4.16)**, there was a marked gender difference. In both DM and VL subdivisions of all rostrocaudal ARC regions the basal percentage of TH-IR neurons expressing FRA-IR nuclei was greater in females than males **(figure 4.17)**. On average, the percentage of FRA expression in TH-IR neurons in females was 4-times greater in the DM-ARC, and 2-7 times greater in the VL-ARC as compared to males. In diestrous female rats the percentage of TH-IR neurons expressing FRA-IR nuclei in the DM-ARC of all rostrocaudal regions was significantly increased 3 h after central administration of prolactin **(figure 4.18)**. Greatest effects of prolactin were

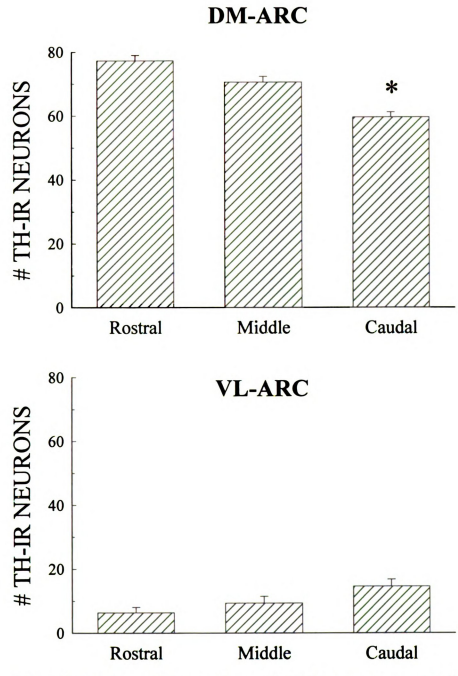


Figure 4.14 Comparison of the numbers of TH-IR neurons in the DM and VL subdivisions of rostral, middle and caudal ARC of diestrous female rats. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of TH-IR and FRA-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 6-8 animals. *, Values that are significantly different ($p < 0.05$) from the rostral ARC.

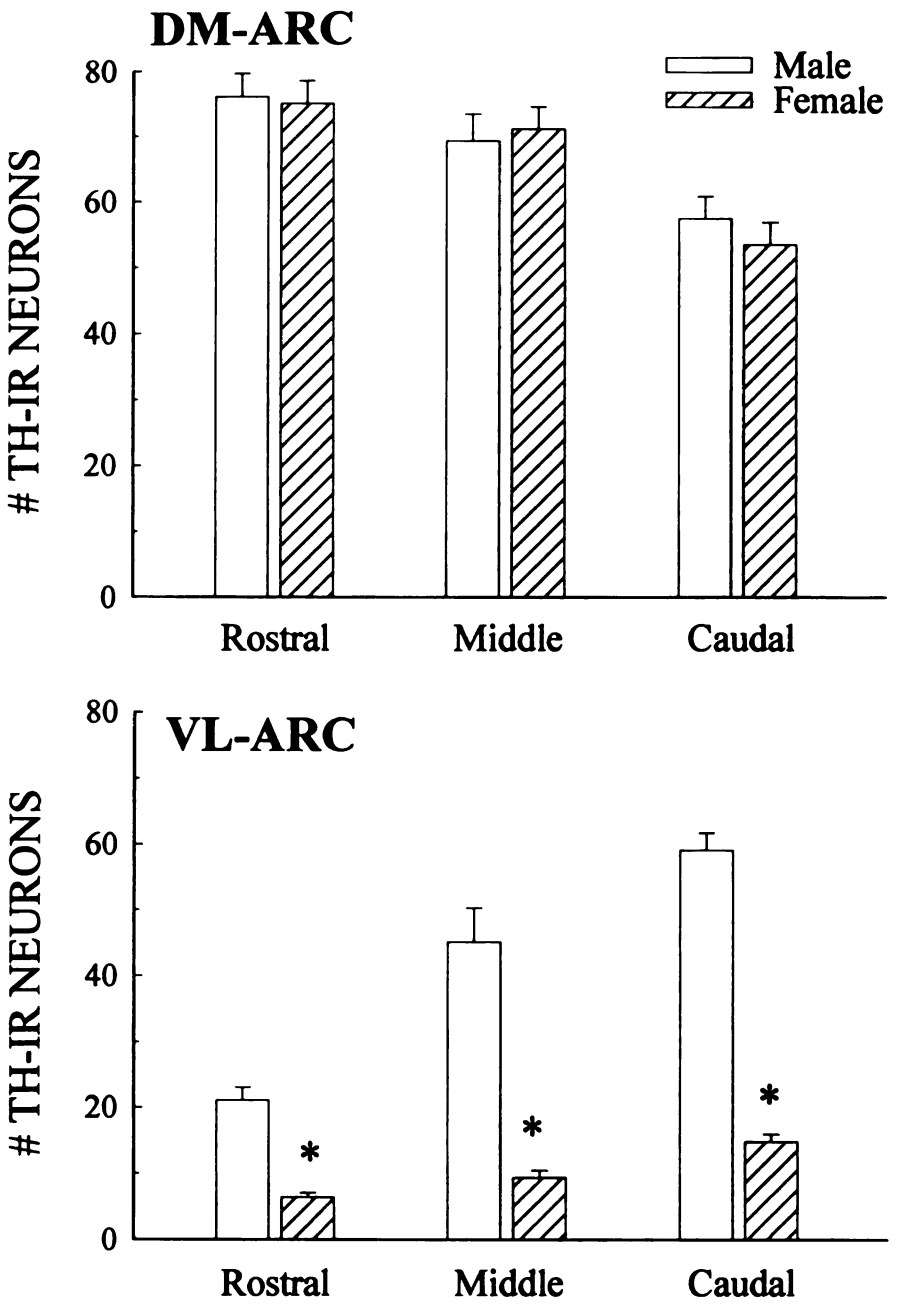


Figure 4.15 Comparison of numbers of TH-IR neurons in the DM and VL subdivisions of rostral, middle and caudal ARC of male and diestrous female rats. Male (open columns) and diestrous female (hatched columns) rats were perfused, brains sectioned and tissue immunohistochemically processed for the detection of TH-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values for diestrous female rats that are significantly different ($p < 0.05$) from region-matched male rats.

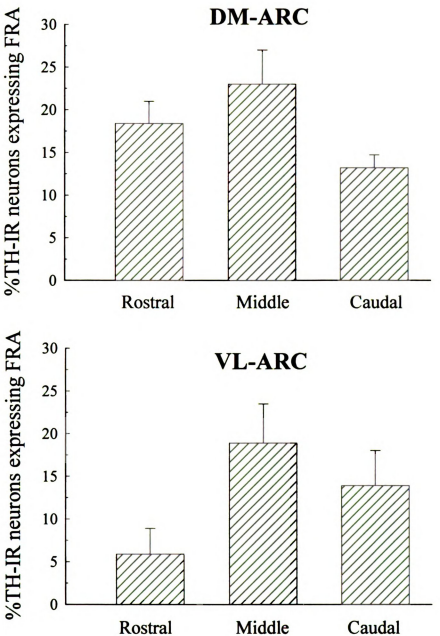


Figure 4.16 Comparison of %TH-IR neurons expressing FRA in the DM and VL subdivisions of rostral, middle and caudal ARC of female rats. Rats were perfused, brains sectioned and tissue immunohistochemically processed for the detection of TH-IR and FRA-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals.

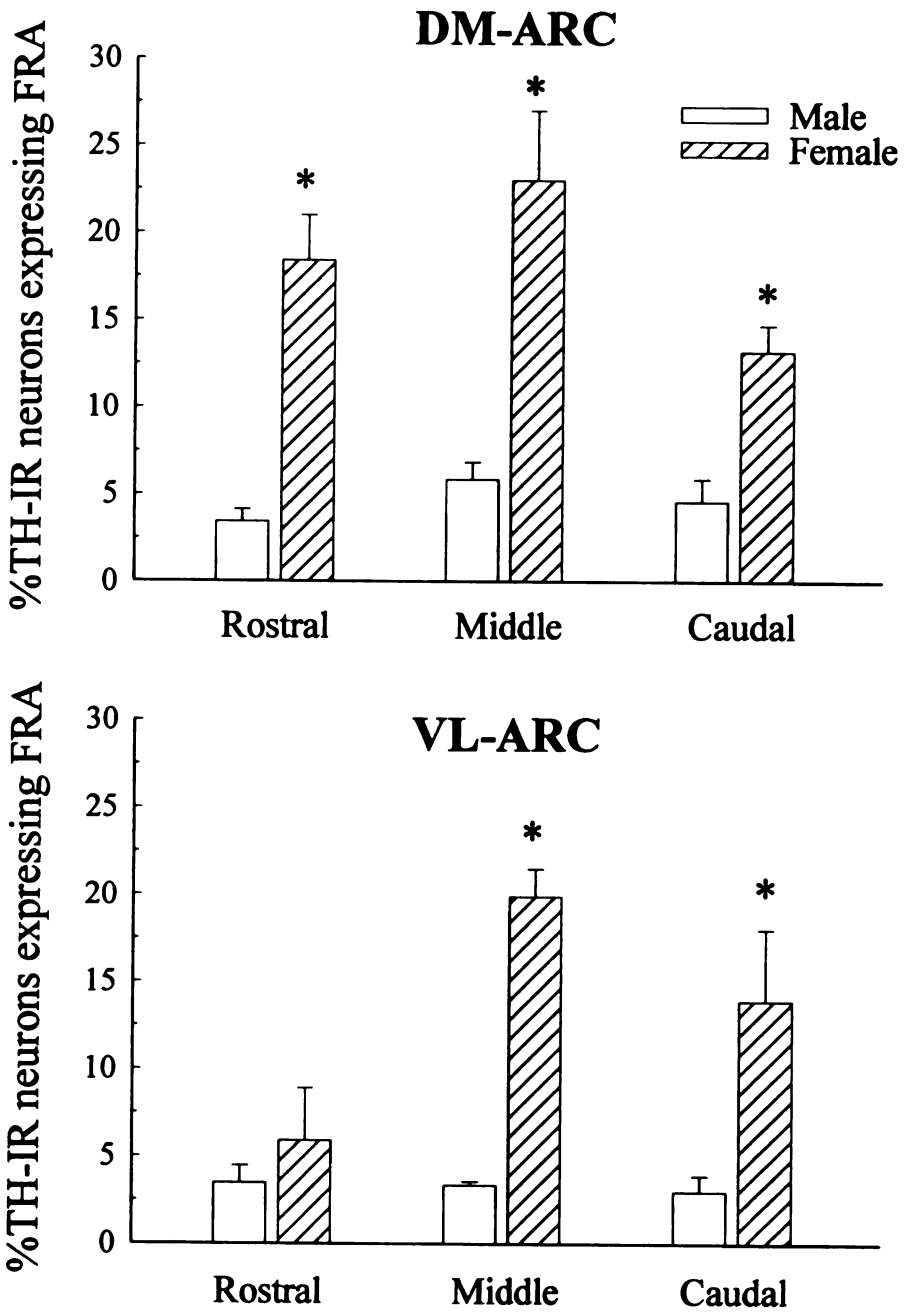


Figure 4.17 Comparison of %TH-IR neurons expressing FRA in the DM and VL subdivisions of rostral, middle and caudal ARC of male and diestrous female rats. Male (open columns) and diestrous female (hatched columns) rats were perfused, brains sectioned and tissue immunohistochemically processed for the detection of TH-IR and FRA-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values for diestrous female rats that are significantly different ($p < 0.05$) from region-matched male rats.

observed in the rostral and middle ARC (40-45% TH-IR neurons expressed FRA) as compared to the caudal ARC (30% TH-IR neurons expressed FRA). Additionally, in female rats, prolactin had no effect at 3 h in the VL-ARC of any rostrocaudal region **(figure 4.18)**.

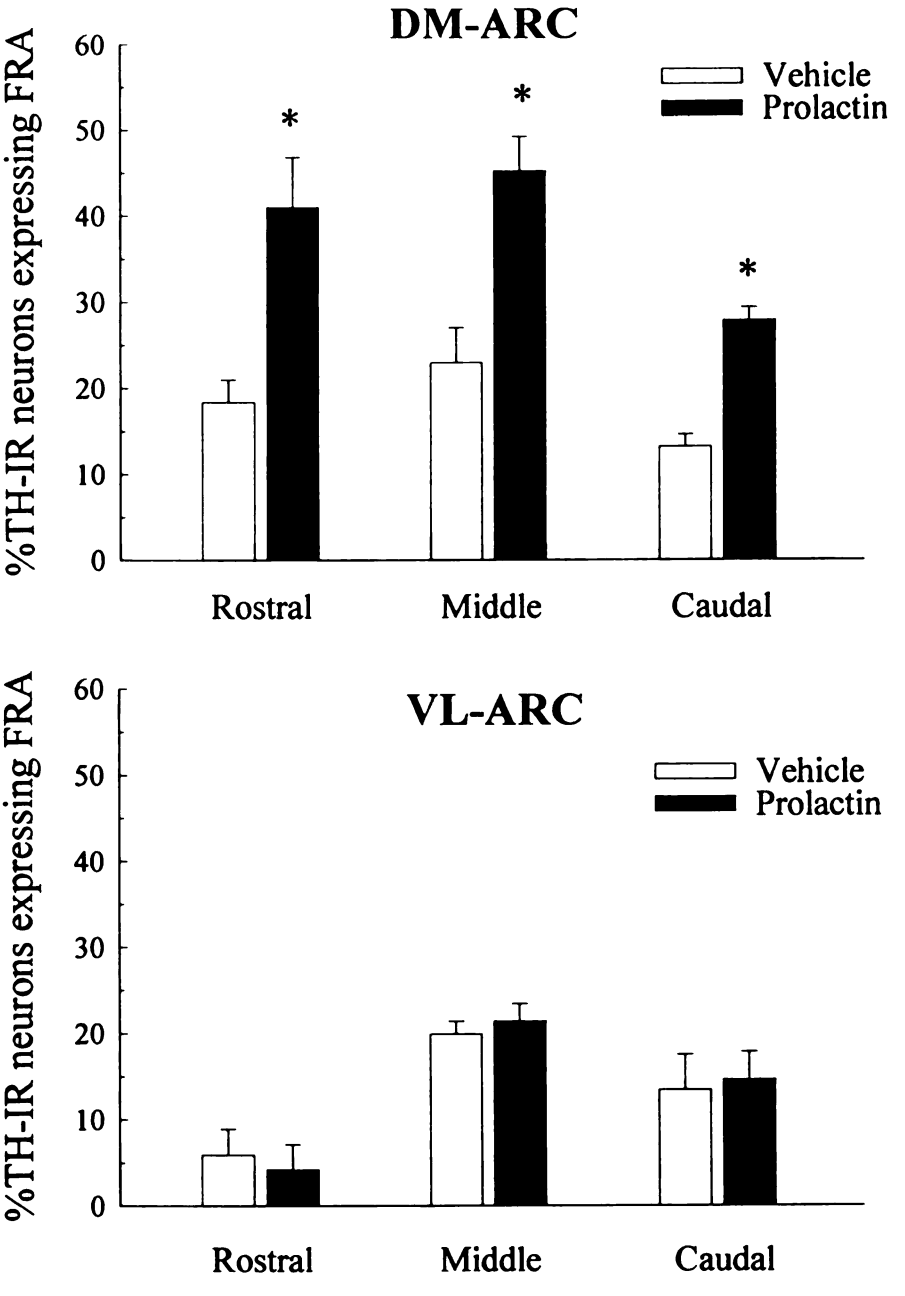


Figure 4.18 Effects of prolactin on %TH-IR neurons expressing FRA in the DM and VL subdivisions of rostral, middle and caudal ARC of diestrous female rats. Rats were injected with prolactin (filled columns; 10 μg/rat; icv) or its water vehicle (open columns; 3 μl/rat; icv) and perfused 3 h later. Brains were removed, sectioned and tissue immunohistochemically processed for the detection of TH-IR and FRA-IR in the DM (upper panel) and VL (lower panel) subdivisions of the rostral, middle and caudal ARC. Approximate distances relative to bregma for these regions were -2.3 mm for rostral ARC, -2.8 mm for middle ARC and -3.3 mm for caudal ARC (Paxinos and Watson, 1997). Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values for diestrous female rats that are significantly different ($p < 0.05$) from regionally-matched vehicle-treated controls.

C. DISCUSSION

The discovery that the immediate early gene *c-fos* is rapidly and transiently expressed in neurons following sensory stimulation (Morgan and Curran, 1989) has spurred interest in the role of AP-1 transcription factors such as FRA and Jun-related antigens in mediating adaptive genomic changes in neurons following both acute and chronic activation (Sheng and Greenberg, 1990; Pennypacker et al., 1995; Harris, 1998). Moreover, mapping of FRA-IR neurons has led to the localization and neurochemical identification of neuronal populations within specific brain regions that are uniquely responsive to a variety of stimuli including seizure, brain injury, pain, and administration of dopamine agonists (Pennypacker et al., 1995; Chaudhuri, 1997). The aim of the present study was to determine if prolactin regulates FRA expression in TIDA neurons located in anatomically distinct subdivisions of the rostral, middle and caudal ARC.

Perikarya of TIDA neurons, originally described as comprising a majority of the A_{12} cell group (Dahlstrom and Fuxe, 1964), are distributed throughout the rostrocaudal extent of the ARC. Two populations of TH-containing neurons have been identified based on their neurochemical phenotypes, and the size and location of their perikarya in the DM and VL subdivisions of the ARC (Everitt et al., 1986). TH-IR neurons in the DM-ARC predominantly innervate the medial portion of the median eminence (Kobayashi and Matsui, 1969; Rethelyi, 1985; Daikoku et al., 1986) and are generally believed to be the primary inhibitors of pituitary prolactin release (Andersson et al., 1981; Reymond et al., 1983; Hoffman et al., 1994; Lee and Voogt, 1999). TH-IR neurons in the VL-ARC innervate the more lateral aspects of the median eminence (Kobayashi and Matsui, 1969; Fuxe et al., 1986), but since these neurons lack AAAD and do not

synthesize dopamine (Meister et al., 1988a; Komori et al., 1991; Misu et al., 1996; Skagerberg et al., 1988), their function is unknown. However, since vascular tissue may express AAAD, it is possible that DOPA released at the median eminence into the hypophysial portal vessels may be converted to dopamine *en route* to the pituitary (Bertler et al., 1964; Constantinidis et al., 1969).

The results of the present study demonstrate an inverse relationship in male rats between numbers of TH-IR neurons in the DM- and VL-ARC in the rostrocaudal plane; i.e. with increasing caudal extent numbers of TH-IR neurons in the DM-ARC decrease, whereas those of the VL-ARC increase. TH-IR neurons projecting to the medial aspect of the median eminence have been shown to have a bimodal rostrocaudal distribution (Kawano and Daikoku, 1987); however, it is possible that this rostrocaudal gradient may be attributed to the inclusion of a small population of tuberohypophysial dopaminergic perikarya (Lookingland et al., 1985) during quantification of the rostral ARC since these neurons could not be distinguished from TIDA neurons in this study. The large number of TH-IR neurons in the caudal VL-ARC of male rats suggests physiological relevance, however, the role of these neurons remains unknown.

In agreement with a previous study (Cheung et al., 1997), there are different numbers of TIDA neurons in the DM- and VL-ARC at any given rostrocaudal region. For example, in male rats there are up to 4 times more TIDA neurons in the rostral DM-ARC than in the adjacent rostral VL-ARC (Cheung et al., 1997). The preponderance of TH-IR neurons in the DM-ARC may imply the relative importance of these neurons in the regulation of prolactin secretion as compared to those of the VL-ARC.

Despite differences in numbers of TH-IR neurons in ARC subdivisions and rostrocaudal regions, the percentage of these neurons expressing FRA-IR nuclei was uniformly low (3-5%) across all ARC subdivisions and rostrocaudal regions in male rats. These results presumably reflect the low level of neurotransmitter release and *de novo* replenishment in TIDA neurons. Furthermore, these results argue against differential levels of TIDA neuronal activity in the DM- and VL-ARC of male rats.

Haloperidol is a D_2 receptor antagonist that rapidly stimulates prolactin secretion via disinhibition of the anterior pituitary lactotrophs. The prolonged hyperprolactinemia that follows feeds back to stimulate TIDA neurons (Moore and Lookingland, 1995). Haloperidol has no direct acute effects on TIDA neurons. Its latent stimulatory effects can be blocked by hypophysectomy (Gudelsky et al., 1977) or prolactin antiserum (Gudelsky and Porter, 1980; Van Loon et al., 1983), demonstrating that the stimulatory effects are mediated by prolactin. In male rats, this "inductive" activation of TIDA neurons is characterized by long latency (12 h), increased TH mRNA in the ARC (Arbogast and Voogt, 1991; Selmanoff et al., 1991) and dependence upon protein synthesis (Johnston et al., 1980). These features suggest that prolactin induces long-term changes in gene expression that result in increased capacity of TIDA neurons to synthesize and release dopamine. Prolactin may elicit these effects through regulation of FRA expression in TIDA neurons.

Consistent with this hypothesis, the results of the present study reveal that in male rats prolactin modulates FRA expression in TIDA neurons located in both DM and VL subdivisions of the ARC without affecting the overall numbers of these neurons. In the DM-ARC, both haloperidol-induced endogenous hyperprolactinemia and central

administration of prolactin cause similar delayed increases in the percentage of TIDA neurons expressing FRA with peak responses occurring by 3 h. This response persists for an additional 3 h in prolactin-treated rats. This is likely due to the central route of administration and the dose of prolactin used in this study which undoubtedly results in greater access of prolactin to its site of action in the mediobasal hypothalamus (Gudelsky et al., 1978) and a more prolonged activation of prolactin receptors within this region (Chiu et al., 1992; Chiu and Wise, 1994).

Since changes in FRA expression in TIDA neurons of the DM-ARC expected at 3 h could be completely blocked by immunoneutralization of circulating prolactin 1 h after haloperidol-injection, it is possible that there exists a stimulus threshold for the induction of FRA expression (requiring duration of hyperprolactinemia greater than 1 h), or continuous hyperprolactinemia is required for maintenance of the response in these neurons.

In the VL-ARC, the stimulatory effect of prolactin on FRA expression in TIDA neurons precedes that in the DM-ARC and is transient; i.e. by 3 h after prolactin administration (when peak responses in TIDA neurons in the DM-ARC were attained) effects in the VL-ARC were no longer observed. Considering that transcriptional activation of *c-fos* and related immediate early genes occurs within minutes, with the accumulation of nuclear protein reaching peak levels on average about 90–120 min later (Harris, 1998). Thus temporal differences in the onset of prolactin-induced changes in expression of FRA by TIDA neurons in the DM- and VL-ARC suggest two possibilities. Either the mechanisms by which prolactin activates these neurons are different (i.e. acute direct versus delayed secondary actions), or TIDA neurons in the VL-ARC are more

sensitive to the stimulatory actions of prolactin than those of the DM-ARC. Differences in sensitivities of these neurons to prolactin could be due to differences in the types, numbers and/or affinities of their prolactin receptors (Kelly et al., 1992; Chiu and Wise, 1994; Arbogast and Voogt, 1997; Bakowska and Morrell, 1997).

In male rats haloperidol suppresses FRA expression in TIDA neurons in the VL-ARC and blocks the stimulatory effects of prolactin on these neurons via a prolactin-independent mechanism. Indeed, immunoneutralization of circulating prolactin in haloperidol-treated rats has no effect on the ability of this D_2 dopamine antagonist to decrease the percentage of TIDA neurons expressing FRA in the VL-ARC, in contrast to the DM-ARC. The inhibitory effect of haloperidol on FRA expression in TIDA neurons in the VL-ARC could due to blockade of D_2 dopamine receptor-mediated tonic regulation of immediate early gene expression in these neurons that is not present in TIDA neurons of the DM-ARC. The significance of this finding remains to be elucidated.

Complementary studies in female rats examined the distribution of TH-IR neurons in ARC subdivisions across rostrocaudal regions and compared these findings with those of male rats. Similar to male rats, females exhibit a descending rostrocaudal gradient in numbers of TH-IR neurons in the DM-ARC subdivision. In the VL-ARC a relatively small subpopulation of TH-IR neurons is evenly distributed throughout the rostrocaudal ARC. Since numbers of these neurons remain constant in females, yet increase caudally in males, a gender difference exists; male rats have 5 times more of these neurons in the caudal VL-ARC than female rats. While the role of TH-IR neurons of the VL-ARC remains elusive, the results suggest a gender-related function. In agreement with previous studies, female rats have several times more TIDA neurons in the DM-ARC than in the

VL-ARC at a given rostrocaudal region (Cheung et al., 1997; Lerant and Freeman, 1997). The preponderance of TH-IR neurons in the DM-ARC, particularly in females, may imply the relative importance of these neurons in the regulation of prolactin secretion as compared to those of the VL-ARC.

There are sexual differences in the basal expression of FRA in TIDA neurons in the DM-ARC (Cheung et al., 1997) that correspond with differences between males and females in the activities of TIDA neurons associated with neurotransmitter release and its replenishment via *de novo* synthesis. Indeed, the synthesis, release and metabolism of dopamine in the median eminence are all elevated in females as compared with males (Demarest and Moore, 1981b; Gudelsky and Porter, 1981; Demarest et al., 1984; Lookingland et al., 1987a), due, in part, to higher levels in females of TH mRNA in perikarya in the ARC (Arbogast and Voogt, 1990) and TH in terminals in the median eminence (Porter, 1986). In agreement, the results of present study reveal that the percentage of TH-IR neurons expressing FRA in both the DM- and VL-ARC of all rostrocaudal regions is uniformly elevated and several-fold higher in non-stimulated female rats as compared to males.

Since sexual differences in neurochemical activity of TIDA neurons are partially due to the feedback effects of circulating prolactin (Moore and Lookingland, 1995) it was likely that prolactin would also regulate FRA expression in females. Indeed, prolactin increased the percentage of TIDA neurons expressing FRA in the DM-ARC, but not the VL-ARC at 3 h in female rats. A comparison of the effects of prolactin on FRA expression in TH-IR neurons of the DM-ARC in male and female rats shows a gender difference in absolute magnitude of effect, i.e. females have a greater response to

prolactin than males. There is neurochemical evidence that removal of endogenous circulating prolactin reduces the responsiveness of TIDA neurons to subsequent prolactin administration (Demarest et al., 1985b). Therefore, it is plausible that the observed gender difference in magnitude of response could be due to the different endogenous levels of circulating prolactin and its effects on TIDA neurons. There is a rostrocaudal gradient in magnitude of response to prolactin at a given dose in both male and female rats, such that effects of prolactin are consistently greater in the rostral and middle ARC than the caudal ARC. This observation could be due to differences in the types, numbers and/or affinities of their prolactin receptors (Kelly et al., 1992; Chiu and Wise, 1994; Arbogast and Voogt, 1997; Bakowska and Morrell, 1997).

In conclusion, these results reveal that: (1) prolactin regulates immediate early gene expression in TIDA neurons in both male and female rats, (2) there are temporal differences in the response of TIDA neurons in DM- and VL-ARC subdivisions to prolactin (i.e. prolactin initially stimulates transient expression of FRA in TIDA neurons of the VL-ARC, followed by a more prolonged activation of FRA expression in TIDA neurons of the DM-ARC), and (3) there is a gender difference in magnitude of response of TIDA neurons to prolactin (i.e. female rats demonstrate a greater response to prolactin than males), and (4) there are rostrocaudal differences in the magnitude of response of TIDA neurons to prolactin (i.e. after correcting for differences in absolute numbers of TIDA neurons, prolactin elicits its greatest response in the rostral ARC and least response in the caudal ARC).

5. EFFECTS OF PROLACTIN ON FRA EXPRESSION IN NON-TH-IR CELLS IN THE ARCUATE NUCLEUS

A. INTRODUCTION

Neural activity mapping with inducible transcription factors (e.g. *c-fos*, FRA, zif268) has led to the localization and neurochemical identification of neuronal populations within specific brain regions that are uniquely responsive to a variety of stimuli including seizure, brain injury, pain, and administration of dopamine agonists (Sheng and Greenberg, 1990; Pennypacker et al., 1995; Chaudhuri, 1997).

In the previous chapter this method was utilized in the characterization of prolactin-induced changes in FRA expression in TIDA neurons (Hentschel et al., 2000b). During the analysis of those experiments, it appeared that changes in FRA expression were also occurring in non-TH-IR cells of the ARC. Furthermore, these changes seemed to temporally precede those in TIDA neurons and were localized within cells in the ARC, consistent with the complete deafferentation study that demonstrated the haloperidol-induced, prolactin-mediated increase in dopamine turnover in the median eminence results from actions of prolactin on neurons within the mediobasal hypothalamus (Gudelsky et al., 1977). Given the 6-12 h latency that precedes the delayed inductive activation of TIDA neurons (Demarest et al., 1984; 1986), the approximate anatomical localization of these cells and their apparent responsiveness to prolactin, it is plausible that these chemically unidentified cells could mediate the effects of prolactin on TIDA neurons.

Accordingly, the first step in this investigation is to verify the purported prolactin-induced changes in immediate early gene expression in chemically unidentified cells in

the ARC. The working hypothesis is that prolactin regulates FRA expression in subsets of non-TH-IR cells within this region. If this is the case, then dual FRA/TH immunohistochemistry will show differential expression of FRA-IR in non-TH-IR cells in the ARC after prolactin treatment. To this end, initial experiments determined the time course effects of haloperidol-induced endogenous hyperprolactinemia on numbers of FRA-IR cells that do not contain TH-IR in the DM and VL subdivisions of the rostral, middle and caudal ARC (Paxinos and Watson, 1997) of male rats. Follow-up experiments examined the ability of PRL-AB to block the effects of haloperidol on FRA expression and compared the effects of haloperidol to those of exogenous prolactin administration in male rats.

B. RESULTS

In the present study changes in FRA expression were reported by ARC subdivision. As shown in **figure 2.9**, the ARC was divided into DM and VL subdivisions by extension of a line dorsolaterally at an angle of 30-50° from the ventrolateral aspect of the third ventricle. The DM subdivision was further separated into two regions based upon the presence (magnocellular) or absence (parvocellular) of large TH-IR perikarya.

Basal FRA expression in non-TH-IR cells of the ARC was relatively low ranging between 5-15 cells per region per section across all ARC subdivisions and rostrocaudal regions in male rats (**figure 5.1**). Within a particular ARC subdivision, levels of FRA expression were uniform throughout the rostrocaudal ARC. However, while FRA expression in non-TH-IR cells in the magnocellular-DM and ventrolateral ARC were not statistically different from one another, both regions had significantly greater FRA expression than that of the parvocellular-DM ARC.

Haloperidol was utilized in these studies to induce endogenous hyperprolactinemia. As established in previous chapters (**figures 3.1 and 4.1**), systemic administration of the D_2 dopamine receptor antagonist haloperidol increased plasma prolactin concentrations to peak levels by 1 h, and maintained endogenous hyperprolactinemia for 12 h post-injection.

Figure 5.2 demonstrates the DM-ARC subdivisions and compares representative photomicrographs of FRA expression in these areas of the middle ARC 3 h after vehicle or haloperidol injection in male rats. Tyrosine hydroxylase-IR perikarya that fluoresce characterize the magnocellular-, but not parvocellular-, DM ARC. Bright-field images show that FRA expression is modestly increased in the magnocellular-DM ARC

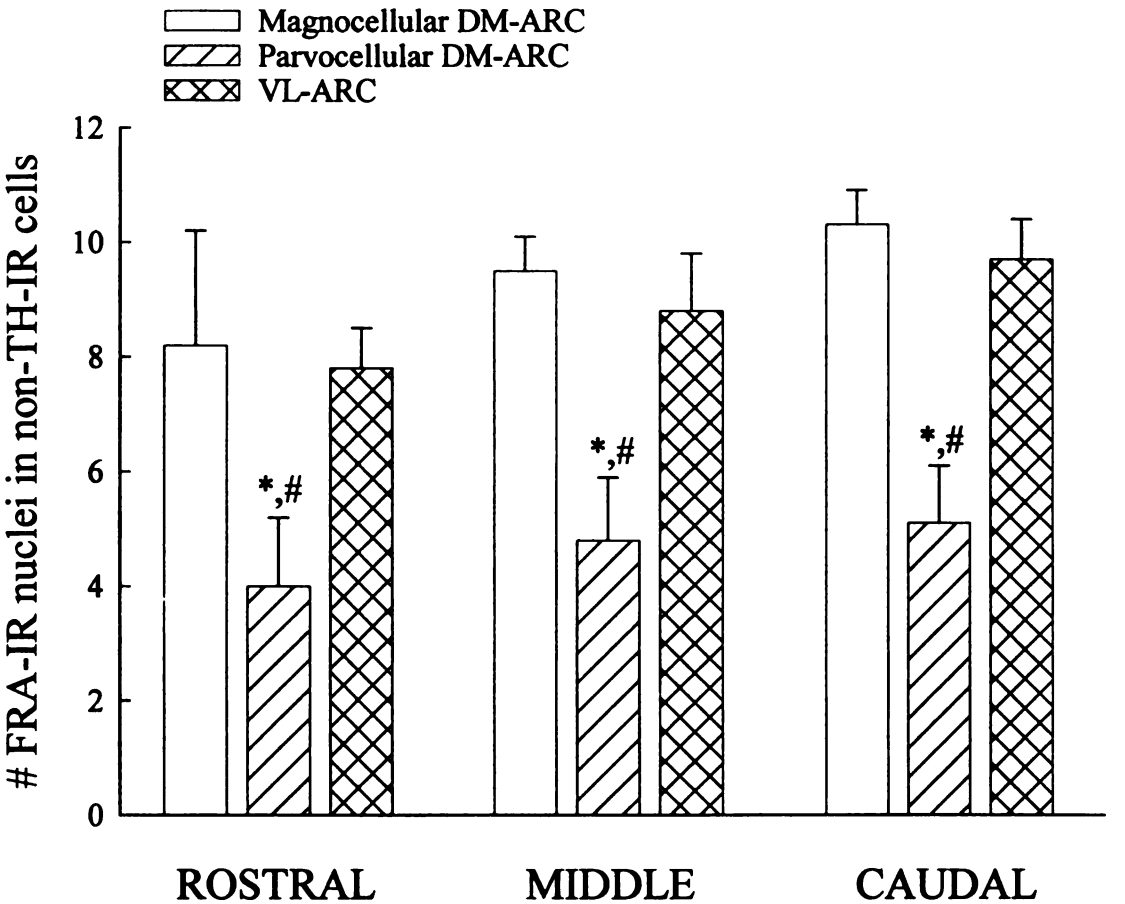


Figure 5.1 Comparison of basal numbers of FRA-IR nuclei in non-TH-IR cells in subdivisions of the rostral, middle and caudal ARC in male rats. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and TH-IR in the magnocellular-DM (open columns), parvocellular-DM (hatched columns) and VL (cross hatched columns) subdivisions of the rostral, middle and caudal ARC. Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from the magnocellular-DM ARC. #, Values that are significantly different ($p < 0.05$) from the VL ARC.

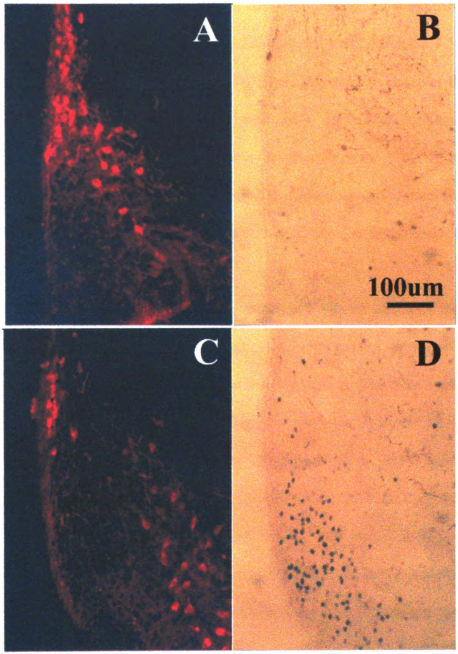


Figure 5.2 Digitized photomicrographs showing increased FRA expression in the middle ARC after haloperidol injection in male rats. Fluorescent TH-IR neurons under dark-field optics (panels A and C) and the same images under bright-field optics (panels B and D, respectively) showing blackened FRA-IR nuclei. Rats were injected with either 0.3% tartaric acid (panels A and B; 1 ml/kg; sc), or haloperidol (panels C and D; 1 mg/kg; sc) and perfused 3 h later.

and greatly increased in the parvocellular-DM ARC after haloperidol administration.

Numbers of FRA-IR nuclei in non-TH-IR cells were increased 1.5–3 h after haloperidol injection in the magnocellular-DM (**figure 5.3**) and parvocellular-DM (**figure 5.4**) ARC of all rostrocaudal regions in male rats. In contrast to the DM-ARC subdivisions, numbers of FRA-IR nuclei in non-TH-IR cells of the VL-ARC subdivision were decreased 3-6 h after haloperidol injection in all rostrocaudal regions (**figure 5.5**). There were no rostrocaudal differences in magnitude of response to haloperidol treatment within any ARC subdivision.

The effects of haloperidol could be mediated by direct action of this drug on central dopamine receptors, or indirectly via endogenous hyperprolactinemia. Accordingly, endogenous prolactin was immunoneutralized to determine if the effects of haloperidol on FRA expression were mediated by prolactin. Prolactin antiserum was administered at a dose known to block the stimulatory effects of estrogen on TIDA neuronal activity (Toney et al., 1992b) and suppress plasma prolactin concentrations for at least 3 h after haloperidol treatment (**figure 3.4**). As shown in **figure 5.6**, co-administration of prolactin antiserum completely blocked the haloperidol-induced increase in numbers of FRA-IR nuclei in non-TH-IR cells (at 3 h) in the magnocellular-DM ARC of all rostrocaudal regions. In contrast, prolactin antiserum merely blunted the haloperidol-induced increase in the numbers of FRA-IR nuclei in non-TH-IR cells (at 3 h) in the parvocellular-DM ARC (**figure 5.7**), and had no effect on the haloperidol-induced decrease in the numbers of FRA-IR nuclei in non-TH-IR cells in the VL-ARC (**figure 5.8**). These observations were consistent across all rostrocaudal ARC regions.

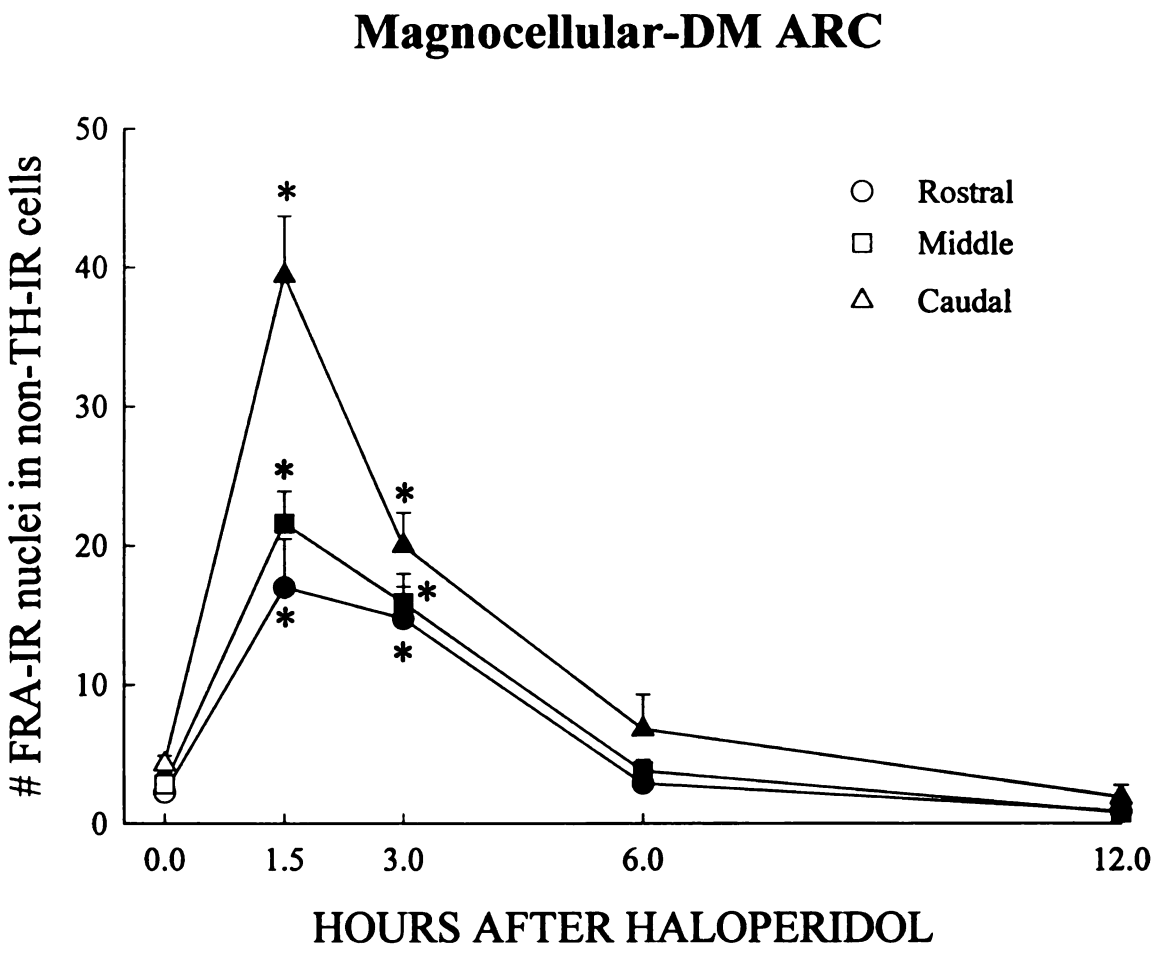


Figure 5.3 Time course effects of haloperidol on numbers of FRA-IR nuclei in non-TH-IR cells in the DM-MC subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. Symbols represent means and vertical lines 1 SEM from 7-9 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

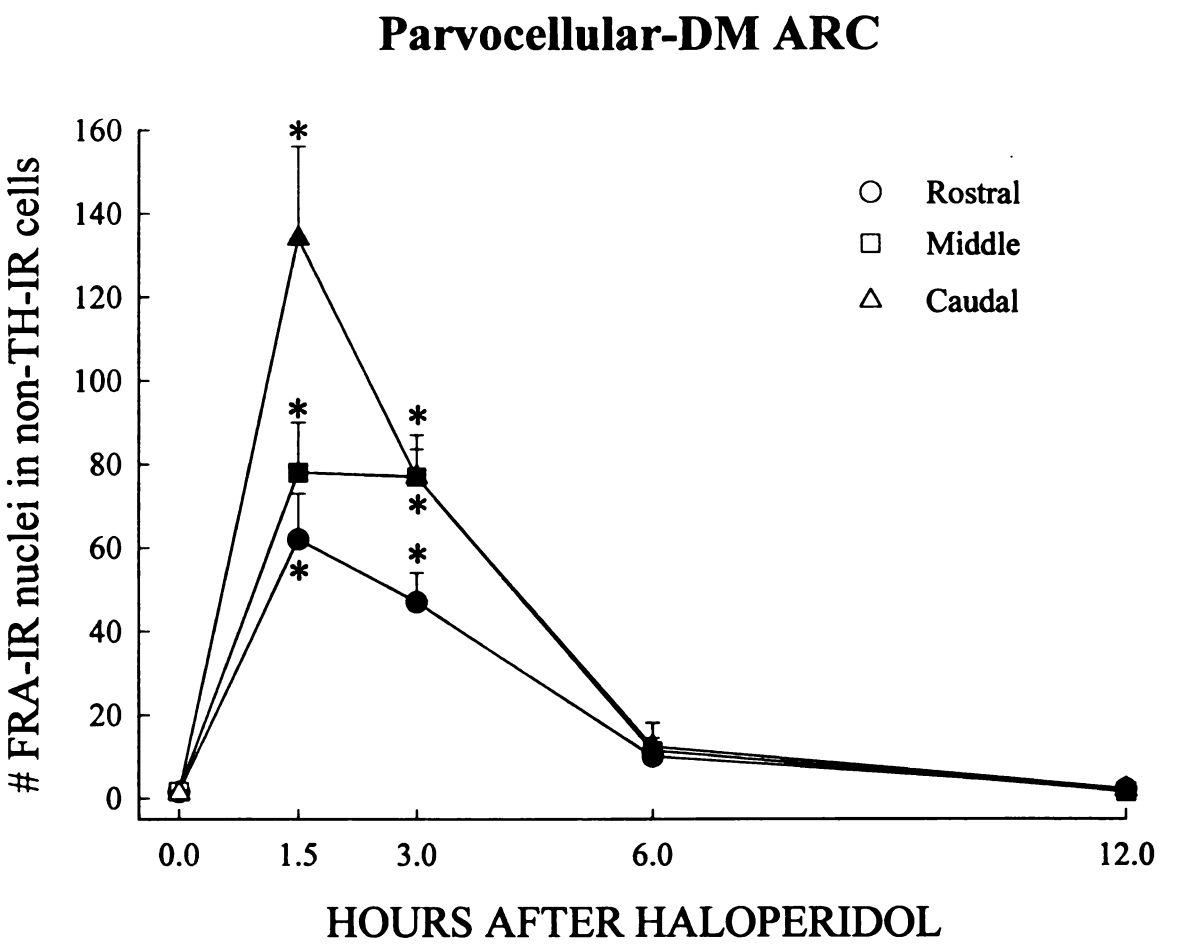


Figure 5.4 Time course effects of haloperidol on numbers of FRA-IR nuclei in non-TH-IR cells in the parvocellular-DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. Symbols represent means and vertical lines 1 SEM from 7-9 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

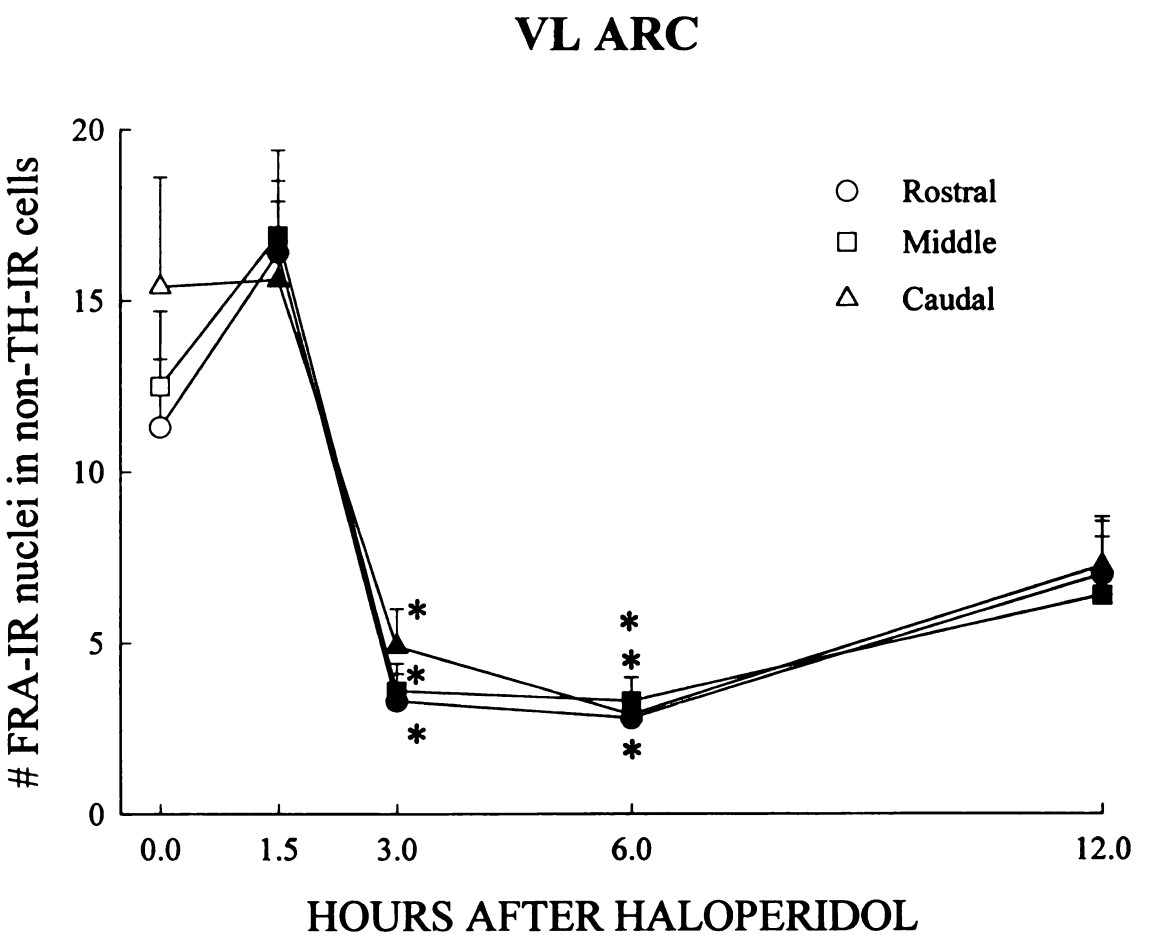


Figure 5.5 Time course effects of haloperidol on numbers of FRA-IR nuclei in non-TH-IR cells in the VL subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. Symbols represent means and vertical lines 1 SEM from 7-9 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

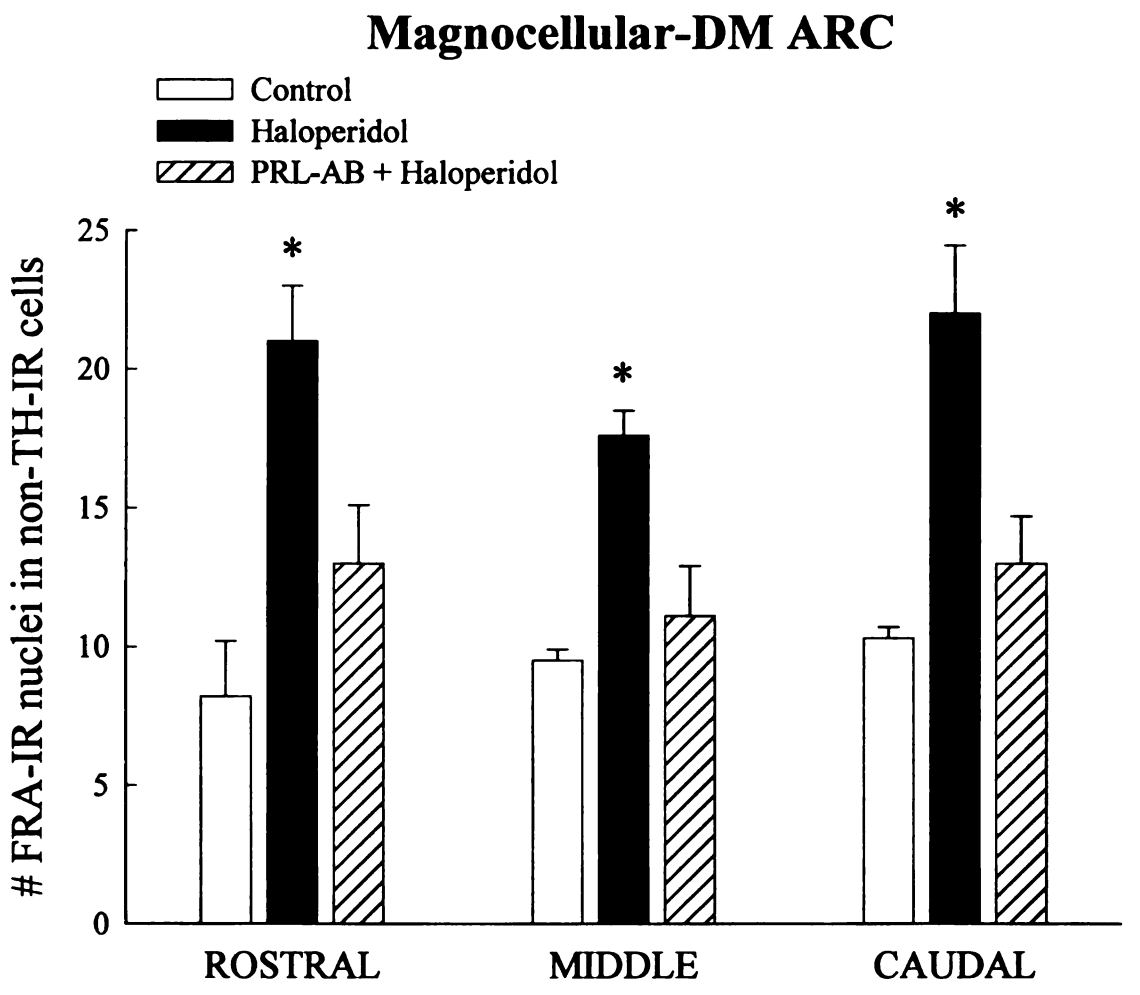


Figure 5.6 Effects of prolactin antiserum on numbers of FRA-IR nuclei in non-TH-IR cells in the magnocellular-DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of haloperidol-treated male rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; sc) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; sc), and with either prolactin antiserum (PRL-AB; hatched columns; 200 µl/rat; iv) or vehicle (normal rabbit serum, 200 µl/rat; iv) 3 h prior to decapitation. Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from vehicle-treated controls.

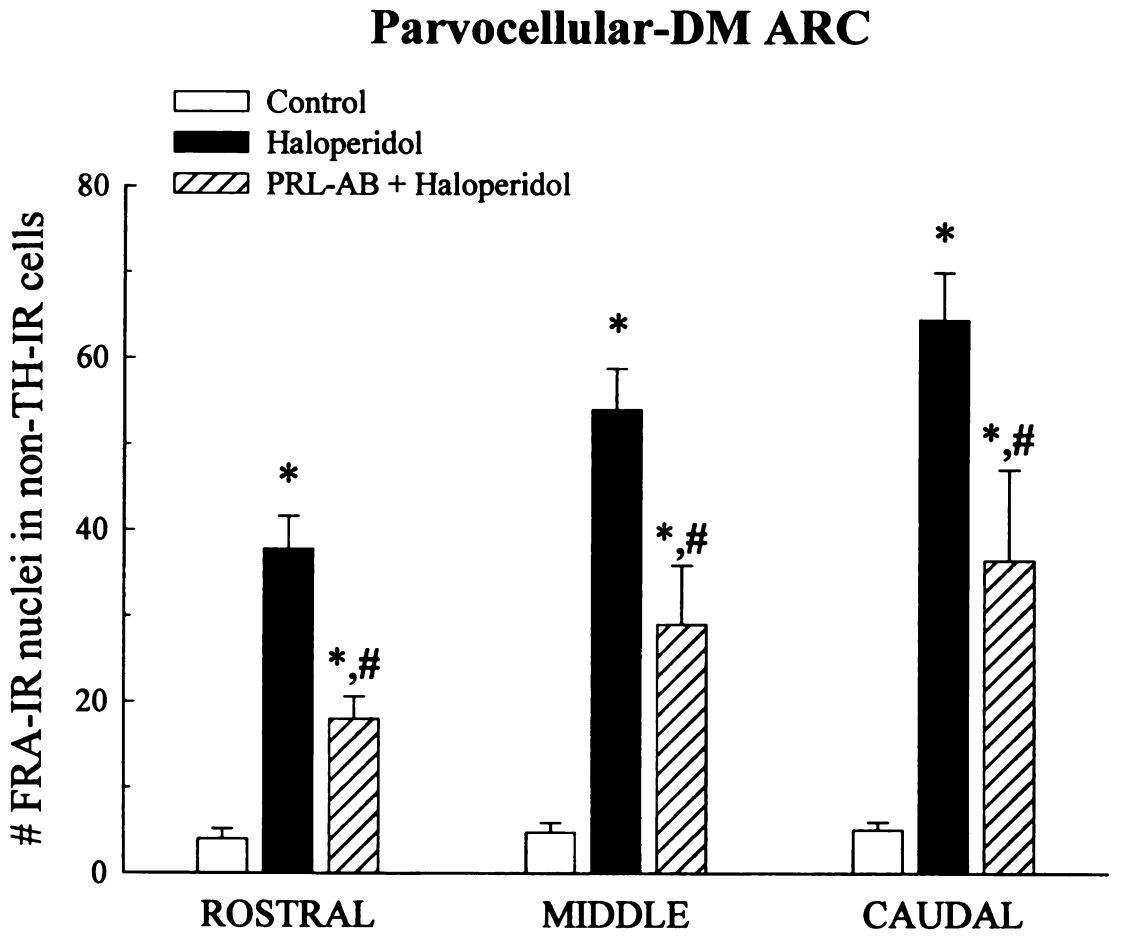


Figure 5.7 Effects of prolactin antiserum on numbers of FRA-IR nuclei in non-TH-IR cells in the parvocellular-DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of haloperidol-treated male rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; sc) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; sc), and with either prolactin antiserum (PRL-AB; hatched columns; 200 µl/rat; iv) or vehicle (normal rabbit serum, 200 µl/rat; iv) 3 h prior to decapitation. Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from vehicle-treated controls. #, Values that are significantly different ($p < 0.05$) from haloperidol-treated controls.

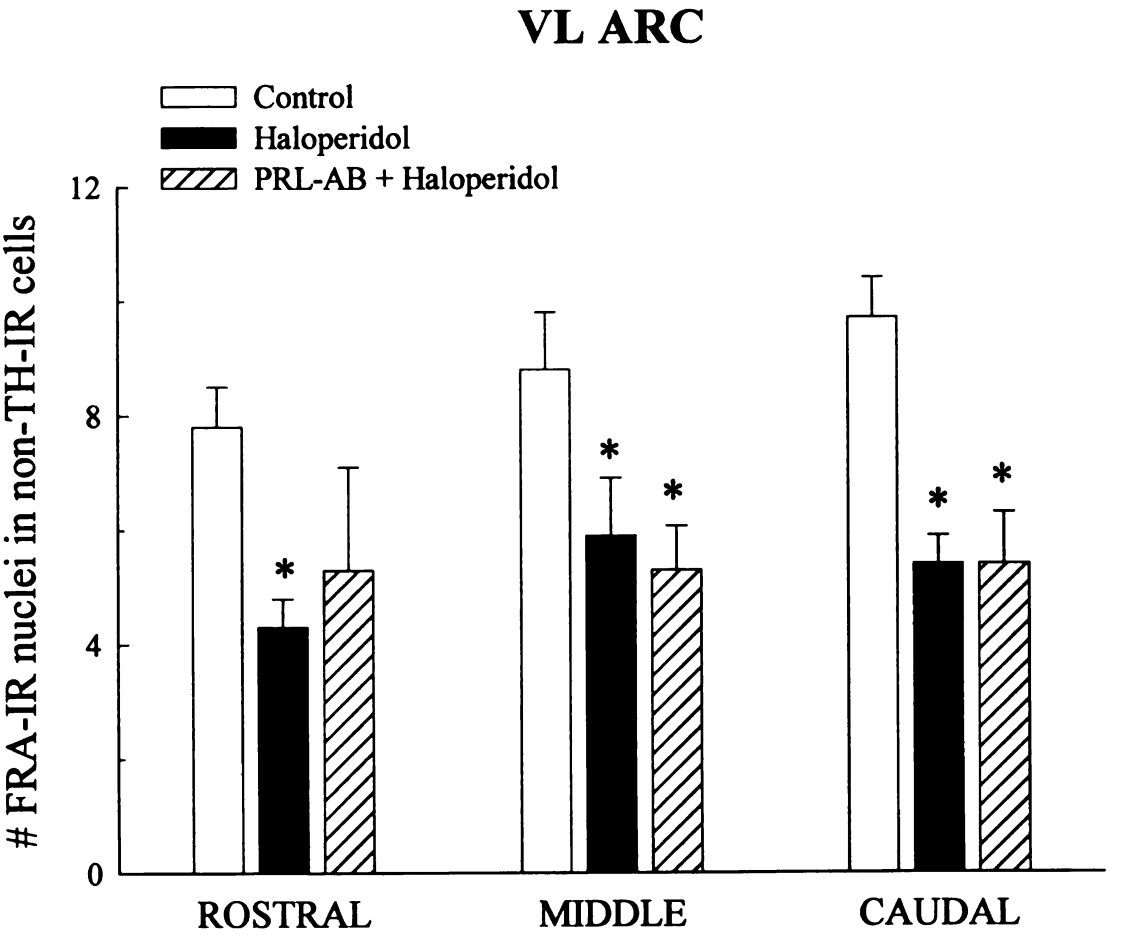


Figure 5.8 Effects of prolactin antiserum on numbers of FRA-IR nuclei in non-TH-IR cells in the VL subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of haloperidol-treated male rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; sc) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; sc), and with either prolactin antiserum (PRL-AB; hatched columns; 200 µl/rat; iv) or vehicle (normal rabbit serum, 200 µl/rat; iv) 3 h prior to decapitation. Columns represent means and vertical lines 1 SEM from 7-9 animals. *, Values that are significantly different ($p < 0.05$) from vehicle-treated controls.

Central administration of prolactin transiently increased numbers of FRA-IR nuclei in non-TH-IR cells at 3 h in the magnocellular (**figure 5.9**) and parvocellular (**figure 5.10**) subdivisions of the DM-ARC of all rostrocaudal regions in male rats. In the VL-ARC, prolactin increased numbers of FRA-IR nuclei in non-TH-IR cells 1.5–3 h after injection in all rostrocaudal regions (**figure 5.11**). There were no rostrocaudal differences in magnitude of response to prolactin treatment within any ARC subdivision.

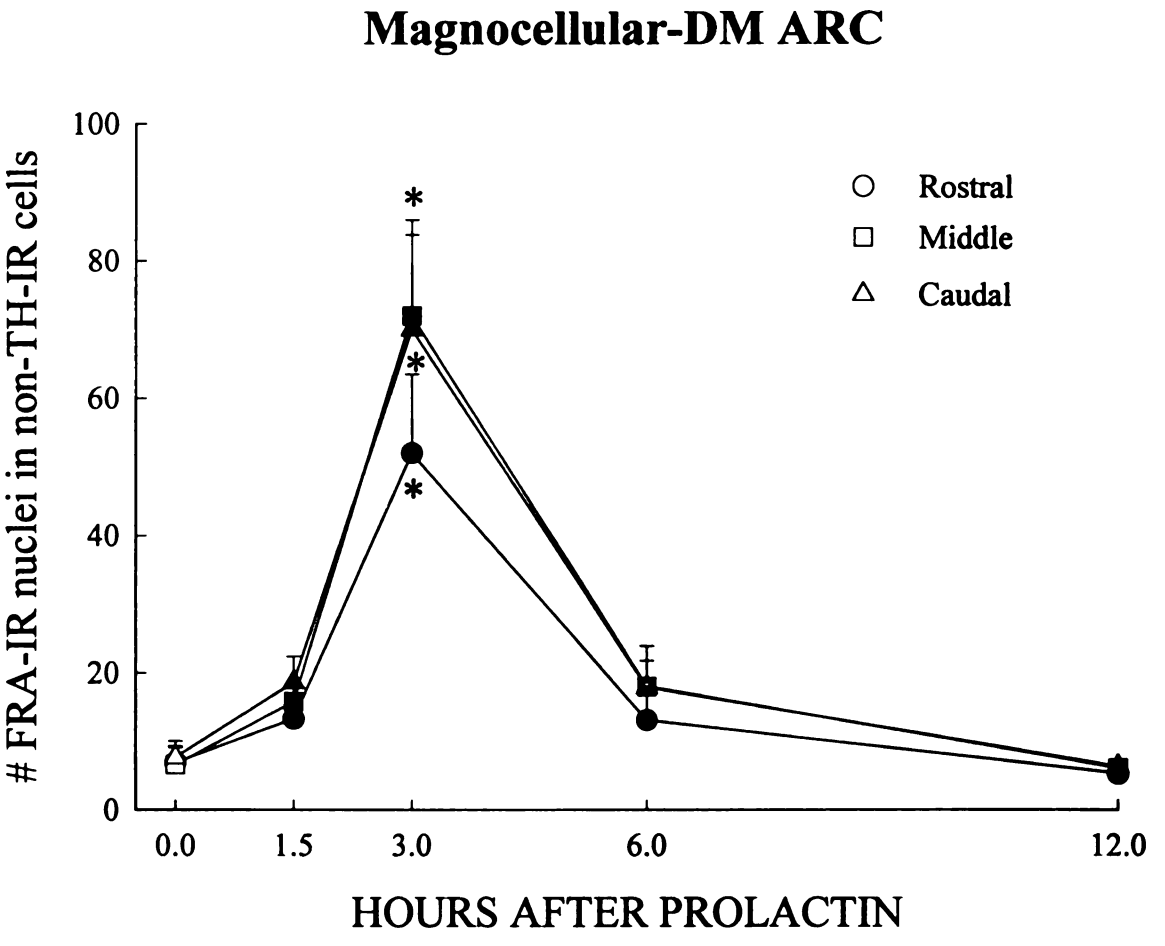


Figure 5.9 Time course effects of prolactin on numbers of FRA-IR nuclei in non-TH-IR cells in the magnocellular-DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (distilled water; 3 μl/rat; icv) 6 h prior to perfusion. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

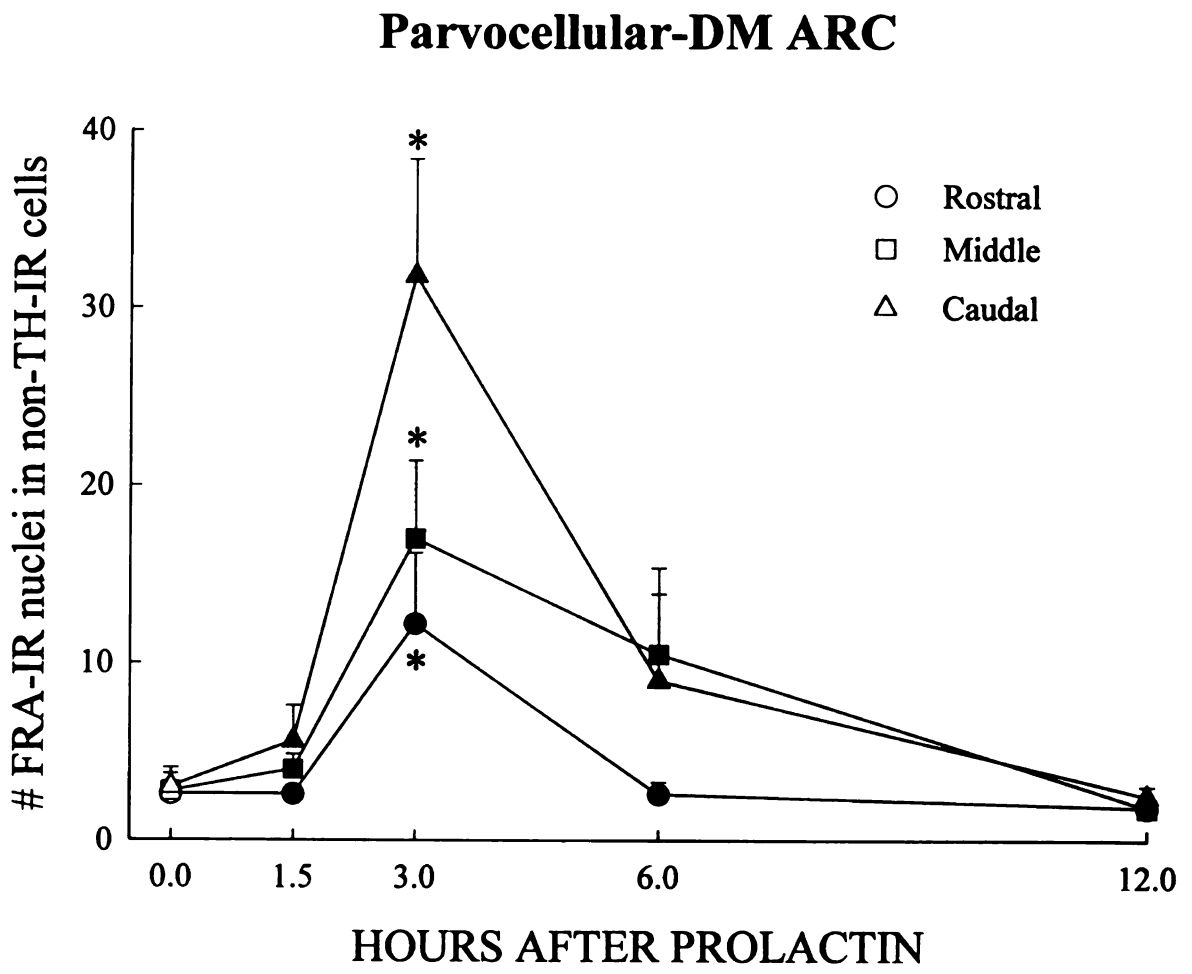


Figure 5.10 Time course effects of prolactin on numbers of FRA-IR nuclei in non-TH-IR cells in the parvocellular-DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (distilled water; 3 μl/rat; icv) 6 h prior to perfusion. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

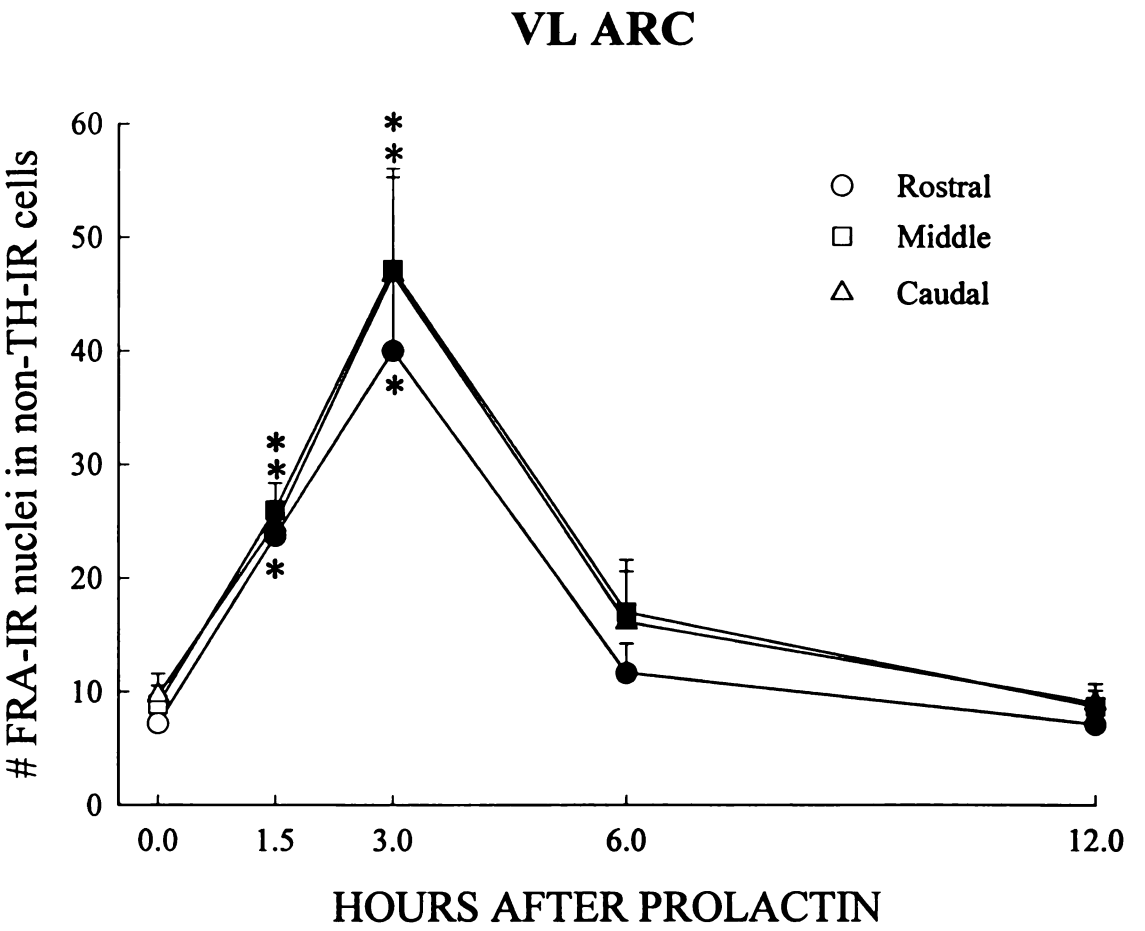


Figure 5.11 Time course effects of prolactin on numbers of FRA-IR nuclei in non-TH-IR cells in the VL subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (distilled water; 3 μl/rat; icv) 6 h prior to perfusion. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

C. DISCUSSION:

While it is well known that TIDA neurons regulate prolactin secretion, little information is available regarding the feedback mechanism underlying the delayed inductive activation of these neurons by prolactin. In the present study a serendipitous observation was further investigated via neural activity mapping with inducible transcription factors.

Neural activity mapping with inducible transcription factors has led to the localization and identification of neuronal populations within specific brain regions that are responsive to a variety of stimuli. While this approach is widely utilized and generally accepted there are two major considerations in this type of study: cell type expression specificity and stimulus coupling uncertainty (Chaudhuri, 1997; Harris, 1998). It is now known that not all cells produce all transcription factors, hence the phenomenon termed cell type expression specificity. Cell type expression specificity demands careful interpretation of negative results. Another complexity of neural activity mapping using inducible transcription factors is the convergence/redundancy of multiple signaling pathways which leads to stimulus-coupling uncertainty. Stimulus-coupling uncertainty can make a causal link between changes in gene expression and a stimulus difficult to establish and highlights the importance of appropriate controls.

In the present study, only positive results are reported, and it was known *a priori* that the cells were capable of FRA expression. Furthermore, specific causal relationships are neither tested nor reported, and similarly-handled controls were employed. In this way, neural activity mapping via immunohistochemical detection of inducible transcription factors (i.e. FRA proteins) was utilized to determine if prolactin regulates

FRA expression in non-TH-IR cells in the DM and VL subdivisions of the ARC in male rats.

ARC subdivisions had low basal expression of FRA-IR in non-TH-IR cells (5-15 cells/region/section) across all ARC subdivisions and rostrocaudal regions in male rats. The lower basal numbers of FRA-IR cells in the parvocellular-DM ARC most likely relates to differences in relative size of ARC subdivisions and hence total cell numbers. Since AP-1 sites are inherent to many genes including "house-keeping genes" these numbers probably reflect their low level of expression. Naturally, any changes in FRA expression due to haloperidol-induced hyperprolactinemia must be detected over this background noise.

Haloperidol rapidly stimulates prolactin secretion via blockade of D_2 receptors on anterior pituitary lactotrophs, and the prolonged hyperprolactinemia that follows feeds back to stimulate TIDA neurons (Moore and Lookingland, 1995). Haloperidol has no direct acute effects on TIDA neurons and its latent stimulatory effects can be blocked by hypophysectomy (Gudelsky et al., 1977) or prolactin antiserum (Gudelsky and Porter, 1980; Van Loon et al., 1983; Hentschel et al, 2000a), demonstrating that the stimulatory effects are mediated by prolactin. While measuring prolactin-induced changes in FRA expression in TIDA neurons, it appeared that changes in FRA expression in non-TH-IR cells of the ARC seemed to temporally precede and/or coincide with those in TIDA neurons. Furthermore, these cells were localized within the ARC, consistent with the complete deafferentation study that demonstrated the haloperidol-induced, prolactin-mediated increase in dopamine turnover in the median eminence results from actions of prolactin on neurons within the mediobasal hypothalamus (Gudelsky et al., 1977). Given

the 6-12 h latency that precedes the inductive activation of TIDA neurons (Demarest et al., 1984; 1986), it is plausible that these cells mediate the effects of prolactin on TIDA neurons. Logically, the first step in this investigation was to verify the purported prolactin-induced changes in immediate early gene expression. The working hypothesis was that prolactin regulates FRA expression in subpopulations of non-TH-IR cells in the ARC.

Consistent with this hypothesis, the results of the present study reveal that in male rats prolactin differentially modulates FRA expression in non-TH-IR cells located in both DM and VL subdivisions of the ARC. In the magnocellular-DM ARC, both haloperidol-induced endogenous hyperprolactinemia and central administration of prolactin cause similar delayed increases in numbers of non-TH-IR cells expressing FRA with peak responses occurring by 1.5-3 h. As described in Chapter 4, the (two-fold) greater response in rats treated with icv prolactin compared to haloperidol is likely dose- and route of administration-related.

The effects of haloperidol could be mediated by direct action of this drug on central dopamine receptors, or indirectly via endogenous hyperprolactinemia. Preliminary experiments examined the ability of PRL-AB to block the effects of haloperidol on FRA expression in male rats. Prolactin antiserum (200µl/rat; iv) decreased plasma prolactin to non-detectable levels as measured by Nb2 lymphoma bioassay (Canon et al., 1991) for up to 3 h in haloperidol-treated male rats. Radioimmunoasssay analyses of binding specificity for this antiserum revealed that PRL-AB used in these experiments was selective for prolactin, having no affinity for luteinizing hormone or growth hormone (Hentschel et al., 2000a). In the present studies,

coadministration of PRL-AB completely blocked the haloperidol-induced changes in FRA expression expected at 3 h in non-TH-IR cells in the magnocellular-DM ARC, indicating that these changes are prolactin-dependent. Furthermore, the peak temporal changes in FRA in non-TH-IR cells of the magnocellular-DM ARC occur 3 h after prolactin treatment, and therefore precede or occur concurrently with the changes observed for regionally matched TIDA neurons (3-6 h; Chapter 4). It is possible that chemically unidentified cells of the magnocellular-DM ARC may have a role in mediating the delayed inductive activation of TIDA neurons by prolactin.

As seen in the magnocellular-DM ARC, both haloperidol-induced endogenous hyperprolactinemia and central administration of prolactin increases the numbers of non-TH-IR cells expressing FRA in the parvocellular-DM ARC with peak responses occurring by 1.5-3 h. However, coadministration of PRL-AB merely blunted the haloperidol-induced changes in FRA expression at 3 h in non-TH-IR cells in the parvocellular-DM ARC, indicating that these changes are only partially prolactin-dependent. Additionally, haloperidol-treated rats demonstrate a 4-fold greater response than prolactin-treated rats which is likely due to D_2 dopamine receptor mediated effects in the parvocellular-DM ARC. These results suggest that the cells of the parvocellular-DM ARC are tonically modulated through a D_2 dopamine receptor-mediated mechanism, unlike the cells of the magnocellular-DM ARC.

In the VL-ARC, the onset of the stimulatory effects of prolactin on FRA expression in non-TH-IR cells precedes that of both regions of the DM-ARC. Considering that transcriptional activation of *c-fos* and related immediate early genes occurs within minutes, with the accumulation of nuclear protein reaching peak levels on

average about 90–120 min later (Harris, 1998), temporal differences in the onset of prolactin-induced changes in expression of FRA in the DM- and VL-ARC suggest two possibilities, either the mechanisms by which prolactin effects these cells are different (i.e. acute direct versus delayed secondary actions), or those non-TH cells of the VL-ARC are more sensitive to prolactin than those of the DM-ARC. Differences in sensitivities of these cells to prolactin could be due to differences in expression of prolactin receptor isoforms and their respective differential densities. Temporal changes in FRA in non-TH-IR cells of the VL-ARC are coincident with the changes in FRA expression within TIDA neurons of the VL-ARC. However, since the changes in FRA expression in both TH-IR and non-TH-IR cells alike of the VL-ARC precede those of all other ARC subdivisions, it is possible that these cells may initiate the prolactin-induced inductive activation of TIDA neurons.

In male rats haloperidol suppresses FRA expression in non-TH-IR cells in the VL-ARC and blocks the stimulatory effects of prolactin on these cells via a prolactin-independent mechanism. Indeed, immunoneutralization of circulating prolactin in haloperidol-treated rats has no effect on the ability of this D_2 dopamine antagonist to decrease the number of non-TH-IR cells expressing FRA in the VL-ARC, in contrast to the magnocellular-DM ARC. The inhibitory effect of haloperidol on FRA expression in non-TH-IR cells of the VL-ARC could due to blockade of D_2 dopamine receptor-mediated tonic regulation of immediate early gene expression in these neurons that is not present in cells of the magnocellular-DM ARC. The significance of this finding remains to be elucidated.

In conclusion, these results reveal that prolactin indeed regulates immediate early gene expression in non-TH-IR cells in the ARC, and that the cells of different ARC subdivisions are temporally differentially responsive to prolactin administration (i.e. prolactin initially stimulates transient expression of FRA in cells of the VL-ARC, followed by FRA expression in cells of the DM-ARC). In the chapters to follow the identity of these prolactin-responsive, non-TH-IR cells is investigated.

6. ROLE OF NEUROTENSIN IN PROLACTIN-INDUCED ACTIVATION OF TIDA NEURONS

A. INTRODUCTION

The feedback activation of TIDA neurons by prolactin consists of tonic and inductive components (Demarest et al., 1984; 1986). The tonic component of prolactin feedback, observed exclusively in females, determines the basal rate of prolactin secretion by regulating short-term changes in TIDA neuronal activity in response to acute changes in concentrations of circulating prolactin. The set point of this neuroendocrine feedback system is higher in females, due in part, to higher circulating prolactin levels caused by a direct stimulatory effect of estrogen on prolactin secretion (Moore and Lookingland, 1995).

The inductive component of prolactin feedback, common to both genders, involves a change in the capacity of TIDA neurons to respond to prolonged periods (3-6 h or more) of hyperprolactinemia (Moore et al., 1987a; Hentschel et al., 2000a), and does not occur until 6-12 h following systemic (Demarest et al., 1984; Demarest et al., 1986; Hokfelt and Fuxe, 1972; Selmanoff, 1981) or central (Annunziato and Moore, 1978) administration of exogenous prolactin, or after experimental manipulations which result in endogenous hyperprolactinemia (Moore et al., 1985). In Chapter 4 we proposed that prolactin induces long-term changes in gene expression that result in increased capacity of TIDA neurons to synthesize and release dopamine, and demonstrated changes in immediate early gene expression in TIDA neurons 3-6 h after prolactin administration. In Chapter 5 neural activity mapping with inducible transcription factors led to the novel identification of non-TH-IR cells in the ARC that are prolactin-responsive. Furthermore,

the changes in immediate early gene expression induced by prolactin in these cells temporally precedes or coincides with those of TIDA neurons. The localization of these cells in the ARC is consistent with mediobasal hypothalamic deafferentation studies (Gudelsky et al., 1978), and suggests that these unidentified cells could be mediators of the delayed inductive activation of TIDA neurons by prolactin.

Certain aspects of prolactin-induced activation of TIDA neurons suggest a role for a peptide mediator. For example, there is temporal dependence in the inductive component of prolactin feedback, i.e. activation of TIDA neurons does not occur until 6-12 h following hyperprolactinemia. Furthermore, the inductive actions of prolactin are protein synthesis-dependent, i.e. activation of TIDA neurons by prolactin can be blocked by cycloheximide (Johnston et al., 1980). These characteristics suggest that the delay in the ability of prolactin to activate TIDA neurons may be due to the time required for *de novo* synthesis, transport and release of a peptide neurotransmitter.

Neurotensin is a tridecapeptide neurotransmitter which fits several criteria as a possible mediator of the effects of prolactin on TIDA neurons. For example, neurotensin-containing neurons are located in the ARC (Emson et al., 1982; Merchanthaler and Lennard, 1991) and TIDA neurons encode mRNA for high-affinity neurotensin receptors (Alexander, 1997). Furthermore, administration of neurotensin produces dose-dependent activation of TIDA neurons both *in vitro* (Herbison et al., 1986; Lin and Pan, 1993) and *in vivo* (Widerlov et al., 1982; Gudelsky et al., 1989; Pan et al., 1992).

The purpose of the studies described in this chapter is to determine if neurotensin mediates the delayed inductive activation of TIDA neurons by prolactin. If this is the case, then the non-peptide neurotensin receptor antagonist SR-48692 (Gully et al., 1993)

will block the stimulatory effects of prolactin on TIDA neuronal activity as estimated by measuring concentrations of DOPAC in termini of these neurons in the median eminence (Lookingland et al., 1987a). In these studies TIDA neuronal responses will be compared to those of NSDA and MLDA neurons.

While NSDA and MLDA systems are not prolactin-responsive, these neurons are activated by haloperidol. Furthermore, there are the many associations between NSDA and MLDA neurons and neurotensin. For example, neurotensin receptors are localized on perikarya and termini of NSDA and MLDA neurons (Young and Kuhar, 1981) and neurotensin administration activates NSDA (Pinnock, 1985) and MLDA neurons (Widerlov et al., 1982) and facilitates basal and potassium-induced dopamine release in these neurons (Okuma and Osumi, 1982). In addition, neurotensin is often colocalized with dopamine in perikarya of NSDA and MLDA neurons (Hokfelt et al., 1984). Hence, this study will consider the effects of a neurotensin receptor antagonist on basal and activated NSDA and MLDA neurons by measuring concentrations of DOPAC in the striatum and nucleus accumbens, regions containing termini of these neurons, respectively. These studies were performed in male and diestrous female rats.

B. RESULTS

SR-48692 is a selective non-peptide neurotensin receptor antagonist which competitively inhibits binding of [^{125}I]NT to high-affinity neurotensin receptors *in vitro* (Gully et al., 1993), and *in vivo* reverses turning behavior induced by intrastriatal injection of neurotensin in both mice and rats (Gully et al., 1993; Steinburg et al., 1994). SR-48692 is devoid of intrinsic agonist activity *in vivo*, and significant antagonistic effects have been demonstrated by 30 min (with maximal effects observed between 1 and 2 h) following peripheral administration of doses between 80 μg/kg (p.o.) (Gully et al., 1993) and 1,000 μg/kg (i.p.; Castagliuolo et al., 1996). As shown in **table 6.1**, 1 h following its systemic injection, SR-48692 (1,000 μg/kg; i.p.) had no effect on DOPAC or dopamine concentrations in the median eminence, striatum or nucleus accumbens of either male or female rats.

Haloperidol is a D_2 dopamine receptor antagonist which induces hyperprolactinemia (**figures 3.1 or 4.3**, top panels) via disinhibition of dopamine suppression of prolactin secretion from adenohypophysial lactotrophs; the resultant hyperprolactinemia, in turn, activates TIDA neurons (Moore and Lookingland, 1995). As shown in **figure 6.1**, 12 h after treatment of male and female rats with haloperidol (1 mg/kg; s.c.) DOPAC concentrations in the median eminence were significantly increased as compared with vehicle-treated controls. The stimulatory effect of haloperidol on median eminence DOPAC concentrations was reversed by SR-48692 (1,000 μg/kg, i.p.; 1 h) in both male and female rats. Injections of SR-48692 (10 - 1,000 μg/kg, i.p.; 1 h) had no effect on dopamine concentrations in the median eminence of either vehicle- or haloperidol-treated male and female rats.

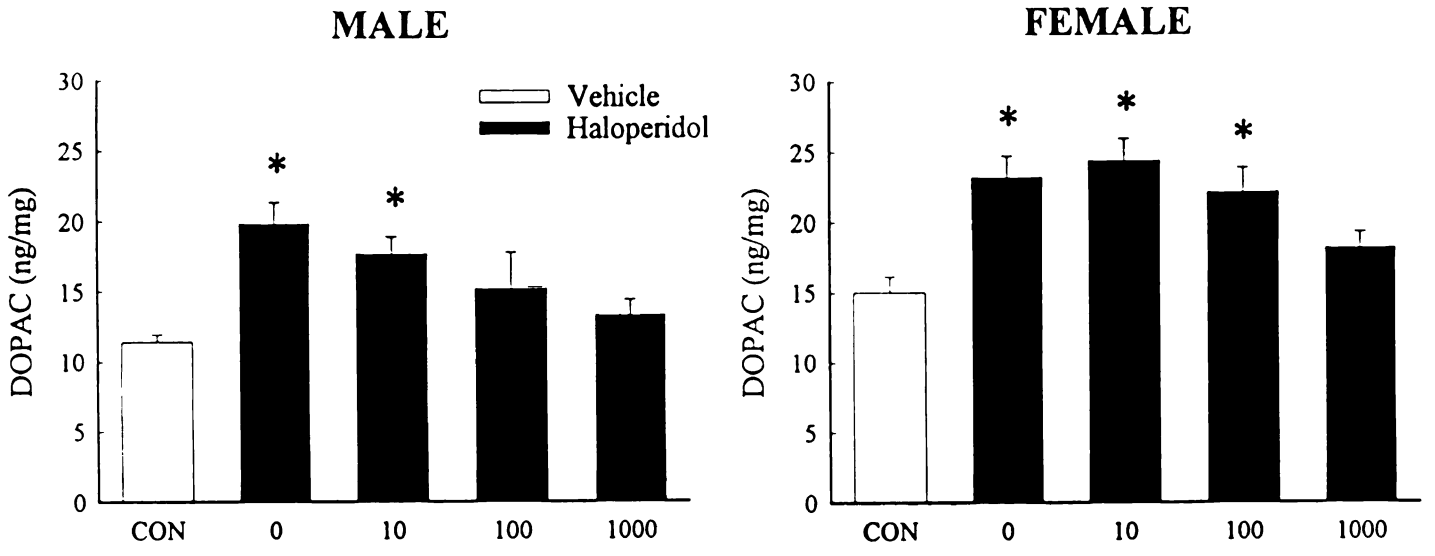
TABLE 6.1 Effects of SR-48692 on DOPAC and dopamine concentrations in median eminence, striatum and nucleus accumbens of male and female rats.

MEDIAN EMINENCE				
GROUP	**GENDER**	**DOPAC ± SEM**	**DA ± SEM**	**N**
Control	Male	15.2 ± 1.0	125.8 ± 3.8	7
SR-48692	Male	17.2 ± 1.4	124.5 ± 2.5	9
Control	Female	18.0 ± 1.6	100.9 ± 6.7	9
SR-48692	Female	18.6 ± 1.3	105.7 ± 5.6	11

STRIATUM				
GROUP	**GENDER**	**DOPAC ± SEM**	**DA ± SEM**	**N**
Control	Male	27.3 ± 1.5	102.5 ± 5.8	8
SR-48692	Male	30.0 ± 2.2	104.5 ± 3.3	9
Control	Female	24.3 ± 0.8	105.2 ± 6.2	9
SR-48692	Female	27.7 ± 1.1	106.9 ± 5.9	9

NUCLEUS ACCUMBENS				
GROUP	**GENDER**	**DOPAC ± SEM**	**DA ± SEM**	**N**
Control	Male	26.0 ± 1.1	100.3 ± 4.8	7
SR-48692	Male	26.1 ± 0.9	105.8 ± 4.9	8
Control	Female	23.2 ± 1.4	91.4 ± 6.5	10
SR-48692	Female	22.5 ± 1.4	87.2 ± 5.1	10

Rats were injected with either SR-48692 (1,000 μg/kg; i.p.) or its 10% DMSO in 0.9% saline vehicle (4 ml/kg; i.p.) 1 h prior to decapitation. Values represent means ± 1 SEM of DOPAC and dopamine concentrations (ng/mg protein) in the median eminence, striatum and nucleus accumbens.



DOPAMINE (ng/mg)		
DOSE SR-48692	MEAN ± SEM	n
Control	125.5 ± 8.5	6
0 ug/kg	126.6 ± 9.6	8
10 ug/kg	131.0 ± 5.3	8
100 ug/kg	125.9 ± 7.8	7
1000 ug/kg	103.6 ± 9.8	6

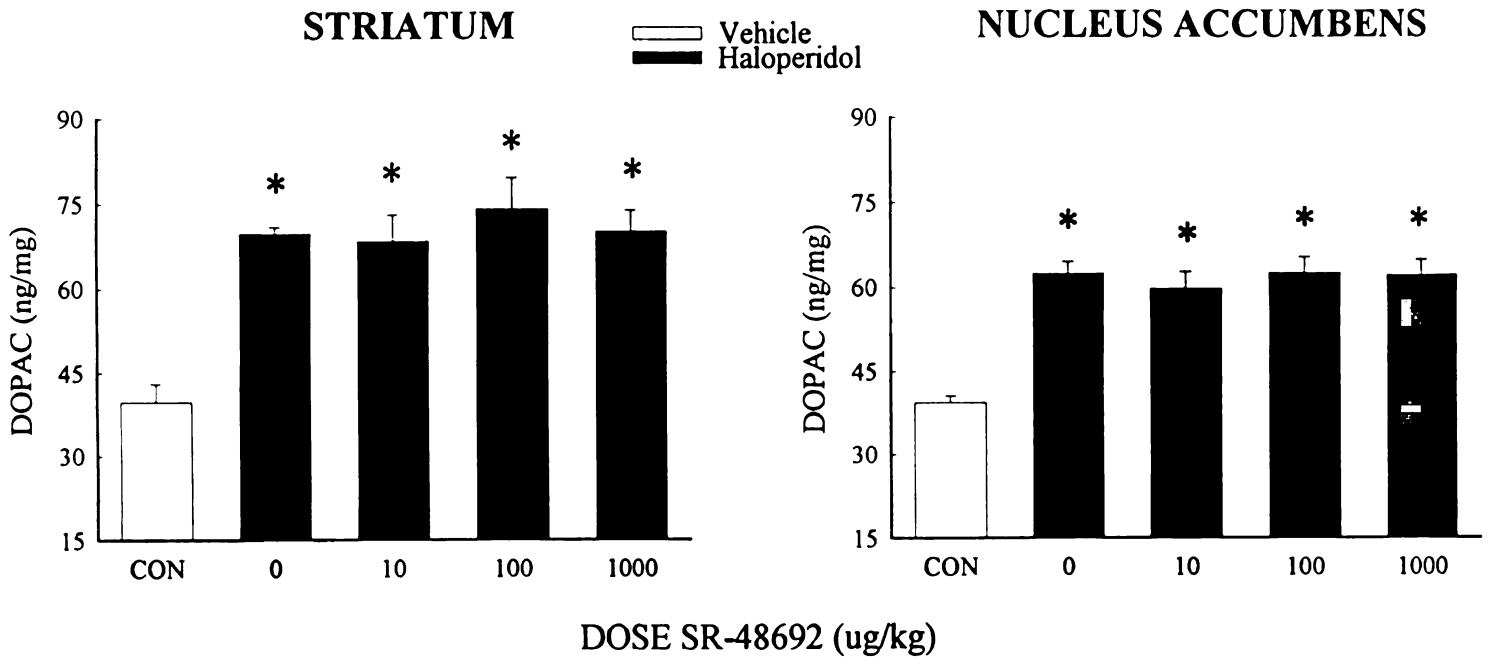
DOPAMINE (ng/mg)		
DOSE SR-48692	MEAN ± SEM	n
Control	114.5 ± 9.0	8
0 ug/kg	120.8 ± 7.4	7
10 ug/kg	100.8 ± 6.7	9
100 ug/kg	102.2 ± 6.1	10
1000 ug/kg	104.2 ± 7.9	8

Figure 6.1 Effects of SR-48692 on DOPAC and dopamine concentrations in the median eminence of haloperidol-treated male and female rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; s.c.) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; s.c.) 12 h prior to decapitation, and with either SR-48692 (10, 100 or 1,000 μg/kg; i.p.) or its 10% DMSO in 0.9% saline vehicle (4 ml/kg; i.p.) 1 h prior to decapitation. TOP: Columns represent means and vertical lines 1 SEM of DOPAC concentrations (ng/mg protein) in the median eminence. BOTTOM: Values represent means ± 1 SEM of dopamine concentrations (ng/mg protein) in the median eminence of male (LEFT) and female (RIGHT) rats. *, $p < 0.05$: values for haloperidol-treated rats that are significantly different from vehicle-treated controls.

Haloperidol activates NSDA and MLDA neurons via blockade of dopaminergic autoreceptors (Nowycky and Roth, 1978) in the striatum and nucleus accumbens, respectively. As shown in **figure 6.2**, 12 h after treatment of male rats with haloperidol (1 mg/kg; s.c.) DOPAC concentrations in the striatum and nucleus accumbens were significantly increased as compared with vehicle-treated controls. The stimulatory effects of haloperidol on striatum and nucleus accumbens DOPAC concentrations were not reversed by SR-48692 (10 - 1,000 μg/kg, i.p.; 1 h), and injections of SR-48692 had no effect on dopamine concentrations in the striatum or nucleus accumbens of either vehicle- or haloperidol-treated male rats.

Results depicted in **figure 6.1** were obtained using an experimental model which employed haloperidol to induce endogenous hyperprolactinemia as a means of indirectly stimulating TIDA neurons. One possible explanation for the dose-related reversal of haloperidol-induced DOPAC concentrations by SR-48692 is that this compound prevents the ability of haloperidol to stimulate prolactin secretion. To test for this, the effects of SR-48692 (1,000 μg/kg, i.p.; 1 h) on haloperidol-induced hyperprolactinemia were investigated in male and female rats. As shown in **figure 6.3**, haloperidol (1 mg/kg, s.c.; 1 h) significantly increased plasma prolactin concentrations in both male and female rats, and SR-48692 (1,000 μg/kg, i.p.; 1 h) had no effect on plasma prolactin concentrations in either vehicle- or haloperidol-treated male or female rats.

As shown in **figure 6.4**, 12 h following central administration of prolactin (10 μg/rat, i.c.v.), concentrations of DOPAC in the median eminence were increased in both male and female rats, and these effects were reversed by administration of SR-48692 (1,000 μg/kg, i.p.; 1 h). Injection of SR-48692 had no effect on dopamine concentrations



DOPAMINE (ng/mg)		
DOSE SR-48692	MEAN ± SEM	n
Control	119.0 ± 5.4	8
0 ug/kg	120.2 ± 5.9	7
10 ug/kg	119.6 ± 4.8	8
100 ug/kg	113.3 ± 3.9	8
1000 ug/kg	125.3 ± 5.9	8

DOPAMINE (ng/mg)		
DOSE SR-48692	MEAN ± SEM	n
Control	110.4 ± 7.5	7
0 ug/kg	92.9 ± 3.6	8
10 ug/kg	91.5 ± 7.1	8
100 ug/kg	92.1 ± 1.9	7
1000 ug/kg	90.7 ± 4.6	8

Figure 6.2 Effects of SR-48692 on DOPAC and dopamine concentrations in the striatum and nucleus accumbens of haloperidol-treated male rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; s.c.) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; s.c.) 12 h prior to decapitation, and with either SR-48692 (10, 100 or 1,000 µg/kg; i.p.) or its 10% DMSO in 0.9% saline vehicle (4 ml/kg; i.p.) 1 h prior to decapitation. TOP: Columns represent means and vertical lines 1 SEM of DOPAC concentrations (ng/mg protein) in the striatum and nucleus accumbens. BOTTOM: Values represent means ± 1 SEM of dopamine concentrations (ng/mg protein) in the striatum (LEFT) and nucleus accumbens (RIGHT). *, $p < 0.05$: values for haloperidol-treated rats that are significantly different from vehicle-treated controls.

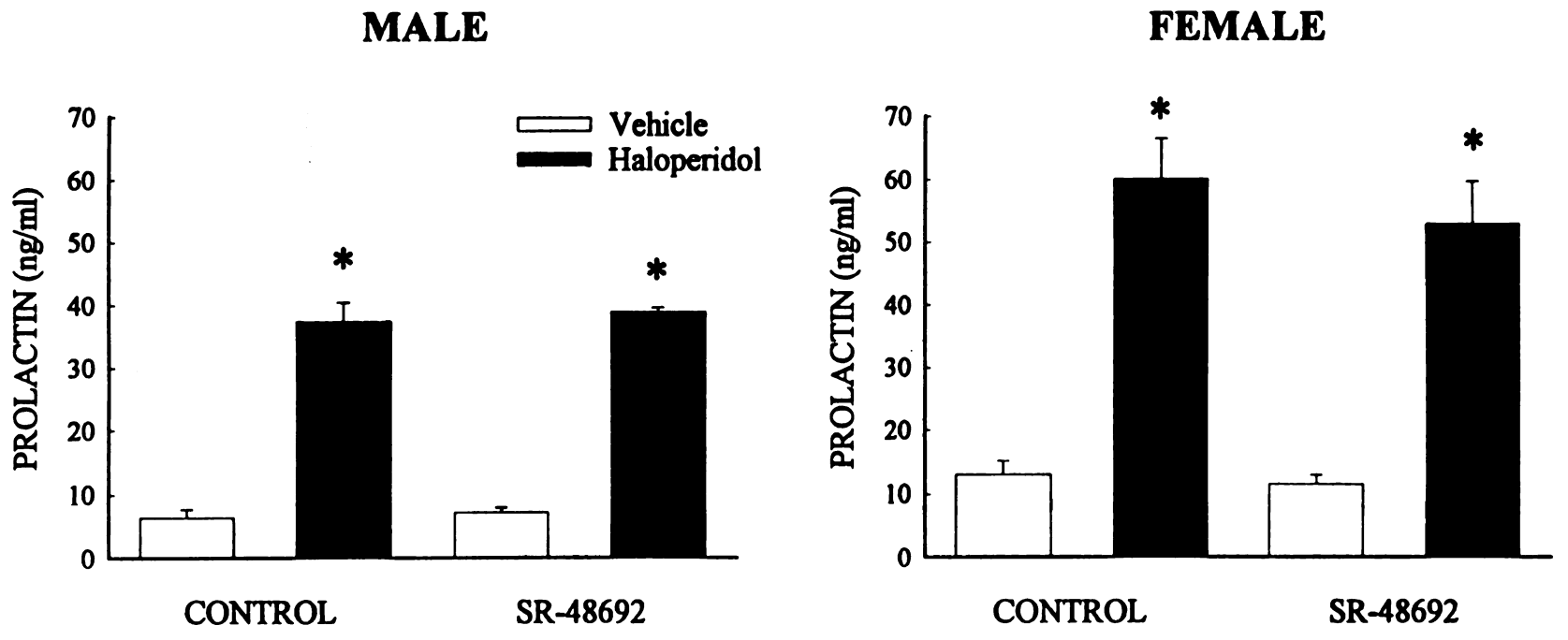
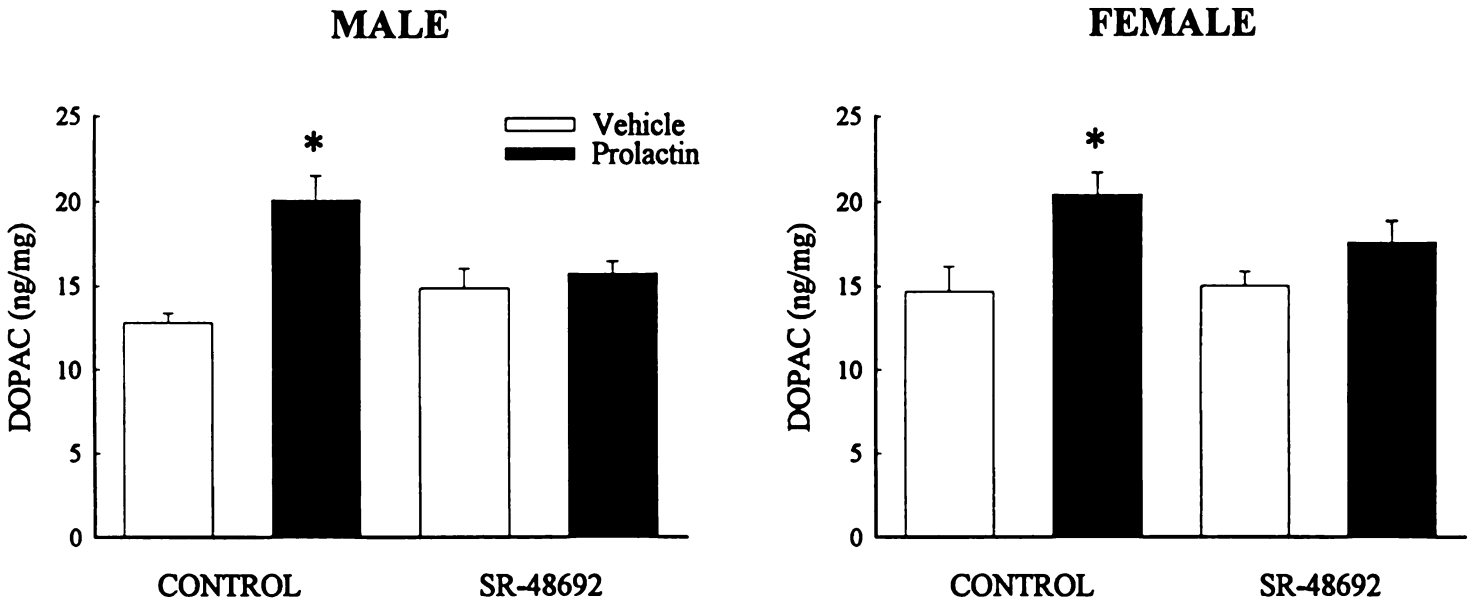


Figure 6.3 Effects of SR-48692 on serum prolactin concentrations in haloperidol-treated male and female rats. Rats were injected with either haloperidol (filled columns; 1 mg/kg; s.c.) or its 0.3% tartaric acid vehicle (open columns; 1 ml/kg; sc) 1 h (males) or 12 h (females) prior to decapitation, and with either SR-48692 (1,000 μg/kg; i.p.) or its 10% DMSO in 0.9% saline vehicle (4 ml/kg; i.p.) 1 h prior to decapitation. Columns represent means and vertical lines 1 SEM of prolactin concentrations (ng/ml plasma) from 7-11 animals. * $p < 0.05$: values for haloperidol-treated rats that are significantly different from vehicle-treated controls.

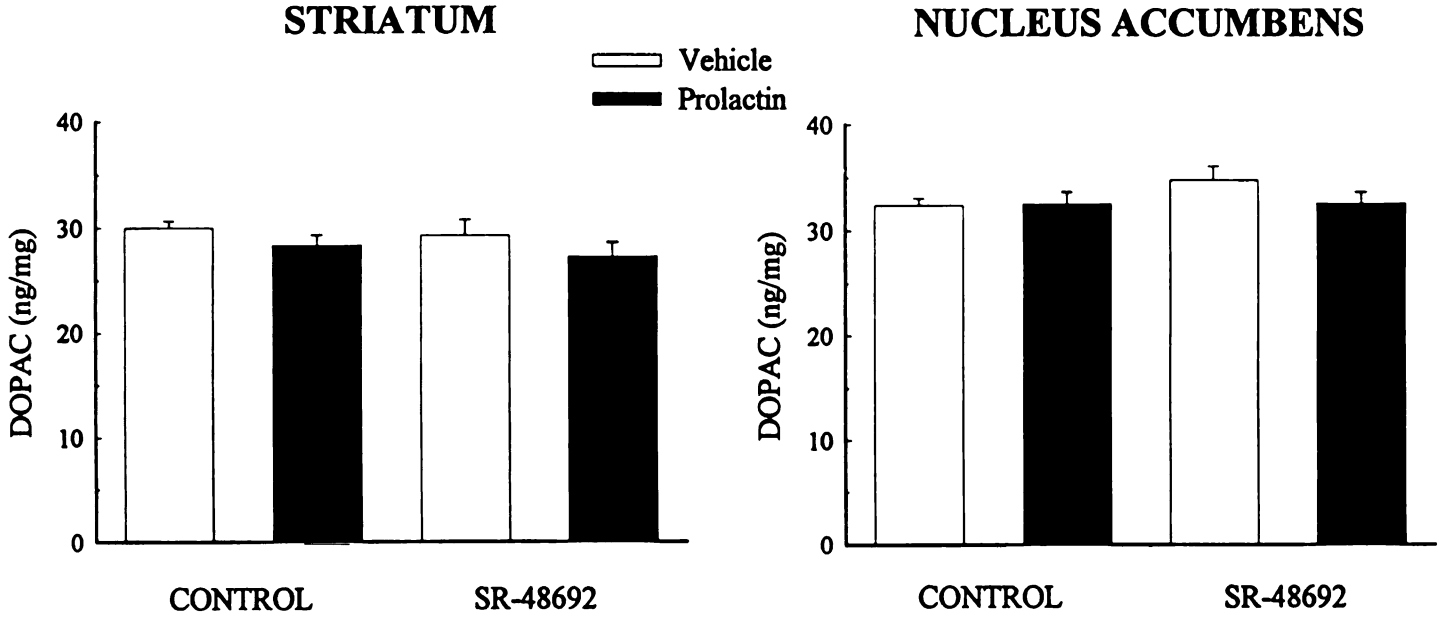


DOPAMINE (ng/mg)		
GROUP	MEAN ± SEM	n
Vehicle + Vehicle	97.7 ± 4.6	7
Prolactin + Vehicle	89.7 ± 4.2	8
Vehicle + SR-48692	94.1 ± 4.3	7
Prolactin + SR-48692	92.8 ± 4.5	8

DOPAMINE (ng/mg)		
GROUP	MEAN ± SEM	n
Vehicle + Vehicle	82.9 ± 3.7	11
Prolactin + Vehicle	92.1 ± 5.4	10
Vehicle + SR-48692	88.4 ± 6.6	10
Prolactin + SR-48692	83.0 ± 5.5	10

Figure 6.4 Effects of SR-48692 on DOPAC and dopamine concentrations in the median eminence of prolactin-treated male and female rats. Rats were injected with either rat prolactin (filled columns; 10 μg/rat; i.c.v.) or its distilled water vehicle (open columns; 3 ul/rat; i.c.v.) 12 h prior to decapitation, and with either SR-48692 (1,000 μg/kg; i.p.) or its 10% DMSO in 0.9% saline vehicle (4 ml/kg; i.p.) 1 h prior to decapitation. TOP: Columns represent means and vertical lines 1 SEM of DOPAC concentrations (ng/mg protein) in the median eminence. BOTTOM: Values represent means ± 1 SEM of dopamine concentrations (ng/mg protein) in the median eminence of male (LEFT) and female (RIGHT) rats. * $p < 0.05$: values for prolactin-treated rats that are significantly different from vehicle-treated controls.

in the median eminence of either vehicle- or prolactin-treated male and female rats. As shown in **figure 6.5**, DOPAC and dopamine concentrations in the striatum and nucleus accumbens were not effected by either central administration of prolactin (10 μg/rat, i.c.v., 12 h), or systemic injection of SR-48692 (1,000 μg/kg, i.p.; 1 h) in male rats.



DOPAMINE (ng/mg)		
GROUP	MEAN ± SEM	n
Vehicle + Vehicle	121.1 ± 2.9	9
Prolactin + Vehicle	117.2 ± 4.5	9
Vehicle + SR-48692	120.8 ± 2.8	9
Prolactin + SR-48692	120.9 ± 3.4	11

DOPAMINE (ng/mg)		
GROUP	MEAN ± SEM	n
Vehicle + Vehicle	106.6 ± 1.5	8
Prolactin + Vehicle	103.7 ± 4.1	9
Vehicle + SR-48692	109.9 ± 6.0	8
Prolactin + SR-48692	113.9 ± 2.8	8

Figure 6.5 Effects of SR-48692 on DOPAC and dopamine concentrations in the striatum and nucleus accumbens of prolactin-treated male rats. Rats were injected with either rat prolactin (filled columns; 10 μg/rat; i.c.v.) or its distilled water vehicle (open columns; 3 ul/rat; i.c.v.) 12 h prior to decapitation, and with either SR-48692 (1,000 μg/kg; i.p.) or its 10% DMSO in 0.9% saline vehicle (4 ml/kg; i.p.) 1 h prior to decapitation. TOP: Columns represent means and vertical lines 1 SEM of DOPAC concentrations (ng/mg protein) in the striatum and nucleus accumbens. BOTTOM: Values represent means ± 1 SEM of dopamine concentrations (ng/mg protein) in the striatum (LEFT) and nucleus accumbens (RIGHT).

C. DISCUSSION

The selective neurotensin receptor antagonist SR-48692 binds both high- and low-affinity forms of the neurotensin receptor (Gully et al., 1993; Betancur et al., 1995; Le et al., 1996) and readily crosses the blood-brain barrier following its systemic administration, reaching pharmacologically active levels between 30 min and 2-3 h following doses of between 80 and 1,000 μg/kg (Gully et al., 1993; Castagliuolo et al., 1996). The results of the present study demonstrate that administration of SR-48692 (1,000 μg/kg; i.p.) has no effect on basal DOPAC and dopamine concentrations in the median eminence of either male or female rats. This lack of effect was not due to the dose or time of administration of SR-48692 since using the same parameters this compound was effective in blocking elevated median eminence DOPAC concentrations in both haloperidol- and prolactin-treated rats. Rather, the inability of this compound to alter median eminence DOPAC and dopamine concentrations is probably due to the absence of intrinsic endogenous agonistic activity at neurotensin receptors under basal conditions when the activity of TIDA neurons in females is tonically regulated by acute changes in circulating prolactin.

On the other hand, blockade of neurotensin receptors with SR-48692 prevents the delayed stimulatory effects of both haloperidol and prolactin on median eminence DOPAC concentrations, without altering the ability of haloperidol to increase prolactin secretion. Taken together, these results suggest that hyperprolactinemia stimulates the release of endogenous neurotensin, which acts via neurotensin receptors to activate TIDA neurons. Hence the inductive activation of TIDA neurons by prolactin in both males and

females is neurotensin-dependent and, in this respect, different from the mechanism underlying the tonic regulation of these neurons in females rats.

The inductive component of prolactin feedback, common to both genders, involves a change in the capacity of TIDA neurons to respond to prolonged periods (3-6 h or more; Hentschel et al., 2000a) of hyperprolactinemia (Moore et al., 1987a) and is characterized by long latency. Prolactin activates TIDA neurons in such a manner that the response is protein synthesis dependent (Johnston et al., 1980). It is possible that this protein synthesis-dependent period corresponds to the induction and *de novo* synthesis of neurotensin. In the present study, blockade of neurotensin receptors 11 h after haloperidol-induced hyperprolactinemia or central prolactin administration prevented the activation of TIDA neurons, a temporal period appropriate in duration for *de novo* synthesis and release of a neurotensin (Rostene and Alexander, 1997).

The source of neurotensin that mediates the stimulatory effect of prolactin on TIDA neurons is located within the mediobasal hypothalamus since complete surgical deafferentation of this region from the rest of the brain does not alter the ability of hyperprolactinemia to activate TIDA neurons (Gudelsky et al., 1978). Since neurotensin mRNA and neurotensin-IR have been localized to neurons within the ARC (including those containing TH-IR; Rostene and Alexander, 1997), prolactin-induced neurotensin synthesis and release could occur in afferent neuropeptidergic neurons (Merchanthaler and Lennard, 1991; Marcos et al., 1996a) and/or in subpopulations of TIDA neurons themselves (Hokfelt et al., 1984; Bachelet et al., 1997). Furthermore, it is plausible that the acutely prolactin-responsive, chemically unidentified cells in the ARC (highlighted in

Chapter 5) are neurotensinergic neurons involved in mediating the delayed inductive activation of TIDA neurons.

The finding that SR-48692 has no effect on basal activity of NSDA and MLDA neurons is consistent with a previous report (Pugsley et al., 1995) and is not dose- or time-dependent since injection of SR-48692 (80 ug/kg; i.p.; 0.5 - 2 h) reversed the contralateral turning behavior induced by a 10 pg intrastriatal injection of neurotensin (Gully et al., 1993). These results suggest that (like TIDA neurons) endogenous neurotensin does not participate in regulation of the basal activity of NSDA or MLDA neurons in male and female rats.

Haloperidol is reported to effect NSDA and MLDA neuronal activity in a variety of ways. The predominant effect is stimulatory via blockade of inhibitory presynaptic D_2 autoreceptors on terminals in the striatum and nucleus accumbens, respectively (Nowycky and Roth, 1978). Haloperidol may also indirectly stimulate these systems through disinhibition as follows. D_2 dopamine receptor antagonism blocks the tonic inhibition of neurotensin neurons (Fuxe et al., 1992; Tanganelli et al., 1993; Brun et al., 1995) thereby increasing activity and release of neurotensin in the striatum and nucleus accumbens (as evidenced by increased extracellular neurotensin content in microdialysates in terminal regions; Wagstaff et al., 1996). Neurotensin, in turn, may bind its receptors localized on terminals of NSDA and MLDA neurons (Quirion et al., 1982; Quirion, 1983; Herve et al., 1986; Kitabgi et al., 1987; Delle Donne et al., 1996) to decrease the sensitivity of D_2 autoreceptors to dopamine (Fuxe et al., 1992; Tanganelli et al., 1989) through an undefined mechanism. Consequently, robust activation is the overall effect of haloperidol on NSDA and MLDA systems.

In the present study, neurotensin receptor blockade by SR-48692 had no effect on NSDA or MLDA neurons. It is possible that SR-48692 had no effect on haloperidol-induced activation of NSDA and MLDA neurons because the effects of endogenous neurotensin are mediated through modulation of the binding constant of NSDA and MLDA autoreceptors which are already blocked by haloperidol or have a differential time course. More likely, regulation of NSDA and MLDA neuronal activity is predominantly mediated via D_2 autoreceptors and neurotensin receptors do not have a significant role.

In conclusion, the results of the present studies are consistent with the hypothesis that neurotensin mediates the delayed inductive activation of TIDA neurons by prolactin in both male and female rats, whereas the tonic activation of TIDA neurons by circulating prolactin in female rats is neurotensin-independent. It remains plausible that the acutely prolactin-responsive, chemically unidentified cells in the ARC (highlighted in Chapter 5) are neurotensinergic neurons which mediate the delayed inductive activation of TIDA neurons in male and female rats. In the following chapter double immunohistochemistry will be utilized to determine if these cells are indeed neurotensinergic.

7. EFFECTS OF PROLACTIN ON EXPRESSION OF FRA IN NEUROTENSIN-IR NEURONS IN THE ARC

A. INTRODUCTION

The inductive component of prolactin feedback involves a change in the capacity of TIDA neurons to respond to prolonged periods of hyperprolactinemia (Moore et al., 1987a; Hentschel et al., 2000a), and does not occur until 6-12 h following systemic (Hokfelt and Fuxe, 1972; Selmanoff, 1981; Demarest et al., 1984, 1986) or central (Annunziato and Moore, 1978; Hentschel et al., 2000a) administration of exogenous prolactin. In Chapter 4 we proposed that prolactin induces long-term changes in gene expression that result in increased capacity of TIDA neurons to synthesize and release dopamine, and demonstrated changes in immediate early gene expression in TIDA neurons 3-6 h after prolactin administration (Hentschel et al., 2000b).

In Chapter 5 neural activity mapping with inducible transcription factors led to the novel identification of non-TH-IR cells in the ARC that are prolactin-responsive. Furthermore, the changes in immediate early gene expression induced by prolactin in these cells temporally precedes or coincides with those of TIDA neurons. The presence of these cells in the ARC is consistent with deafferentation studies that localized the actions of prolactin within the medial basal hypothalamus (Gudelsky et al., 1978), and suggests that these chemically unidentified cells could be mediators of the inductive activation of TIDA neurons.

In Chapter 6, the role of neurotensin in prolactin-induced activation of TIDA neurons was investigated since several characteristics suggested a role for a neuropeptide mediator. The delayed inductive activation of TIDA neurons by prolactin has a 6-12 h

latency and is protein synthesis-dependent (Johnston et al., 1980). Neurotensin is a neuropeptide that activates TIDA neurons acutely (Widerlov et al., 1982; Herbison et al., 1986; Gudelsky et al., 1989; Pan et al., 1992 Lin and Pan, 1993) and is localized within the ARC (Emson et al., 1982; Merchanthaler and Lennard, 1991). Thus, it was proposed that neurotensin mediates the delayed inductive activation of TIDA neurons by prolactin, and consistent with this hypothesis, the non-peptide neurotensin receptor antagonist SR-48692 (Gully et al., 1993) blocked the activation of TIDA neurons in neurochemical studies (Hentschel et al., 1998).

It remains plausible that the acutely prolactin-responsive, chemically unidentified cells in the ARC are neurotensin neurons, and that these neurons mediate the delayed inductive activation of TIDA neurons by prolactin. Logically, if these neurotensin neurons mediate the effects of prolactin, then they should be responsive to hyperprolactinemia. In this series of experiments we tested the hypothesis that neurotensin neurons in the ARC are prolactin-responsive using neural activity mapping with inducible transcription factors. This technique has not been previously described for use in neurotensin neurons. However, several lines of evidence suggest that FRAs play a predominant role in the regulation of neurotensin gene expression. For example, the rat neurotensin gene contains an AP-1 consensus sequence in its promoter (Kislauskis et al., 1988; Rostene and Alexander, 1997), antisense phosphothioate oligodeoxynucleotides to *c-fos* attenuate induction of neurotensin gene expression (Merchant, 1994; Robertson et al., 1995; Svennigsson et al., 1997) and mutations in the AP-1 site of this gene decreases responses to all inducers of neurotensin gene expression by at least an order of magnitude

(Harrison et al., 1995). Therefore, FRA proteins are reasonable transcription factors to target for investigation of this hypothesis.

If neurotensin neurons in the ARC are prolactin-responsive, then prolactin will induce changes in immediate early gene expression in these neurons. To determine if this is the case, initial experiments examined the time course of effects of haloperidol-induced endogenous hyperprolactinemia on FRA expression in neurotensin neurons in DM and VL subdivisions of the rostral, middle and caudal ARC in male rats as estimated by dual immunohistochemistry for the detection of FRA- and neurotensin-IR. Subdivisions of the DM ARC (i.e. magnocellular and parvocellular regions) were not analyzed separately in these studies since these regions are defined by TH-IR perikarya and can not be reliably estimated in the absence of TH staining. Follow-up experiments compared the time course effects of haloperidol to those of exogenous prolactin, and determined the localization of neurotensin-IR neurons relative to TH-IR neurons in the ARC in male rats.

B. RESULTS

Neurotensin-IR neurons are not homogeneously distributed throughout the ARC. Rather, there exists a rostrocaudal gradient in numbers of neurotensin-IR neurons within ARC subdivisions. As shown in **figure 7.1**, there are successively more neurotensin-IR neurons in the DM and VL subdivisions as ARC regions extend caudally. Furthermore, for any rostrocaudal ARC region there were nearly twice as many neurotensin-IR neurons in the VL-ARC as compared to the DM-ARC (**figure 7.1**).

In order to test the hypothesis that neurotensin-IR neurons are responsive to hyperprolactinemia, the time course of effects of hyperprolactinemia on expression of FRA in neurotensin-IR neurons was examined using dual immunohistochemistry. Logically, any hyperprolactinemia-induced changes in immediate early gene expression must be detected over basal levels in these neurons. The basal FRA expression in neurotensin-IR cells of the ARC was relatively low with the highest percentage of neurotensin-IR neurons expressing FRA in the rostral VL-ARC. As shown in **figure 7.2**, the percentage of neurotensin-IR neurons expressing FRA in the VL-ARC decreased from 6% to 2% as ARC regions extended caudally. For all rostrocaudal ARC regions basal FRA expression in neurotensin-IR cells in the VL-ARC was greater than that in the DM-ARC (**figure 7.2**).

Figure 7.3 compares representative photomicrographs of FRA expression in neurotensin-IR neurons of the VL subdivision in the middle ARC 3 h after vehicle or haloperidol injection in male rats. Bright-field images show that FRA expression in neurotensin-IR neurons is increased in the VL-ARC after haloperidol administration. The percentage of neurotensin-IR neurons expressing FRA was increased after haloperidol

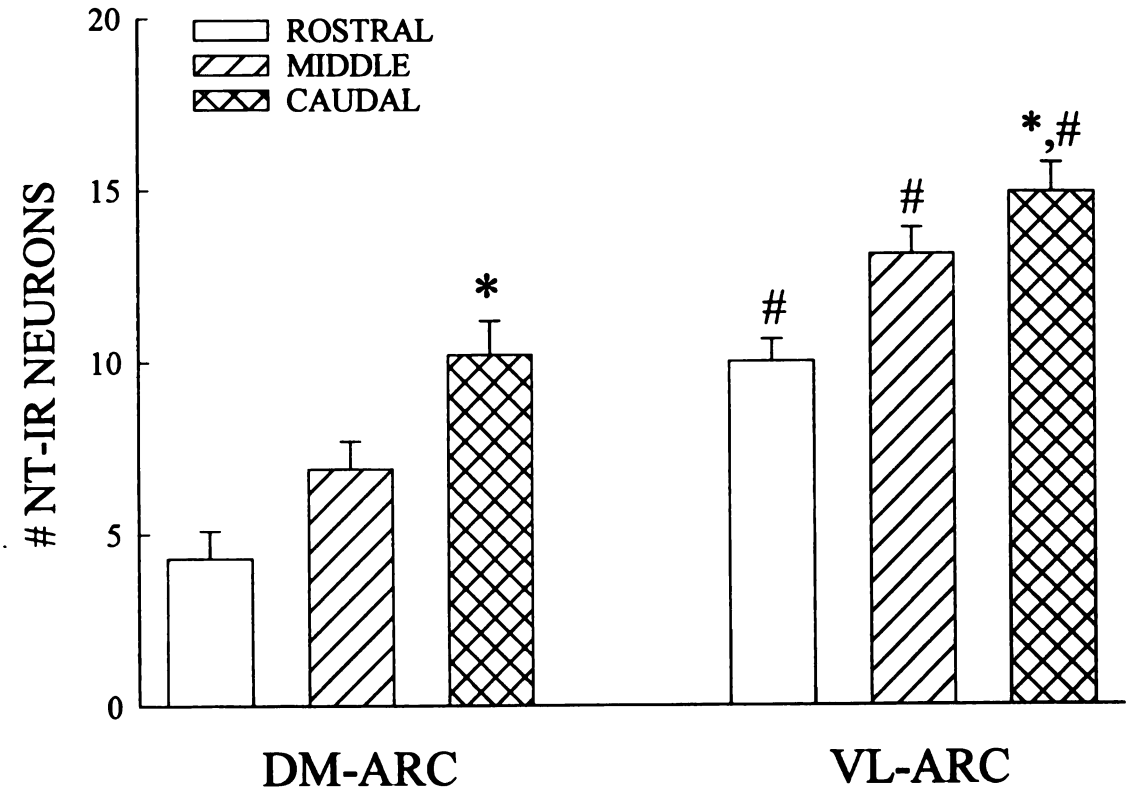


Figure 7.1 Comparison of numbers of NT-IR neurons in the DM and VL subdivisions of rostral, middle and caudal ARC of male rats. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of NT-IR in the DM and VL subdivisions of the rostral (open columns), middle (hatched columns) and caudal (cross-hatched columns) ARC. Columns represent means and vertical lines 1 SEM from 6-8 animals. *, Values within an ARC subdivision that are significantly different ($p < 0.05$) from the rostral ARC. #, Values within a rostrocaudal region that are significantly different ($p < 0.05$) from the DM-ARC.

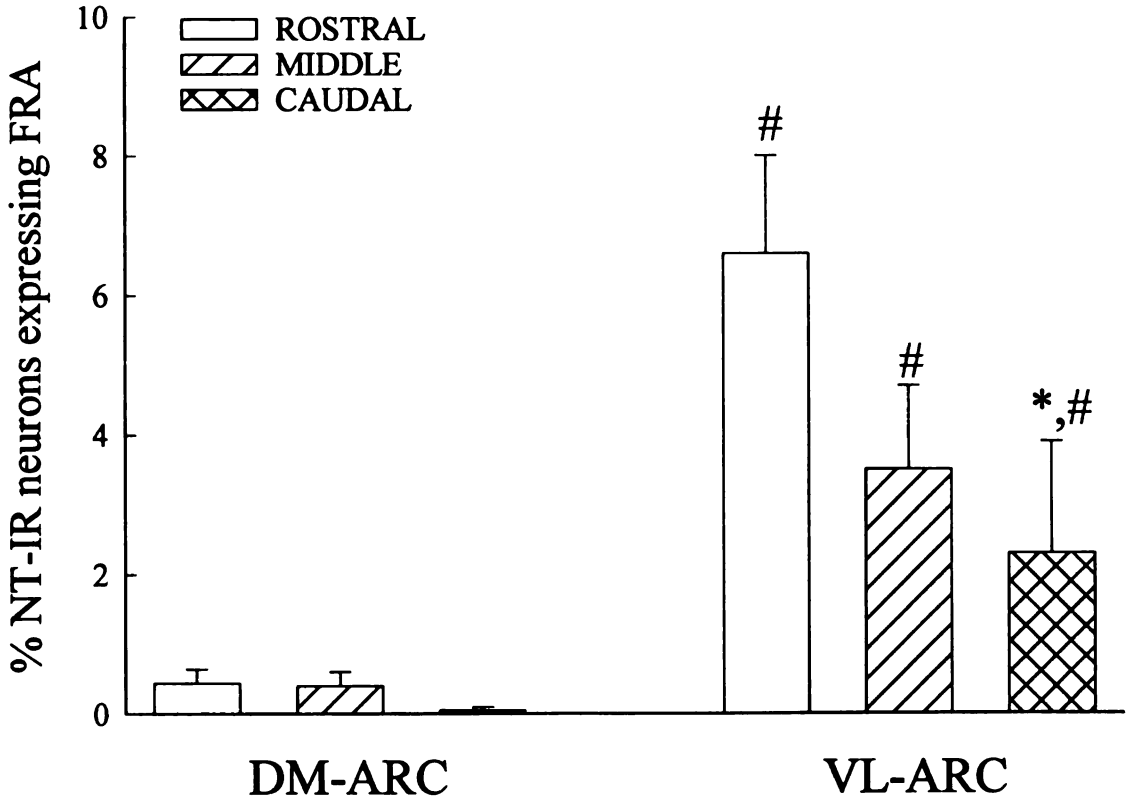


Figure 7.2 Comparison of percentages of NT-IR neurons expressing FRA-IR nuclei in the DM and VL subdivisions of the rostral, middle and caudal ARC in male rats. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR in the DM and VL subdivisions of the rostral (open columns), middle (hatched columns) and caudal (cross-hatched columns) ARC. Columns represent means and vertical lines 1 SEM from 6-8 animals. *, Values within an ARC subdivision that are significantly different ($p < 0.05$) from the rostral ARC. #, Values within a rostrocaudal region that are significantly different ($p < 0.05$) from the DM-ARC.

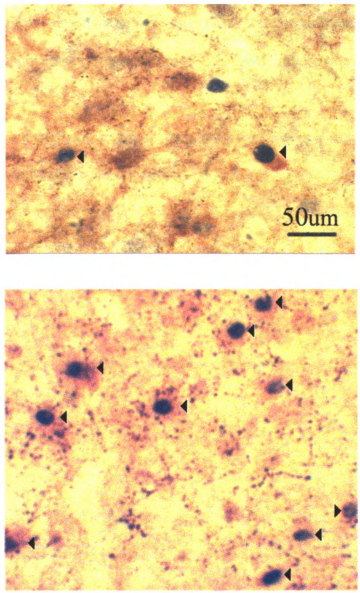


Figure 7.3 Digitized photomicrographs showing increased FRA expression in NT-IR neurons in the middle ARC after haloperidol injection in male rats. Animals were injected with either vehicle (TOP PANEL; 0.3% tartaric acid, 1 ml/kg; sc), or haloperidol (BOTTOM PANEL; 1 mg/kg; sc) and perfused 3 h later. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR. Neurons with blackened FRA-IR nuclei and brown NT-IR perikarya are indicated with an arrowhead.

injection for 1.5–6 h in the DM-ARC (**figure 7.4**) and for 1.5-3 h in the VL-ARC (**figure 7.5**) in all rostrocaudal regions in male rats. There were no differences in the magnitude of the response to haloperidol with regard to either ARC subdivision or rostrocaudal ARC region.

Figure 7.6 compares representative photomicrographs of numbers of neurotensin-IR neurons in the middle ARC after vehicle or haloperidol injection in male rats. Bright-field images show that numbers of neurotensin-IR neurons were increased after haloperidol administration. Haloperidol increased numbers of neurotensin-IR neurons in all rostrocaudal regions in male rats 12 h after injection in the DM-ARC (**figure 7.7**, top panel) and 6-12 h after injection in the VL-ARC (**figure 7.7**, bottom panel). The haloperidol-induced increase in total numbers of neurotensin-IR neurons was greatest in middle to caudal VL-ARC.

Central administration of prolactin increased the percentage of neurotensin-IR neurons expressing FRA at all time points (i.e. 1.5-12 h) in the DM (**figure 7.8**) and VL (**figure 7.9**) subdivisions of all rostrocaudal ARC regions in male rats. Moreover, there were no differences in the magnitude of response to prolactin with regard to ARC subdivision or rostrocaudal ARC region. Prolactin increased numbers of neurotensin-IR neurons in all rostrocaudal regions in male rats 12 h after injection in the DM-ARC (**figure 7.10**, top panel) and 6-12 h after injection in the VL-ARC (**figure 7.10**, bottom panel). The prolactin-induced increases in numbers of neurotensin-IR neurons were greatest in VL subdivisions and caudal ARC regions.

To determine if the increased expression of neurotensin-IR following prolactin treatment (**figure 7.10**) was occurring within dopamine neurons in the ARC, dual

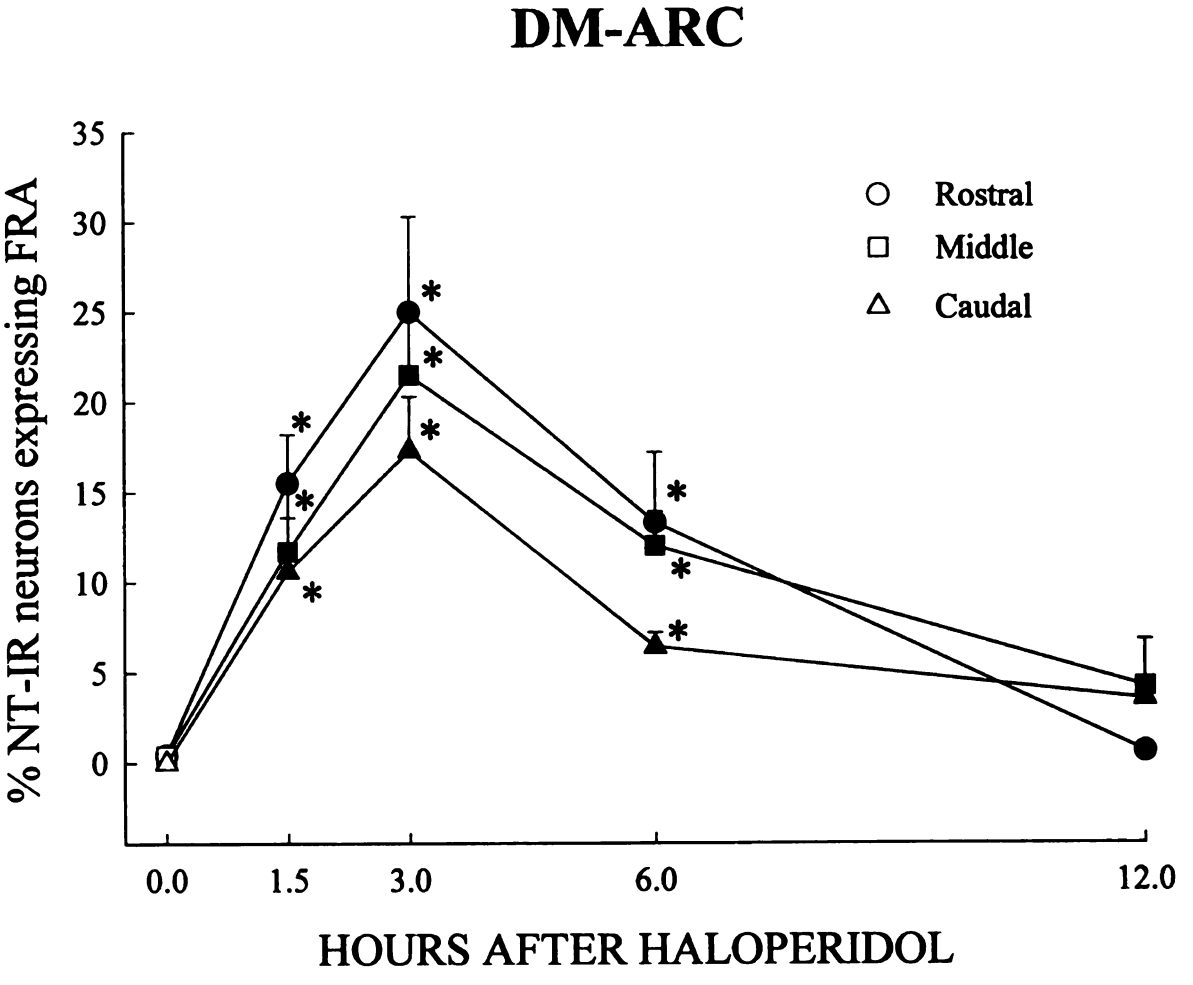


Figure 7.4 Time course effects of haloperidol on percentages of NT-IR neurons expressing FRA in the DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR in the DM-ARC. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

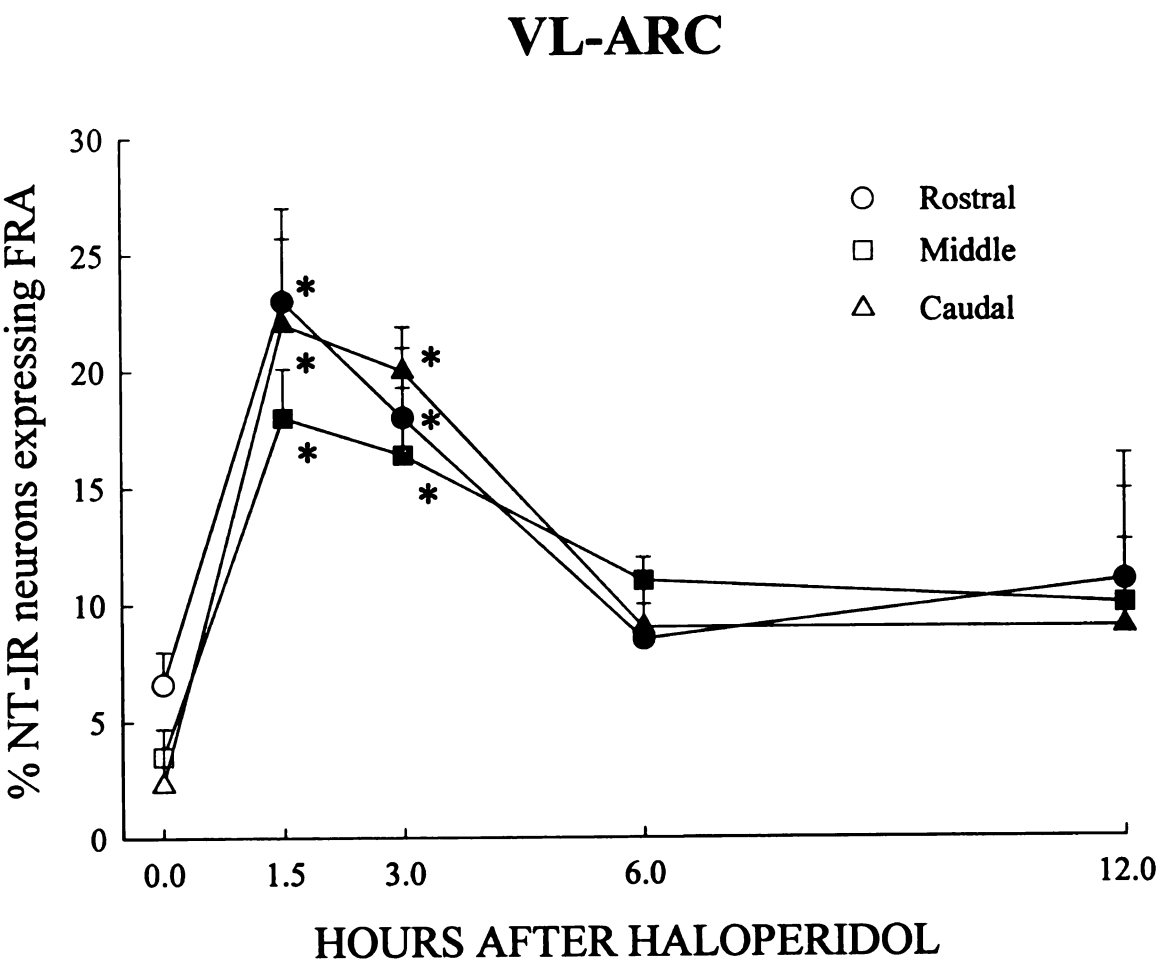


Figure 7.5 Time course effects of haloperidol on percentages of NT-IR neurons expressing FRA in the VL subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR in the VL-ARC. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

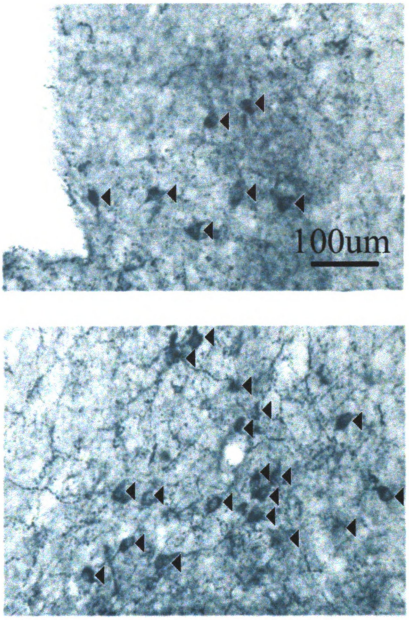


Figure 7.6 Digitized photomicrographs showing increased numbers of NT-IR neurons in the middle ARC after haloperidol injection in male rats. Animals were injected with either vehicle (TOP PANEL; 0.3% tartaric acid, 1 ml/kg; sc), or haloperidol (BOTTOM PANEL; 1 mg/kg; sc) and perfused 3 h later. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of NT-IR. Neurons with NT-IR perikarya are indicated with an arrowhead.

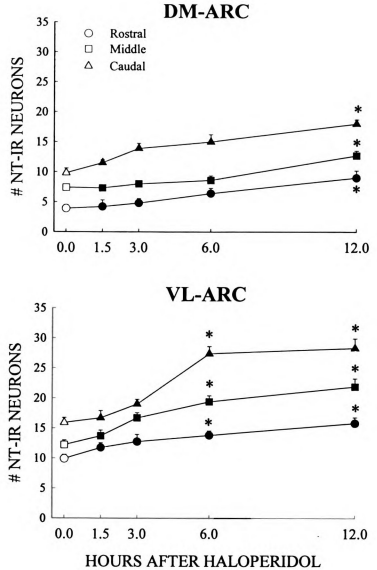


Figure 7.7 Time course effects of haloperidol on numbers of NT-IR neurons in the DM and VL subdivisions of rostral (circles), middle (squares) and caudal (triangles) ARC in male rats. Rats were injected with haloperidol (filled symbols; 1 mg/kg; sc) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (0.3% tartaric acid; 1 ml/kg; sc) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR in the DM-ARC (TOP PANEL) and VL-ARC (BOTTOM PANEL). Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for haloperidol-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

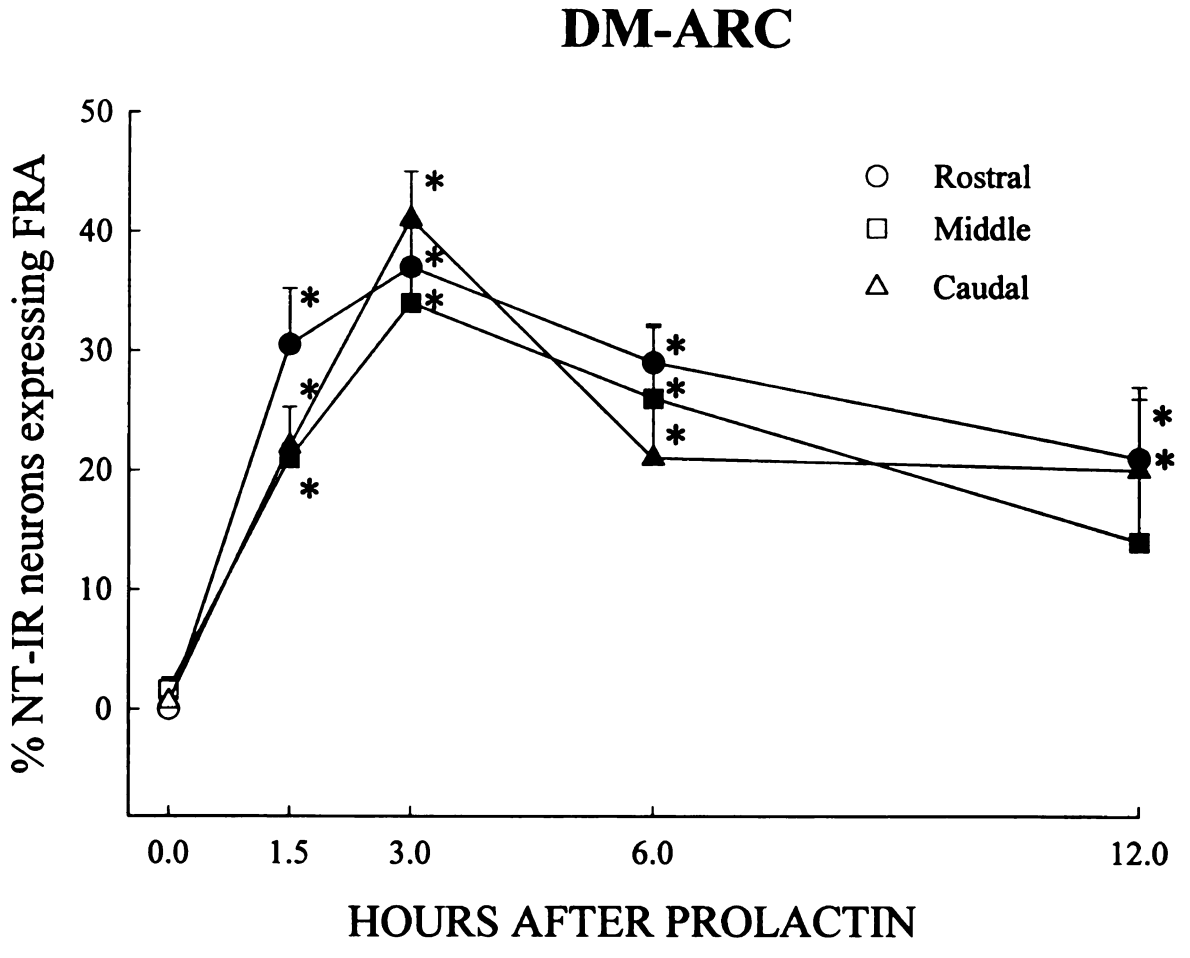


Figure 7.8 Time course effects of prolactin on percentages of NT-IR neurons expressing FRA in the DM subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (water; 3 μl/rat; icv) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

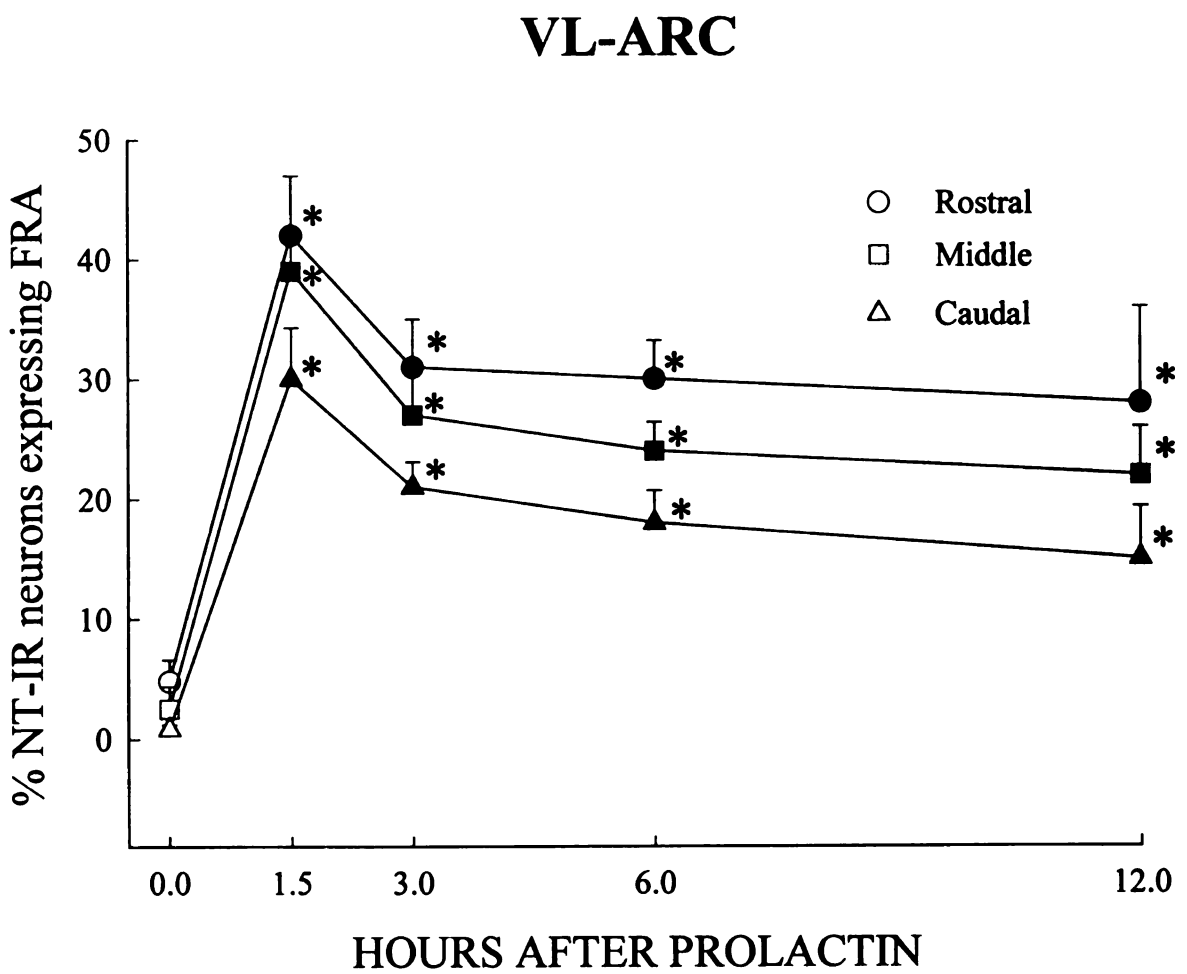


Figure 7.9 Time course effects of prolactin on percentages of NT-IR neurons expressing FRA in the VL subdivision of rostral (circles), middle (squares) and caudal (triangles) ARC of male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (water; 3 μl/rat; icv) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of FRA-IR and NT-IR. Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

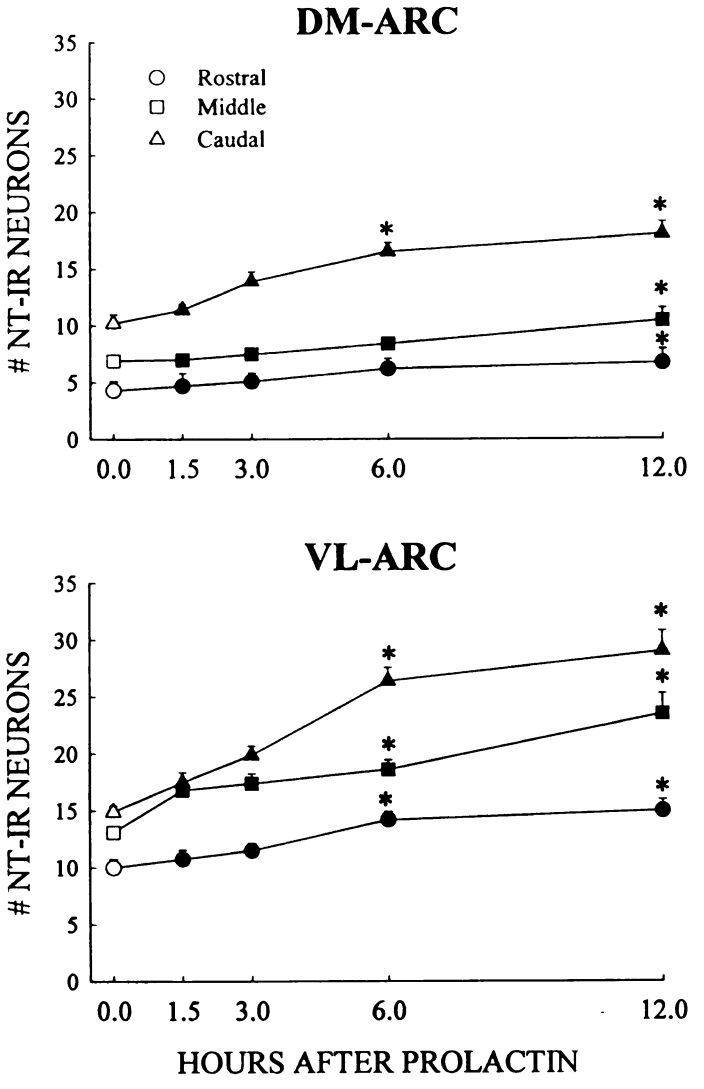


Figure 7.10 Time course effects of prolactin on numbers of NT-IR neurons in the DM and VL subdivisions of rostral (circles), middle (squares) and caudal (triangles) ARC in male rats. Rats were injected with prolactin (filled symbols; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbols) were injected with vehicle (water; 3 μl/rat; icv) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of NT-IR in the DM-ARC (TOP PANEL) and VL-ARC (BOTTOM PANEL). Symbols represent means and vertical lines 1 SEM from 6-8 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

immunohistochemistry for the detection of TH and neurotensin was performed on tissue sections derived from the prolactin time course. **Figure 7.11** depicts the localization of neurotensin-IR neurons relative to TH-IR neurons in the VL-ARC in male rats. Under bright-field optics the outlines of neurotensin-IR neurons stained black were traced and superimposed upon the fluorescent images of TH-IR neurons in the same section under dark-field optics. Immunoreactivity for neurotensin and TH was very rarely colocalized within neurons in the ARC. However, neurotensin-IR neurons were regularly adjacent to TH-IR neurons in the DM-ARC and VL-ARC. As shown in **figure 7.12,** the numbers of neurotensin-IR neurons in the middle DM-ARC were increased 12 h after prolactin injection. However, no increase in colocalization of neurotensin and TH was observed at any time after prolactin injection. Similarly, in the middle VL-ARC, numbers of neurotensin-IR neurons were increased 6-12 h after prolactin injection while no increase in colocalization of neurotensin and TH was observed through 12 h (**figure 7.13**).

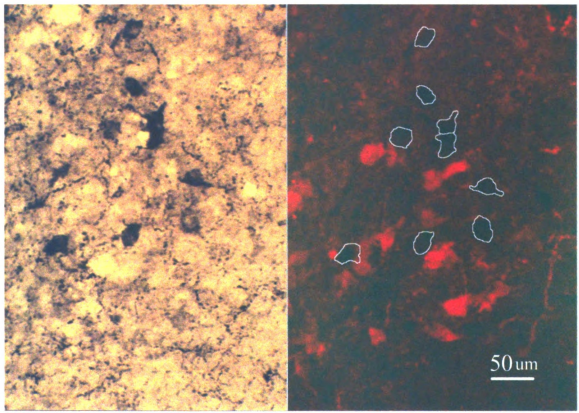


Figure 7.11 Digitized photomicrographs depicting the localization of NT-IR neurons relative to TH-IR neurons in the VL-ARC in male rats. Brains were perfused, removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of NT-IR and TH-IR. Black NT-IR neurons under bright-field optics (LEFT PANEL) were traced and superimposed on the same image under dark-field optics which shows fluorescent TH-IR neurons (LEFT PANEL).

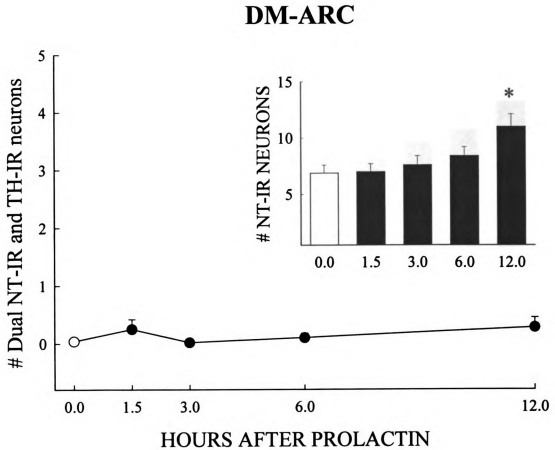


Figure 7.12 Time course effects of prolactin on numbers of dual NT-IR and TH-IR neurons, and numbers of NT-IR neurons (INSET) in the DM subdivision of the middle ARC in male rats. Rats were injected with prolactin (filled symbols and columns; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbol and column) were injected with vehicle (water; 3 μl/rat; icv) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of NT-IR and TH-IR. Symbols and columns represent means and vertical lines 1 SEM from 6-7 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

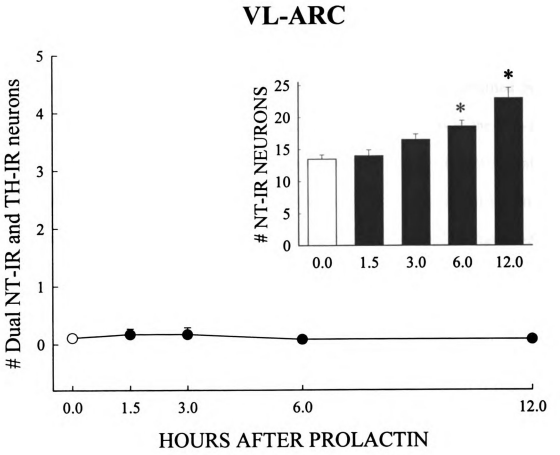


Figure 7.13 Time course effects of prolactin on numbers of dual NT-IR and TH-IR neurons, and numbers of NT-IR neurons (INSET) in the VL subdivision of the middle ARC in male rats. Rats were injected with prolactin (filled symbols and columns; 10 μg/rat; icv) and perfused 1.5, 3, 6 or 12 h later. Zero time controls (open symbol and column) were injected with vehicle (water; 3 μl/rat; icv) 3 h prior to perfusion. After perfusion, brains were removed, sectioned in the frontal plane (30 μm) and immunohistochemically processed for the detection of NT-IR and TH-IR. Symbols and columns represent means and vertical lines 1 SEM from 6-7 animals. *, Values for prolactin-treated rats that are significantly different ($p < 0.05$) from vehicle-treated controls.

C. DISCUSSION:

In the present study neural activity mapping using inducible transcription factors was applied to neurotensin-IR neurons in the ARC to further characterize the role of neurotensin in the delayed inductive activation of TIDA neurons by prolactin.

Perikarya of neurotensin neurons are heterogeneously distributed throughout the ARC. Two populations of neurotensin-containing neurons have been identified in the ARC based on location of perikarya and termini. Neurotensin neurons in the DM-ARC terminate within the ARC, whereas neurotensin neurons in the VL-ARC terminate predominantly in the median eminence (Meister et al., 1989). The results of the present study indicate that the majority of neurotensin neurons are localized in the VL-ARC consistent with a previous report (Lantos et al., 1995) and that there exists a rostrocaudal gradient in numbers of neurotensin neurons such that there are more neurotensin neurons in subdivisions in the caudal extent of the ARC. Since TIDA neurons of the DM-ARC have been shown to have neurotensin receptor mRNA (Alexander, 1997; Rostene and Alexander, 1997) and neurotensin neurons in this ARC subdivision terminate locally (Meister et al., 1989), it is possible that these neurotensin neurons are involved in the feedback activation of TIDA neurons by prolactin.

Neurotensin neurons in the VL-ARC terminate in the external zone of the median eminence, but neurotensin is not secreted into the hypophysial portal blood (Clarke et al., 1993). Thus it is possible that these neurons are involved in axon-axonal interactions with TIDA neurons to facilitate the secretion of dopamine. However, the role of these neurons and the significance of their anatomy remains to be elucidated.

Despite differences in numbers of neurotensin neurons in ARC subdivisions and rostrocaudal regions, the percentage of these neurons expressing FRA-IR nuclei was low (<8%) across all ARC subdivisions and rostrocaudal regions in male rats. In agreement with previous neurochemical studies (Chapter 6), data suggest that the majority of neurotensin neurons in the ARC are not tonically active.

Consistent with the hypothesis that prolactin induces immediate early gene (i.e. FRA) expression in neurotensin neurons in the ARC, both haloperidol-induced endogenous hyperprolactinemia and central administration of prolactin cause rapid increases (by 1.5 h) in the percentage of neurotensin neurons expressing FRA in the DM and VL subdivisions of the ARC in all rostrocaudal regions. This response persists 3-6 h in haloperidol-treated animals and 12 h in prolactin-treated rats. The longer response in icv prolactin-treated rats is likely related to the pharmacokinetics of central dosing (as discussed in Chapter 4).

Changes in immediate early gene expression in neurotensin neurons are followed by delayed increases in total numbers of neurotensin neurons. In all rostrocaudal regions the numbers of neurotensin-IR neurons in the DM-ARC are increased 12 h after injections of haloperidol or prolactin. Similarly, total numbers of neurotensin-IR neurons in the VL-ARC are increased 6-12 h after injections of haloperidol or prolactin. The discrepancy between the DM-ARC and VL-ARC in the temporal characteristics of neurotensin expression may be related to the shorter latency to peak changes in immediate early gene expression in the VL-ARC (1.5 h) compared to that of the DM-ARC (3 h), or to differential post-translational processing of proneurotensin (Carraway and Mitra, 1990; Checler et al., 1991; Woulfe et al., 1994) in the VL-ARC as compared

to the DM-ARC. The increased capacity of TIDA neurons that temporally coincides with the increase in numbers of neurotensin neurons in the ARC could be due to the effects of this neuropeptide as a neuromodulator of dopaminergic neurotransmission. For example, neurotensin has been shown to facilitate dopamine release in the NSDA system (Battaini et al., 1986; Tanganelli et al, 1993).

Activation of TIDA neurons is blocked following administration of a neurotensin receptor antagonist 11 h after induction of hyperprolactinemia, suggesting that the long protein synthesis-dependent period could correspond to the *de novo* synthesis and release of neurotensin. In the present study, neurotensin neurons were localized within the ARC in close proximity to TIDA neurons. Immediate early gene expression in these neurotensin neurons is induced by hyperprolactinemia and the time course of these changes precede those in TIDA neurons (i.e. numbers of neurotensin cells are increased 6-12 h following hyperprolactinemia). These data support the hypothesis that delayed inductive activation of TIDA neurons by prolactin is mediated by neurotensin neurons in the ARC.

Since proneurotensin mRNA (Kiyama and Emson 1991) and neurotensin-IR (Chronwall, 1985; Palkovits, 1992) have been localized in neurons within the ARC including those containing TH-IR (Ibata et al., 1983; Halasz et al., 1985), prolactin-induced neurotensin synthesis could occur in afferent neurotensin neurons in the ARC (Merchanthaler and Lennard, 1991; Marcos et al., 1996a) and/or in subpopulations of TIDA neurons themselves (Hokfelt et al., 1984; Bachelet et al., 1997). Dual immuno-histochemistry for the detection of neurotensin-IR and TH-IR was utilized to determine if the prolactin-induced synthesis of neurotensin occurs in TIDA neurons. Consistent with

one report (Bachelet et al., 1997) yet contrasting others (Kahn et al., 1980; Ibata et al., 1983; Hokfelt et al., 1984; Ciofi et al., 1993), neurotensin-IR is rarely colocalized with TH-IR neurons in the ARC. Moreover, no increase in colocalization is observed at any time following prolactin administration for up to 12 h. Instead, neurotensin-IR neurons are often located adjacent to TH-IR neurons in the DM-ARC and VL-ARC. Thus, it is more likely that neurotensin mediates prolactin-induced activation of TIDA neurons via local afferent input within the mediobasal hypothalamus.

The immunohistochemical detection of neurotensin-IR perikarya in the ARC has inherent technical challenges. Neurotensin is synthesized *de novo* as a proneuropeptide and is proteolytically processed to its active form during axonal transport (Hook et al., 1990; Rostene and Alexander, 1997). Furthermore, neurotensin-IR fibers heavily ramify over the entire ARC (Kahn et al., 1980; Jennes et al., 1982). Consequently, the staining of neurotensin-IR perikarya may be weak and must be detected over the high background of neurotensin-IR fibers. To evade this technical difficulty many investigators pretreat animals with colchicine, an inhibitor of axonal transport. Accordingly, colchicine increases neuropeptide content in perikarya and decreases fiber background (Kahn et al., 1980). However, recent studies demonstrate that colchicine treatment *per se* rapidly and strongly induces expression of some peptides, including that of neurotensin, in certain cell populations in the vicinity of the ventricles (Kiyama and Emson, 1991; Alexander and Leeman, 1994; Rostene and Alexander, 1997). Consequently, experimental interpretations may be complicated/confounded by the use of colchicine. Thus reports of extensive colocalization of neurotensin and TH in studies utilizing colchicine may have been overestimated.

In conclusion, the results of this study reveal that prolactin acutely regulates immediate early gene expression in neurotensin neurons in the ARC, and induces a delayed increase in neurotensin synthesis in neurons in the ARC that is not colocalized in TIDA neurons. These results provide additional evidence that the delayed inductive activation of TIDA neurons by prolactin is indeed mediated by local afferent neurotensin neurons in the ARC.

8. SUMMARY AND DISCUSSION

A. SUMMARY

It has been more than ten years since the discovery of the delayed-inductive activation of TIDA neurons by prolactin. However, the mechanisms underlying the effects of prolactin remain largely unknown. The overall aim of the experiments described in this dissertation was to gain insight into these mechanisms. To this end, studies herein detailed: the temporal effects of prolactin on neurochemical estimates of TIDA neuronal activity, the temporal effects of prolactin on immediate early gene expression in cells of the ARC including TIDA neurons, and the role of neurotensin as a mediator of the effects of prolactin. The major points from these studies are summarized below.

A single injection of haloperidol elicits an early (by 1 h) and prolonged (through 12 h) state of hyperprolactinemia. Hyperprolactinemia subsequently activates TIDA neurons after a delay of six or more h. Immunoneutralization of circulating prolactin blocks the delayed activation of TIDA neurons by haloperidol, confirming that prolactin mediates the stimulatory effects of haloperidol on these neurons.

In the temporal characterization of the delayed response, experiments demonstrate that the activation of TIDA neurons is dependent upon a period of sustained hyperprolactinemia for initiation of the response. A period greater than 3 h, but less than 6 h of hyperprolactinemia is required for its initiation. Furthermore, hyperprolactinemia is required to maintain the response as immunoneutralization of circulating prolactin blocks the activation of TIDA neurons in delayed response-initiated animals.

Prolactin regulates the expression of FRA in TH-IR neurons in the ARC. In female rats, the basal expression of FRA in TH-IR neurons in the ARC is 4 times greater than that in males, in agreement with the gender difference established by neurochemical estimates. Hyperprolactinemia causes transient increases in the percentage of TH-IR neurons expressing FRA after 1.5 h in the VL-ARC and 3 h in the DM-ARC (**figure 8.1**). Immunoneutralization studies indicate that these changes are dependent upon a sustained period of hyperprolactinemia greater than 1 h for induction.

Prolactin also regulates the expression of FRA in chemically unidentified neurons in the ARC. In non-catecholaminergic cells, prolactin causes transient increases in numbers of FRA-IR nuclei after 1.5-3 h in the VL-ARC, and 3 h in the DM-ARC (**figure 8.1**). Additionally, the changes in FRA expression occur in both magnocellular and parvocellular subdivisions of the DM-ARC.

Prolactin regulates the expression of FRA in neurotensin-IR neurons in the ARC. Prolactin increases the percentage of neurotensin-IR neurons expressing FRA from 1.5-12 h in the ARC, and causes a delayed increase in the total number of neurotensin-IR neurons after 6 h in the VL-ARC and 12 h in the DM-ARC (**figure 8.1**). Immunohistochemistry demonstrates that the neurotensin-IR is only rarely colocalized with TH-IR, and that the incidence is not affected by up to 12 h of hyperprolactinemia. However, the same studies also demonstrate that neurotensin neurons are often closely associated with TIDA neurons.

The neurotensin receptor antagonist SR-48692 has no effect on neurochemical estimates of basal TIDA neuronal activity or plasma prolactin concentrations. However, this antagonist blocks the delayed stimulatory effects of prolactin on dopamine release

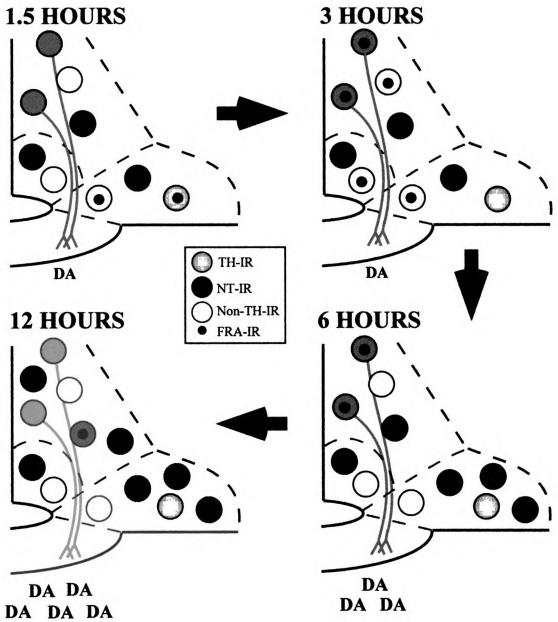


Figure 8.1 Schematic summary of the temporal effects of prolactin on FRA expression in cells, and recruitment of neurotensin neurons in ARC subdivisions during the inductive activation of TIDA neurons. Abbreviations: DA, dopamine; FRA, Fos-related antigens; IR, immunoreactive; NT, neurotensin; TH, tyrosine hydroxylase.

from TIDA neurons. The results suggest that neurotensin mediates the delayed, but not tonic stimulatory effects of prolactin on TIDA neurons.

B. CONCLUDING DISCUSSION

To review, the inductive component of TIDA neuronal activation by prolactin involves a change in the capacity to respond to prolonged periods of hyperprolactinemia (Moore et al., 1987a) and is observed in both genders. It stimulates TIDA neuronal activity to levels 2-3 times basal (Demarest et al., 1985a; Selmanoff, 1985) 6-12 h following systemic (Hokfelt and Fuxe, 1972; Selmanoff, 1981; Demarest et al., 1984; 1986) or central (Annunziato and Moore, 1978) administration of exogenous prolactin, or after manipulations which elicit endogenous hyperprolactinemia (Moore et al., 1985; Hentschel et al., 2000a). This component of prolactin feedback is protein synthesis-dependent (Johnston et al., 1980) and is associated with increased gene expression [i.e. TH mRNA] in TIDA neurons (Arbogast and Voogt, 1991; Selmanoff et al., 1991).

Prolactin exerts multiple effects on TIDA and other non-catecholaminergic neurons in the ARC to elicit the change in capacity and increased responsiveness of TIDA neurons. Data from the experiments in this dissertation have provided insight into the mechanisms underlying the delayed-inductive activation of TIDA neurons by prolactin. The data summarized early in this chapter are interpreted and mechanisms leading to the inductive activation of TIDA neurons in the 6th to 12th hour are temporally presented as they theoretically occur.

Following 1.5 h of elevated prolactin, changes are occurring in gene expression in neurotensin neurons throughout the ARC. Perhaps these neurons begin to upregulate synthetic enzymes for preproneurotensin. However, it is unlikely that these neurons directly stimulate TIDA neurons (at this time) as TIDA neurons are acutely activated by

central injections of neurotensin within 1 h which is inconsistent with the neurochemical time course of TIDA neuronal activation.

At the same time, prolactin selectively elicits changes in gene expression in catecholaminergic neurons in the VL-ARC. These neurons may be involved in the stimulation of the TIDA neurons in the DM-ARC, and/or may be recruited and induced to synthesize AAAD.

After 3 h of elevated prolactin, there is sustained immediate early gene expression in neurotensin neurons throughout the ARC. Presumably these neurons are molecularly active as they continue to synthesize neuropeptide and perhaps begin to recruit other neurons to synthesize neurotensin.

TIDA neurons of the DM-ARC show transient changes in immediate early gene expression after 3 h of elevated prolactin. These changes are dependent on sustained hyperprolactinemia for initiation. The changes likely involve modulation of late response genes such as the rate limiting enzyme, TH which could ultimately contribute to the increased capacity of this system. Since data show that the total number of catecholaminergic neurons in the ARC is constant, it is likely that the level of activity of existing neurons is elevated. This idea is consistent with data from investigators who note increased TH mRNA in the ARC after chronic hyperprolactinemia (Selmanoff, 1991; Arbogast and Voogt, 1991).

The increased immediate early gene expression in TIDA neurons of the DM-ARC at 3 h is unlikely representative of increased neurotransmitter release since it is inconsistent with the neurochemical time course of activation. Increases in immediate early gene expression may also be elicited from afferent neuronal input that modulate

second messenger systems, protein kinase activity and the expression of constitutive transcription factors or their localization. For example, it has been demonstrated that some growth factors and neuropeptides may affect levels of second messengers and induce changes in immediate early genes, like FRA, without affecting neurotransmitter release (Hoffman and Murphy, 2000).

Following 6 h of elevated prolactin, there is sustained elevation of immediate early gene expression in neurotensin neurons throughout the ARC as well as evidence of recruitment of neurotensin neurons in the VL-ARC. In addition, neurochemistry demonstrates the onset of activation of TIDA neurons that is mediated through neurotensin receptors (Hentschel et al., 1998). The data suggest that neurotensin neurons have a role proximal to TIDA neurons in the circuitry of the inductive component (**figure 8.2**). In agreement with the well-known facilitatory role of neurotensin neurons in dopaminergic systems, neurotensin could act to enhance dopamine release from TIDA neurons.

After 12 h of increased prolactin levels, immediate early gene expression in neurotensin neurons remains elevated and total numbers of neurotensin-IR neurons throughout the ARC are increased. The data are consistent with recruitment of neurotensin neurons into an active pool and ongoing neuropeptide synthesis. Given the intimate proximity of neurotensin and TIDA neurons in the ARC, it is plausible that stimulatory input from neurotensin neurons to TIDA neurons is maintained by elevated prolactin and mediated through neurotensin receptors (**figure 8.2**). The increased capacity of TIDA neurons could be from several mechanisms: (1) increased synthetic capacity of existing TIDA neurons by induction of synthetic enzymes such as the rate limiting

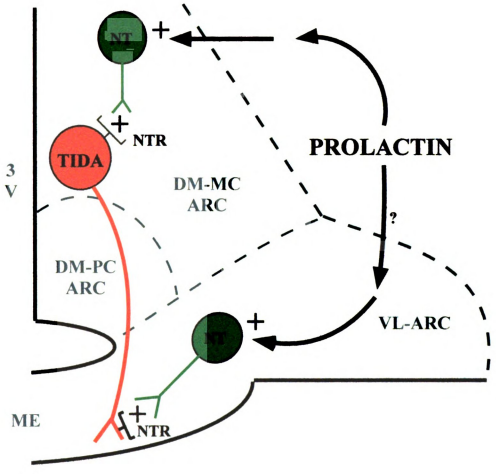


Figure 8.2 Schematic representation of the theoretical *distal* circuitry of the inductive activation of TIDA neurons by prolactin. Abbreviations: 3V, third ventricle; ARC, arcuate nucleus; DM, dorsomedial; MC, magnocellular; ME, median eminence; NT, neurotensin neuron; NTR, neurotensin receptor; PC, parvocellular; TIDA, tubero-infundibular dopamine neuron; VL, ventrolateral.

enzyme, TH; (2) the facilitative effects of neurotensin neuromodulation of TIDA neuronal activity; and/or (3) the recruitment of the TH-IR neurons of the VL-ARC to synthesize AAAD, and release dopamine. Such hypotheses could be tested using *in situ* hybridization histochemistry.

In situ hybridization histochemistry (ISHH) could be utilized to test for increased synthetic capacity of TIDA neurons via induction of TH and other synthetic enzymes by examining the time course effects of central prolactin administration on synthetic enzyme mRNA expression in TIDA neurons. Similarly, if the increased capacity of TIDA neurons is due to the recruitment of TH-IR neurons of the VL-ARC to synthesize AAAD and release dopamine, then the time course effects of central prolactin administration should demonstrate the induction of AAAD mRNA expression in neurons in the VL-ARC containing TH mRNA as detected using dual ISHH. These data should next be verified using dual immunohistochemistry for the translated (protein) products.

The function of the inductive activation of TIDA neurons by prolactin is not known. However, since prolactin exerts actions on reproduction, fluid balance, growth, metabolism and immune function, the regulation of its secretion from the pituitary is very important. TIDA neurons are the primary regulators of prolactin secretion. It's possible that the inductive component as a conserved mechanism for control of pathologic hypersecretion of prolactin. The characteristics of the inductive component are consistent with this: (1) the inductive component acts as the gain of a system, increasing the capacity of the TIDA neuronal system to control prolactin secretion, if necessary, (2) prolonged hyperprolactinemia is required for initiation of the response, and (3) sustained hyperprolactinemia is required to sustain the response. The inductive component appears

to act as a fail-safe system for the regulation of the secretion of prolactin and hence maintaining normal reproductive, growth, metabolic and immunologic functions.

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