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The Effects of Ethanol on Growth Hormone and Prolactin Gene Expression in Male Rats

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THE EFFECTS OF ETHANOL ON GROWTH HORMONE AND PROLACTIN GENE EXPRESSION IN MALE RATS

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LIST OF ABBREVIATIONS

ADU	arbitrary densitometer units
α	alpha
β	beta
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine 3′, 5′-monophosphate
cc	cubic centimeter
cDNA	complimentary deoxyribonucleic acid
Ci	curie
cpm	counts per minute
Da	daltons
DA	dopamine
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
g	gram

GH	growth hormone
GHRH	growth hormone releasing-hormone
GI	gastro-intestinal
GIT	guanidinium isothiocyanate
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
ip	intraperitoneal
kb	kilobase
kDa	kiloDalton
L	liter
M	molar
μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
mg	microgram
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar

mm	millimeter
mRNA	messenger ribonucleic acid
MOPS	3-(N-morpholino) propanesulfonic acid
ng	nanogram
nM	nanomolar
O.D.	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
Prl	prolactin
RIA	radioimmunoassay
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate buffer
Taq	*thermus aquaticus* DNA polymerase
TBE	tris-borate-EDTA buffer

TE	tris-EDTA
TRH	thyrotropin-releasing hormone
tRNA	transfer RNA
TSH	thyroid-stimulating hormone
UV	ultraviolet
v/v	percent volume per volume
x g	times gravity

CHAPTER I

INTRODUCTION

Alcohol (ethyl alcohol, or EtOH) is the most important drug of dependence in all industrialized countries. The social and clinical problems that arise from alcohol's widespread abuse are now well documented. In the U.S. alone, approximately 18 million adults exhibit some form of alcoholism, alcohol dependence, or alcohol abuse (Van Natta, *et al* 1984). Recent studies of current trends in alcohol drinking behavior indicate that bouts of high level alcohol consumption over short periods of time, termed "binge-drinking" episodes, are becoming more common, especially amongst young adults in the U.S. (Meilman *et al* 1990). Binge drinking is believed to be one of several behavioral characteristics involved in the development of chronic alcoholism (Richards, *et al* 1990). While many studies have been undertaken to determine the effects of chronic alcohol consumption on health in alcoholic subjects and in animal models of alcoholism, less is known about the deleterious effects of short-term, binge alcohol consumption.

Several elements of the endocrine system are known to be adversely affected by alcohol, including the hypothalamic-pituitary-growth hormone (GH) axis, and the hypothalamic-pituitary-prolactin (PRL) axis (Zakhari, 1992). GH and PRL are closely

related pituitary hormones which subserve a wide variety of essential functions in mammals, such as regulation of growth, reproduction, maintenance of lactation, intermediary metabolism and immune responsiveness (Hadley, 1988). The suppressive effect of EtOH on GH secretion and its stimulating effect on PRL release have been previously investigated, and both a pituitary (Redmond, 1980; Dees, *et al* 1988; Emanuele, *et al* 1989; Sontag and Boyd 1989) and a hypothalamic/central nervous system locus of EtOH action have been postulated (Conway and Maureci, 1991). Despite this research, there is at present no consensus on EtOH's precise site of action, nor is there a complete mechanistic explanation for the differential effects of EtOH on these two hormones. The hypothesis to be tested is that, similar to effects on other pituitary hormone systems, EtOH acts at numerous levels within the GH and PRL axes and exerts its deleterious effects through numerous mechanisms.

Therefore, the specific aims of this dissertation research are: 1) to investigate the potential site(s) of action of acute EtOH within the GH and PRL axes, and 2) to further elucidate the biochemical and molecular mechanisms responsible for the EtOH-induced fall in serum GH and the concomitant rise in serum PRL l evels. To address the aims of this research, an *in vivo* rat model approximating binge alcohol consumption was created. Adult male Sprague-Dawley rats, aged 60-90 days, were given acute, high level alcohol doses (3 g/kg) via an intraperitoneal (i.p.) injection, sacrificed at various time points after injection, and assessed for various endocrine parameters, compared to saline-injected controls. These "single binge" experiments were followed up with "double binge" studies to determine if the system reacts in a

similar fashion after repeated EtOH exposure.

This dissertation describes the effects of EtOH at the pituitary level, on both GH andPRL synthesis and secretion, and at the hypothalamic level on GHRH and SRIF synthesis and secretion. At the molecular level, steady-state messenger RNA (mRNA) levels for these hormones were measured, as well as the transcription rates for the piuitary genes. Additional mechanisms were investigated, including EtOH's effects on second messenger systems involved in GH and PRL gene expression.

CHAPTER II

REVIEW OF RELATED LITERATURE

A. The Growth Hormone (GH) Axis

Growth hormone, (GH) is an important anterior pituitary hormone required for normal postnatal growth, particularly long-bone growth, and is also involved in a variety of other physiological functions such as maintenance of nitrogen, mineral, lipid, and carbohydrate metabolism (Hadley, 1988). While GH has long been recognized as a regulator of body growth, it is produced even in adult non-growing animals and humans, and evidence is increasing that this hormone is needed throughout life for the normal function of tissues and organs (Thorner and Vance, 1988). Two of the more recent and intriguing additions to the growing list of GH functions which are carried out into adulthood are modulation of the immune system (Berczi, *et al* 1991), and reversal of some of the physical aspects of aging, such as improved muscle tone and increase in lean body mass, and reversal of the age-related decrease in bone density in elderly patients (Rudman, *et al* 1990).

In mammals, GH is synthesized and secreted from a subpopulation of anterior pituitary cells known as the somatotropes, the most abundant cell type in the gland (Martin, 1985). Somatotropes can store a significant amount of GH in cytoplasmic

secretory vesicles. The adult human gland contains 5 to 10 mg of the GH protein (Murray, *et al* 1990). Upon stimulation from the hypothalamus and higher brain centers, GH is released into the general circulation where it binds to specific GH receptors, located predominantly on liver cells, and also to a lesser degree on bone, cartilage, kidney, pancreas, testes and hypothalamic cells (Hadley, 1988). Upon binding to liver cell receptors, GH stimulates the release of liver-derived growth factors, collectively known as the somatomedins. The primary somatomedin released in response to GH is somatomedin C, or insulin-like growth factor-1 (IGF-1) (Martin, 1985). Like many endocrine systems, the GH axis is under highly complex regulation by short and long feedback loops. These feedback loops ensure that hormone levels remain within certain homeostatic boundaries, which are determined by the biochemical and physiologic needs of the organism.

It is believed that the majority of GH's growth-promoting effects are mediated through IGF-1, while the effects on carbohydrate, lipid and protein metabolism and the proliferative effect on lymphocytes are due to the direct action of GH itself (Martin, 1985; Berczi, *et al* 1991). The GH protein exists as a 191-amino acid monomer with a molecular weight of 22 kDa, although 20 kDa and 36 kDa variants have been reported (Sinha, *et al* 1987; Bollengier, *et al* 1988). In the circulation GH has a half-life of about 20 minutes (Taylor, *et al* 1969). While in the circulation, it is attached to a specific GH-binding protein (GHBP) (Martin 1985). Plasma IGF-1 is also bound to carrier proteins. In contrast to GH, the half-life of IGF-1 in the circulation is about 24 hours. There is little little diurnal variation in the plasma levels of IGF-1 (Martin,

1985).

GH release is episodic and pulsatile in all species in which it has been examined. Serum GH may change as much as 10-fold within a few minutes (Murray, *et al* 1990). One of the largest increases occurs shortly after the onset of sleep. Other factors which can stimulate GH release include stress, exercise, hypoglycemia or fasting, a protein meal, and the amino acid arginine (Murray, *et al* 1990). The pattern of GH secretion in rats is sexually dimorphic and greatly influenced by androgens both during the neonatal and adult periods (Jansson, *et al* 1985). In male rats, secretory bursts of GH occur at 3.3 hour intervals interspersed by trough periods during which GH levels are virtually undetectable (Tannenbaum, *et al* 1993). In contrast, female rats exhibit GH pulses that are irregular in timing, more frequent, and of lesser magnitude than in males. It has been speculated that such differences in pulse amplitude may account for the larger size of adult male rats versus adult female rats (Bercu, *et al* 1991). The pulsatile nature of GH release is essential to its effectiveness. A study utilizing young hypophysectomized (surgical removal of the pituitary) rats fitted with chronic venous cannulae showed that pulsatile infusions of GH produced a sustained growth response, whereas continuous infusion of GH led to growth retardation (Robinson and Clark, 1987). Thus, a plasma GH profile which resembles the physiological 3-hour episodic secretory pattern in male rats is most effective in promoting growth.

B. Regulation of GH Synthesis and Secretion

GH secretion is regulated by a complex neuroendocrine control system that includes both neurotransmitters and feedback by hormonal and metabolic substrates. The final common pathway for the integration of these signals involves two hypothalamic neuropeptides, which are hypophysiotropic hormones. GH-releasing hormone (GHRH), a 40-44 residue peptide, exerts stimulatory effects on GH secretion while somatostatin (SRIF), a tetradecapeptide, exhibits an inhibitory influence (Hadley, 1988). There are several negative feedback actions in the operation of the GH axis (Abe, *et al* 1983). GHRH inhibits its own secretion by hypothalamic neurons ("ultra short-loop" feedback), and simultaneously may augment the release of somatostatin. GH stimulates the production of somatostatin by the hypothalamus ("short-loop" feedback). IGF-1 tends to inhibit the release of GH by actions both on the hypothalamus (increased SRIF production) and on the pituitary somatotropes through diminished responsiveness to GHRH ("long-loop" feedback). A diagrammatic representation of the mammalian GH axis and its feedback loops is depicted in figure 1.

C. Growth Hormone Releasing- Hormone (GHRH)

In humans and rats, the majority of GHRH cell bodies are located in the medial basal hypothalamus (MBH), specifically in the lateral zone of the arcuate nucleus (Martin, 1985). These neurons project to nerve endings in the median eminence which abut next to capillaries of the pituitary portal vessel circulation, the vascular link between the hypothalamus and the pituitary (Maclean and Jackson, 1988). .The entire

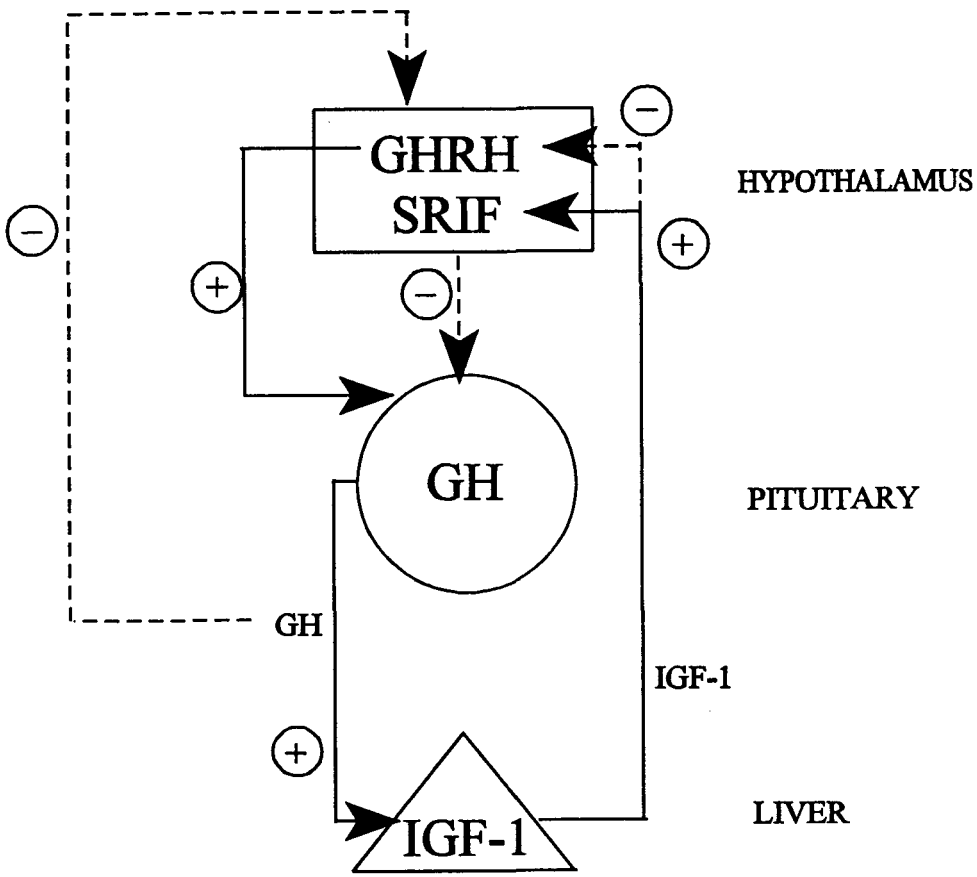


Figure 1. Diagrammatic representation of the hypothalmic-pituitary-growth hormone axis, showing positive and negative feedback loops.

GHRH gene has been isolated and sequenced from genomic libraries. The gene spans 10 kb, including 5 exons and 4 introns. Exons 1 and 2 encode the 5′ untranslated regions, exons 2-4 encode most of the 108 amino acid prepro-hormone and exon 3 encodes almost all of the bioactive portion of mature GHRH (Mayo, *et al* 1985). GHRH expression is believed to be regulated by a number of factors, including a short-loop negative feedback of GH on the hypothalamus and a long-loop negative feedback of IGF-1. There is also evidence for paracrine regulation within the hypothalamus by SRIF (Frohman, *et al* 1990). The regulatory sequences in the 5′ untranscribed regions of the GHRH gene are still being determined, however there are reports describing regions with partial homology to cAMP response elements (Frohman and Jansson, 1986). The GHRH gene is expressed primarily in the hypothalmus, but mRNA and protein secretion have also been reported in the placenta (Baird, *et al* 1985) and testis (Berry and Pescovitz, 1988). The exact function of this "ectopic" expression of GHRH is at this point unclear.

GHRH released from the hypothalamus travels via the pituitary portal vessels, and binds to specific receptors on the pituitary somatotroph. Most evidence suggests that these receptors are coupled directly to the stimulatory GTP (guanine nucleotide) binding protein, G_s, which in turn activates the the catalytic subunit of adenylate cyclase (AC) to form cAMP (Maclean and Jackson, 1988). Treatment of primary cultures of pituitary cells with GHRH results in a rapid rise in intracellular levels of cAMP (Bilezikjian and Vale, 1983). Furthermore, activators of cAMP such as forskolin stimulates not only GH release and synthesis but also growth of somatotropes

(Billestrup, *et al* 1986). Calcium influx also occurs independently and may be further enhanced by cAMP-mediated phosphorylation of calcium channels (Frohman and Jansson, 1986). Through these and possibly other mechanisms, GHRH begins a cascade of events that ultimately leads to phosphorylation and activation of transcription factors responsible for increased GH gene transcription as well as events leading to increased GH release from intrapituitary stores (Hadley, 1988). There is considerable evidence that in the male rat, GHRH and SRIF are released in reciprocal 3 to 4 hour cycles into the portal circulation (Plotsky and Vale, 1985) to act on the pituitary somatotropes and generate the ultradian rhythm of GH secretion (Tannenbaum, *et al* 1993).

The rat GH gene is organized into 5 exons and 4 introns and spans 2.1 kb in length on chromosome 17 (Chien and Thompson, 1980). The mature mRNA for growth hormone is about 1 kb in length. Within the GH promoter, two *cis* acting elements, centered around -80 and -122 were found to bind a trans-acting pituitary-specific factor termed Pit-1 (also known as GHF-1) (Nelson, *et al* 1988; Bodner and Karin, 1987). As determined by RNA and immunohistochemical analysis of mouse and rat, Pit-1 expression is pituitary-specific. Recent reports indicate, however, that a possible exception to this rule may be hemopoetic and lymphoid tissue (Delhase, *et al* 1993). Pit-1 was one of the first identified members of the POU (pit-1, oct-1,2, unc-86) homeodomain family of developmentally important transcriptional regulators which are characterized by conservation of a 60 amino acid homeodomain and a second region of about 75 amino acids located N terminally to the homeodomain called the POU-

specific domain (Theill and Karin, 1993). POU domain genes constitute a subclass of the homeobox genes, which exert critical developmental and transcriptional functions (Finney, *et al* 1988). Both the POU-specific and the homeodomain regions are important for sequence-specific DNA binding of Pit-1 homodimers to the AT-rich Pit-1 binding sites in the GH promoter (Ingraham, *et al* 1988). A transactivation domain is found near the N-terminus containing a 72 amino acid serine-threonine rich region, and is required for high level transcriptional activity (Ingraham, *et al* 1990). The binding to and transactivation of the GH promoter by Pit-1 was one of the first demonstrations that homeodomain proteins are indeed cell type-specific transcriptional regulators (Bodner, *et al* 1988). In adult rats and humans, Pit-1 expression is restricted to three cell types within the pituitary: the GH-, prolactin (PRL)- and thyroid stimulating hormone (TSH)-producing cells (thyrotropes). Although there has been some controversy concerning the target genes that are activated by Pit-1, several studies have shown that Pit-1 can transactivate both the GH and PRL genes and is also important for regulation of the gene for the β subunit of TSH by TRH and cAMP (Steinfelder, *et al* 1991). Pit-1 also autoregulates its own expression (McCormick, *et al* 1990).

Studies have shown that in addition to Pit-1, other transcription factors may be targets of the GHRH-induced intracellular signal in anterior pituitary cells, including the proto-oncogene product C-FOS. Billestrup, *et al* (1987) demonstrated that GHRH stimulation of primary somatotropes resulted in a transient increase in *c-fos* mRNA and protein and that this stimulation was diminished by somatostatin. C-FOS is known to

be important in specific differentiation events but more recent studies with growth factors and mitogens have suggested that the expression of *c-fos* is a more general response of cells to activation of protein kinase C (PKC) or increases in intracellular levels of cAMP (Mitchell, *et al* 1986). The activation of *c-fos* in the hypothalamus and pituitary is also associated with the stress response (Kononen, *et al* 1992) and has been correlated with elevations with elevations in the stress hormone adrenocorticotropic hormone (ACTH) (Handa, *et al* 1993). It is also worth noting that experimental evidence is accumulating for a role of *c-fos* expression in PRL gene regulation as well (Davis, 1990).

D. Somatostatin (SRIF)

Somatostatin or SRIF (somatotropin release inhibitory factor) is a 14 amino acid cyclic peptide isolated and sequenced by Brazeau and coworkers in 1973. Besides inhibiting GH release, SRIF also subserves a physiologic role as a TSH-release inhibitory factor, and also has inhibitory effects on the release of PRL from the normal (Maclean and Jackson 1988). The discovery of somatostatin led to a new way of thinking in the field of neuroendocrinology, for it was the first hypothalamic hormone to be localized extensively outside that region. It has been detected in the rest of the CNS as well as in extra-neuronal locations especially the gastrointestinal (GI) tract and pancreas. Coincident with this widespread distribution is a diversity of functions, besides its inhibitory effects on pituitary and GI hormones (Reichlin, 1987). SRIF protein is first synthesized as a preprosomatostatin of 116 amino acids and is cleaved

posttranslationally to a prosomatostatin form, which is finally processed to a 28 amino acid form and the active 14 amino acid SRIF. In most neural tissue, SRIF-28 and and SRIF-14 are co-secreted. The biochemical or physiologic function(s) of the pro-SRIF fragment remain unknown (Goodman, 1983).

Somatostatinergic neurons are localized within the periventricular and anterior zones of the hypothalamus (Maclean and Jackson, 1988). Regulation of the SRIF release is under both neurotransmitter and hormonal control. Short and long feedback loops have been demonstrated in response to both GH and IGF-1. Hybridization studies to quantify steady-state SRIF mRNA levels confirm that GH exerts positive feedback on SRIF content and synthesis (Rogers, *et al* 1988). Corticotropin-releasing hormone (CRH) may stimulate SRIF release as part of its GH inhibitory effect in some species (Rivier and Vale, 1985). Studies of the effects of neurotransmitters have yielded conflicting results, perhaps because of diverse experimental models.

Somatostatin's effect on target neuronal or endocrine cells is invariably inhibitory (Maclean and Jackson, 1988). SRIF reaches the somatotropes via the portal circulation and binds to receptors which are linked to the G_i subset of G proteins, also known as the inhibitory G proteins. The activation of this G protein leads to an inhibition of AC and hence a decrease in the intracellular levels of cAMP (Murray, *et al* 1988). However, experiments have shown that the reduced cAMP formation cannot entirely account for SRIF inhibition of GH release. Rather, in somatotropes as in all other studied SRIF target tissue, binding of SRIF to its receptor inhibits stimulated increases in intracellular calcium, which in turn prevents neurotransmitter or hormone

release. Although SRIF leads to a decrease in secreted GH, there is no evidence that SRIF has any affects on GH gene transcription (Maclean and Jackson, 1988). Other hypothalamic neuropeptides, monoamines, and hormones of peripheral endocrine organs such as thyroid hormone, adrenal glucocorticoid and gonadal steroid can be demonstrated to exert effects on GH secretion, however, there is no convincing evidence that they act directlyat the level of the pituitary. They may however, exert their effects by modifying the secretion of GHRH and SRIF (Frohman, *et al* 1987).

To summarize, the hypothalamic regulatory peptides GHRH and SRIF up- and down-regulate, respectively, intracellular cAMP levels and thereby affect GH expression. This response is likely to be mediated, at least in part, via the Pit-1 binding sites, although other factors such as calcium influx and *c-fos* expression may also play important roles.

E. The Prolactin (PRL) Axis

PRL is an anterior pituitary hormone which is prevalent in all vertebrates. It is produced in and secreted from a subpopulation of pituitary cells known as somatotropes. It is a 198 amino acid protein with an apparent molecular weight of 24 kDa (Martin, 1985). However, there is now evidence that PRL exists as a heterogeneous molecule, having several isoforms of varying molecular weights (Clapp, *et al* 1988, DeVito, 1988; Greenan, *et al* 1990). The function of these PRL "variants" is the subject of much current research. Although not normally glycosylated, recent reports indicate that PRL can undergo this type of post-translational modification

(Bollengier, *et al* 1988). The biological significance of the glycosylated form of PRL is not well understood at this time. PRL was first isolated from the pituitary, but has subsequently been found in a variety of tissues such as the placenta (Gellerson, *et al* 1991), brain (Emanuele, *et al* 1986), prostate (Nagasawa, 1989), testes (Einson, 1978), and lymphocyte (Clevenger, *et al* 1991). Like GH, PRL is involved in a wide spectrum of functions including maintenance of lactation, reproduction, osmoregulation, promotion of growth, and support of metabolism (Hadley, 1988). Recently, PRL has also been implicated in regulation of the immune system (Berczi, *et al* 1981; Yu-Lee, 1990), and in transcriptional activation (Clevenger, *et al* 1991). Thus, unlike other pituitary hormones, PRL was not committed early in evolution to the control of one or few related processes, but remained diversified and adaptive in nature. In rats and humans, PRL is secreted episodically and has a half-life in the blood of about 15 to 20 minutes (Hadley, 1988). There is a nighttime surge of PRL secretion, which like GH, is associated with the onset of sleep. The times of onset and duration of the PRL peak are not identical to GH, however (Neill, 1988). Pulsatile release of PRL was thought to reflect an overlying hypothalamic control, but recent reports indicate that such pulses originate within the pituitary gland itself (Shin and Reifel,1981). These observations were made on hypophysectomized rats bearing renal grafts of anterior pituitary tissue. Pulsatile PRL release was observed even when the pituitary is physically disconnected from hypothalamic influence. Similar observations of pulsatile PRL release from primary pituitary cells *in vitro* have also been reported

(Ben-Jonathan, 1985).

F. Regulation of PRL Synthesis and Secretion

PRL gene transcription is influenced by a number of hormones including estrogen, thyroid hormone and glucocorticoids, which act through nuclear receptors, and dopamine (DA), thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide (VIP) and epidermal growth factor (EGF), which through membrane receptors coupled to a variety of second messenger systems, such as the calcium-calmodulin/protein kinase C (PKC) pathway and cAMP/protein kinase A (PKA) pathway (Davis, 1990). PRL secretion from the anterior pituitary lactotrope cells is under tonic inhibitory control by the catecholamine dopamine (DA) which is released by the tuberoinfundibular dopaminergic neurons of the hypothalamus (Ben-Jonathan, *et al* 1989). Therefore, as opposed to GH regulation, under normal physiologic conditions PRL synthesis is under negative rather than positive control. Although a number of hormones, including glucocorticoids, are inhibitory, the primary negative regulator is dopamine, acting through D2 receptors on pituitary lactotropes which are negatively linked to AC via guanine nucleotide binding proteins (Elsholtz, *et al* 1991). Thus the primary mechanism of DA action appears to be inhibition of cAMP production. *In vivo* experiments have shown that transcription of the prolactin gene is suppressed by the potent dopaminergic agonist ergocryptine (Pritchett, *et al* 1987), which implies that the decrease in cAMP levels may have effects that are mediated, at least in part at the level of transcription. Another reported prolactin inhibitory factor is

gonadotropin-releasing-hormone associated peptide (GAP) (Nikolics, *et al* 1985). This 56 amino acid peptide is the carboxy-terminal portion of the peptide precursor for gonadotropin releasing hormone (GnRH), which itself is represented by the amino terminal 10 amino acids of the precursor. The peptide is reported to be exceedingly potent at inhibiting prolactin secretion (Nikolics, *et al* 1985). However, at this point, very little is known about GAP's mechanism of action on PRL release.

One of the best characterized positive regulators of PRL is the hypothalamic tripeptide, thyrotropin-releasing hormone (TRH). Early studies with the GH lines of rat pituitary cells showed that TRH is an activator of secretion of PRL and also increases PRL mRNA levels in these cells (Tashjian, *et al* 1990; Dannies and Tashjian, 1976). Subsequent studies showed that the stimulation by TRH of PRL gene expression is exerted at the level of transcription (Murdoch, *et al* 1983). Extensive investigations have examined the characteristics of the TRH receptor and have produced compelling evidence that calcium and phosphoinositides act as second messengers in its action, and that Pit-1 is most likely the final acceptor of the signal (Gershengorn, 1982; Martin, 1985).

The PRL gene is composed of five exons separated by four introns. The gene contains larger introns than GH, spans 2.1 kb in length, and produces a mature mRNA of approximately 900 bp (Chien and Thompson, 1980). The activity of the PRL gene is influenced by two promoter regions: a distal enhancer region (-1713 to -1495 bp), and the proximal promoter region containing the first approximately 200 bp of the promoter (Lufkin and Bancroft, 1987; Day and Maurer, 1989). The distal enhancer

region contains elements conferring responsiveness to TRH, cAMP, EGF, and contains an estrogen response element (ERE), while the proximal promoter region shows responsiveness to calcium, TRH, EGF, and phorbol esters (Day and Maurer, 1989; Keech and Gutierrez-Hartmann 1989). Positive transcriptional regulation of the prolactin gene by polypeptide hormones including TRH is conferred by short promoter sequences that contain binding sites for the pituitary-specific transactivating factor Pit-1/GHF-1 (described in detail above) (Davis, 1990). Pit-1 interacts with four binding sites within the distal enhancer and four sites within the proximal enhancer (Nelson, *et al* 1986) in response to phosphorylation of the protein.

G. Similarities Between GH and PRL

GH, PRL, chorionic somatomammotropin or placental lactogen (CS), and proliferin all belong to a family of related hormones that regulate a number of diverse and essential physiologic processes. It is now widely accepted that these hormones arose approximately 350 million years ago by a duplication event from a common ancestral gene (Miller and Eberhardt, 1983). Today, the GH and PRL genes are located on different chromosomes (Owerbach, *et al* 1980) and are regulated in a cell-specific manner. GH and PRL are related by structure (Catt, *et al* 1967), immunologic reactivity, and overlapping biologic activities (Niall, *et al* 1973). Indeed, GH has lactogenic effects and PRL has growth-promoting effects (Murray, *et al* 1988). Both are important in intermediary metabolism and both act as modulators of the immune

system (Berczi, *et al* 1991). GH and PRL range in size from 190 to 199 amino acids in different species. Each has a single tryptophan residue and each has 2 homologous disulfide bonds. The amino acid homology between GH andPRL is 35%. At the gene level, the coding sequences of GH andPRL are each organized into 5 exons interrupted by 4 introns. The genes are highly homologous in the 5′ flanking regions and the coding sequence areas and diverge in the 3′ flanking regions (Theill and Karin 1993). There is an overlap in populations of transcriptional regulators binding to the GH and PRL gene promoters (Pit-1, for example). The splice junctions are highly conserved, even though the introns in the PRL gene are much longer (Murray, *et al* 1988). It was because of the structural and functional similarities between these two pituitary hormones that the scope of this study was broadened to include the effects of acute EtOH on PRL as well as GH gene expression.

H. Pharmacology of Ethanol

Ethanol (ethyl alcohol or EtOH) is rapidly absorbed from the gastrointestinal tract or the intraperitoneal space into the bloodstream and the general circulation. It becomes widely distributed throughout the body according to the water content of tissue, easily penetrating the both the blood-brain and placental barriers (Leonard, 1992). The liver is the primary organ involved with the oxidation of alcohol. Alcohol dehydrogenase (ADH) initiates the first step in the oxidation process by converting alcohol to acetaldehyde and hydrogen ions. Acetaldehyde is then rapidly oxidized to carbon dioxide and water by aldehyde dehydrogenase. More than 90% of the drug is

oxidized in the liver, while the remainder is excreted unchanged through the lungs, skin and kidneys (Goldstein, *et al* 1983). Damage to tissues and organs, especially in the liver, occurs when heavy alcohol consumption results in excess protons produced by ADH leading to increased conversion of pyruvate to lactate, and the synthesis of saturated fatty acids, which accumulate in the liver, impairing normal function. An additional consequence of the excess protons is hypoglycemia, a condition which is common amongst alcoholics (Goldstein, *et al* 1983).

I. Effects of Ethanol on Growth Hormone

The effects of EtOH on GH have been studied previously and the some of the deleteriou effects alcohol exerts on this axis have been documented. Several *in vivo* studies in animals (Redmond, *et al* 1980; Dees *et al*, 1988; Maureci and Conway, 1991) and humans (Valimaki, *et al* 1987; Valimaki *et al*, 1990; Aliev, 1991) have shown that EtOH decreases serum GH after both acute and chronic administration. The site of the acute effects of ethanol has not been precisely determined, although there is now some evidence that suggests an alteration of hypothalamic function (Dees, *et al* 1988; Dees, *et al* 1990; Conway and Maureci, 1991). This is similar to findings in other endocrine axes such the LHRH-LH axis (Cicero, *et al* 1982) and the CRH-ACTH axis (Rivier, *et al* 1990) in which a hypothalamic effect of ethanol was noted. Conway and Maureci (1991) used the adrenergic α-2 receptor agonist clonidine, which stimulates the GH system centrally (at the level of GHRH expression), in conjunction

with acute EtOH exposure in male rats, and showed that EtOH could block the clonidine-induced rise in GH, leading them to conclude that this was the site of ethanol's action. Redmond (1980) showed that in addition to a suppressive effect on serum GH, acute EtOH at high doses (3 or 4 g/kg) also suppressed GH pulse amplitude. Since GH pulse generation has both a hypothalamic and pituitary component, this implies a dual locus of EtOH action. Additionally, it has been shown that doses of 100-300 mg% EtOH added to primary pituitary cells *in vitro* also has the capability of blunting GH secretion, another piece of evidence suggesting that the pituitary is also a potential site of action (Emanuele *et al* 1989). Soszyinski and Frohman (1992a) followed up the *in vitro* studies of Emanuele *et al* (1989), by examining the effects of EtOH on signal transduction mechanisms. While differences in results were noted, this group concluded that disruptions in cAMP-dependent pathways were responsible for diminished GH release. Finally, there is evidence for effects of EtOH at the liver since Sonntag and Boyd (1989) found that ethanol could diminish levels of IGF-1 in the plasma of male rats. This result however, was only seen after long term EtOH exposure (6 weeks). They attributed this effect, in part, to EtOH-induced alterations in GH pulsatility, which is essential for maximal response of IGF-1 producing liver cells to GH.

J. Effects of Ethanol on Prolactin

To date there are few studies examining the effects of prolactin in either humans or animals. In these limited reports, however, the consensus is that EtOH results in

significantly elevated serum PRL levels in both humans (Ylikhari, *et al* 1978; Ellingboe, *et al* 1980; Bertello, *et al* 1983; Phipps, *et al* 1987; Soyka, *et al* 1991) and rats (Chapin, *et al* 1980, Salonen and Huhtaniemi 1990; Seilcovich, *et al* 1982; Emanuele, *et al* 1987; Dees, *et al* 1984). The exact mechanism(s) of this EtOH-induced hyperprolactinemia is unclear, but several explanations have been proposed. At the hypothalamic level, increased hypothalamic β-endorphin (Schulz, *et al* 1980), and reduced dopaminergic inhibition (Seilcovich, *et al* 1985) have been noted. Also, substance P, which has been found to be decreased in the mediobasal hypothalamus and increased in the pituitary with alcohol, has been postulated to account for the rise in prolactin (Seilicovich, *et al* 1990). *In vitro* exposure of dispersed normal male rat pituitary cells to alcohol has consistently effected a rise in prolactin secretion (Seilicovich, *et al* 1984; Emanuele, *et al* 1987). This rise indicates that at least part of alcohol's effects on PRL is a direct pituitary effect. Nonetheless, this does not rule out an additional hypothalamic locus of action. Seilcovich, *et al* (1988) found that the enhancement of PRL release by ethanol from the pituitary *in vitro* is a calcium dependent process since omission of Ca^{++} from the medium results in a complete cancellation of the ethanol effect. Finally, Sato *et al* (1990) proposed that the elevated PRL may be due to ethanol-induced cell swelling, which subsequently led to a calcium influx, a known stimulator of PRL release.

K. Alcohol, Biological Membranes, and Second Messenger Systems

Meyer in 1901 was the first to suggest that alcohol had effects similar to general anesthetics by dissolving into cell membranes and disrupting the lipid network that comprises the cell wall (Hunt, 1985). It is now known that, at pharmacologically relevant concentrations of 25-100 mM, an acute dose of EtOH increases the fluidity of cell membranes following its acute administration, and that these changes correspond to the sedative effects of the drug (Tabakoff, *et al* 1988). This suggests that EtOH produces its effects in a relatively non-specific manner, but it is now known that biological membranes are structurally and functionally heterogeneous and that certain regions of the membrane are more sensitive to the disordering effects of EtOH than others (Tabakoff, *et al* 1988). Thus, EtOH may affect calcium flux across a membrane, or disrupt the intracellular phosphatidylinositol system, which in turn, may affect the intracellular availability of calcium. This could have a serious effects on hormone release. Thus, while it is generally believed that EtOH does not produce its effects via a specific "alcohol receptor", some lipids do show a particular vulnerability to the disorganizing effects of the drug (Chin and Goldstein, 1981). With regard to its effect on hormone release, EtOH increases AC activity, and hence, cAMP accumulation in a wide variety of cells, possibly via the membrane-bound G protein complex (Tabakoff, *et al* 1988). cAMP increases protein kinase A activity which leads to phosphorylation of various soluble and membrane-bound proteins and results in the physiologic effects of hormones and neurotransmitters (Alberts, 1989). The effect of EtOH on this second messenger system appears to depend on its location. An

example of this is the noradrenaline-linked cyclase in the cerebral cortex of the brain. This system seems to be directly affected by the drug, whereas the dopamine-linked enzyme in the basal ganglia region of the brain appears to be altered by a combination of changes in the membrane fluidity together with those in the G protein-cyclase complex (Hunt 1985). While EtOH administration is associated with enhanced membrane fluidity due to the disordering effects of the drug, after chronic administration the membranes become more rigid due to an increased replacement of the unsaturated by saturated fatty acids (Hunt, 1985).

In conclusion, GH and PRL are essential pituitary hormones with diversephysiological roles. As such they are under highly complex regulation which functions at numerous levels within their respective axes. Alcohol disturbs this homeostasis, and most evidence indicates that EtOH acts in a specific, not global fashion, and at multiple loci.

CHAPTER III

MATERIALS AND METHODS

A. Animals

All animals used in this study were adult male Sprague-Dawley rats, aged 60-90 days, obtained from Harlan Laboratories, Indianapolis, IN. Rats were received approximately 4 days before experiments began to acclimate to surroundings so as to minimize the effects of stress. The animals were housed in individual cages with 12 hour light: 12 hour dark regime at 22-24° C. They were given food and water *ad libitum*.

B. Acute Ethanol (EtOH) Administration

Animals were treated at the same time of the day (0900) in order to minimize the effects of ultradian rhythms in hormone levels. Animals were given a single intraperitoneal (i.p.) injection of either sterile saline (0.9%) or EtOH (30% v/v in sterile saline) at a dose of 1 ml per 80 g body weight (3g ethanol/kg). Control animals were handled identically and received an injection of saline at a dose of 1 ml per 80 g body weight. All rats were then immediately returned to their home cages in a quiet room and given free access to food and water until time of sacrifice at 1.5, 3, 6, or 24 hours after injection. Animals involved in the repeat ethanol exposure, or "double

binge" experiments were treated as above, then given a second injection of 3g/kg EtOH or saline 24 hours after the first injection, and subsequently sacrificed at various time points post-injection.

C. Tissue Collection

Animals were sacrificed by decapitation, calvaria removed, and the brain was rapidly removed and dissected on ice. The hypothalamic dissection was bounded by the optic chiasm anteriorly and the mammillary bodies posteriorly. Dissection laterally followed the groove which separates the hypothalamus from the olfactory tubercle rostrally, the hypothalamus from the amygdala at mid-levels, and the hypothalamus from the optic tract caudally. Each hypothalamus was 3 mm thick, encompassing tissue to the dorsal edge of the vertical portion of the third ventricle. The hypothalami were placed in an autoclaved microcentrifuge tube and stored at -80°C.

Pituitary glands were carefully removed from the sella turcica and placed on a glass plate on ice. The anterior pituitary was then gently separated from the posterior pituitary with a blunt forceps. The posterior pitutiary was discarded. The anterior pituitaries were immediately placed in an autoclaved microcentrifuge tube and frozen on a dry ice/methanol bath (-80° C). The tissues were stored at -80° C until assayed.

D. Blood Ethanol Determination

At the time of sacrifice, trunk blood was collected into borosilicate glass test tubes and kept on ice until centrifugation. Serum was obtained by centrifuging the

trunk blood for 15 minutes at 2000 x g. Blood EtOH levels were determined using a commercially available enzymatic kit (Sigma, St Louis, MO. cat no. 330-1). The kit is based on the reduction of nicotinamide adenine dinucleotide (NAD) to NADH by alcohol dehydrogenase in the presence of EtOH which results in an increase in absorbance at 340 nm.

Three milliliters of glycine buffer (0.5 mol/L, pH 9.0) was added to each NAD-ADH assay vial. The vial was then capped and mixed by inversion. Once the vial reached room temperature, 10 μl of serum was added, mixed by inversion, and incubated at room temperature for 10 minutes. The sample was transferred to a cuvette and covered with parafilm. Absorbance was read at 340 nm on a Spectronic 21D spectrophotometer (Milton Roy, Rochester, N.Y.). Blood ethanol concentration was calculated from a standard curve.

E. GH Radioimmunoassay (RIA)

The GH RIA was conducted using materials contributed by the National Institute of Diabetes, Digestive, and Kidney disorders (NIDDK) and the National Hormone and Pituitary Program through Dr. A.F. Parlow. Each assay tube contained 100 μl of either guinea pig anti-rabbit antibody (initial dilution 1:10,000) or buffer (PBS 0.01 M, 1% BSA, 1% normal guinea pig serum, pH 7.4) for detection of nonspecific binding, and 100 μl of standard or unknown. GH was iodinated using the chloramine T method. Samples were incubated at room temperature for 72 hours. Goat anti-guinea pig second antibody, (100 μl) was then added to each tube and

incubated for 4 hours at room temperature. Then 1 ml of normal saline (0.9%) was added to each tube, centrifuged at 2,500 rpm for 30 minutes, decanted, and the pellets were counted on a Packard gamma counter. The intraassay coefficient was 4% while the interassay coefficient was 8%.

F. PRL Radioimmunoassay (RIA)

The PRL RIA was conducted using materials supplied by the National Hormone and Pituitary Program and by Dr. A.F. Parlow. Each assay tube contained 100 μl of either rabbit anti-rat antibody (initial dilution 1:2,500) or buffer (PBS 0.01 M, 1% BSA, 3% normal rabbit serum, pH 7.4) for detection of nonspecific binding, and 100 μl of standard (NIADDK rat prolactin RP-3) or unknown. Prolactin was iodinated using the chloramine T method. I^{125} prolactin, (100 μl) containing approximately 10,000 cpm/tube, was added to all assay tubes and these were then incubated at room temperature for 72 hours. Thereafter, 100 μl of goat anti-rabbit IgG, diluted 1:20 (Pel-Freeze Biologicals), was added and samples were incubated at room temperature for 4 hours. Then 1 ml of normal saline was added, after which the tubes were centrifuged at 2,000 rpm at 4° C for 30 min., decanted, and pellets counted in a Packard gamma counter. Assay sensitivity was 156 pg/ml, or 15.6 pg/ tube. The interassay coefficient of variation was 23%. The intraassay coefficient of variation was 3.8%.

G. GHRH Radioimmunoassay (RIA)

GHRH RIA was performed using an antibody donated by Dr. W.B.

Wehrenberg (University of Wisconsin, Milwaukee). Each assay tube contained 200 μl of either rabbit anti-rat GHRH (1:40,000 working dilution) or buffer (0.01 M PBS, 0.05 M EDTA, 1% normal rabbit serum), 200 μl of standard (synthetic GHRH, Sigma, St Louis,MO), or unknown, and 100 μl of I^{125} GHRH (10,000 cpm/tube). The GHRH was iodinated by the chloramine T method. The mixture was incubated at 4°C for 48 hours. On day three, 200 μl of goat anti-rabbit IgG (1:10 working dilution) was added to each tube. On day 4, 300μl of 20% polyethylene glycol (PEG, M.W. 8000, Sigma) was added to each tube, vortexed and centrifuged at 2000 x g at 4°C for 30 min. The supernatant was aspirated and the pellets counted on a Packard gamma counter for 1 min. each. Assay sensitivity was 156 pg/ml. Interassay coefficient of variation was 8% and intraassay coefficient of variation was 12%.

H. cAMP Radioimmunoassay (RIA)

Intracellular pituitary cAMP levels were measured using a commercially available RIA kit (Amersham, Arlington Heights, IL, #TRK 432), based on competition with tritium-labelled cAMP. Individual pituitaries were homogenized in 200 μl ice-cold 10 mM PBS, with 4mM EDTA added as a phosphodiesterase inhibitor. Samples were deproteinized by boiling for 3 min. and then centrifuged at 12,000 rpm for 10 min. The supernatant was removed to a fresh tube to be assayed. All samples were measured in triplicate. Radioactivity was quantified by liquid scintillation counting, and cAMP levels were calculated from a standard curve.

I. Tissue RNA Extraction

Total cellular RNA was isolated from pituitaries and hypothalami using a modification of a previously published procedure (Chomczynski and Sacchi, 1987). Tissue was collected as described above and stored at -80°C until needed. Pituitaries or hypothalami were homogenized in 500 μl of 4 M guanidium isothiocyanate (GIT) buffer (4M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl and 0.1 M 2-mercaptoethanol). The homogenate was extracted with 50 μl 2 M sodium acetate, pH 4.0, 500 μl phenol, and 100 μl chloroform:isoamyl alcohol 49:1, mixed, and incubated ice for 15 minutes. The sample was centrifuged at 10,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and the nucleic acid precipitated by the addition of an equal volume of isopropanol and maintained on dry ice for 30 minutes. The nucleic acid was pelleted by centrifugation at 10,000 x g for 15 minutes at 4 °C. The isopropanol was aspirated and the resulting pellet resuspended in 100 μl of GIT. The pellet was fully dissolved by heating to 65°C with mixing. RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 5.2, and an equal volume of isopropanol, and by incubating on dry ice for 15 minutes. The nucleic acid was pelleted. The pellet was washed with 500 μl 70% EtOH, dried at 65°C for 2 minutes, and resuspended in 200 μl of diethylpyrocarbonate (DEPC, 0.2%) treated water. The pellet was once more dissolved by heating to 65°C. The final RNA sample was stored at -80°C until needed. To determine the concentration of RNA in each sample, absorbance at 260 nm was measured using 4 μl of RNA in 1 ml of deionized, distilled water (dd H_2O).

J. Northern Blot Analysis

Total RNA (5-10 μg) was precipitated with 0.1 volume 3 M sodium acetate and 2.5 volumes of 100% EtOH on dry ice for 15 minutes. RNA was pelleted by centrifugation at 10,000 x g at 4°C for 15 minutes, the supernatant aspirated and the nucleic acid washed with 70% EtOH. The resulting pellet was dried at 65°C until all residual ethanol was evaporated. The RNA was resuspended in 20 μl of RNA loading dye (50% formamide, 1X MOPS, 15% formaldehyde, 10% glycerol) and 1 μl of ethidium bromide (1mg/ml) by heating at 65°C for 15 minutes with intermittent mixing. After incubation at 65°C, the sample was loaded onto an agarose-formaldehyde gel and electrophoresed. Total RNA was electrophoresed through agarose-formaldehyde gels. RNA electrophoresis was performed at 80 V in 1X MOPS buffer. Gels were prepared with 1X MOPS buffer (0.2M MOPS, pH 7.0; 50mM sodium acetate, 10 mM EDTA), 1.1% ultra pure agarose, and 5% formaldehyde in a fume hood. The formaldehyde was added just prior to pouring the gel.

The gel was prepared for Northern blotting by soaking in 10X SSC at room temperature for two 20 minute periods, with gentle shaking in order to remove formaldehyde. During the gel washing period, a nylon reinforced nitrocellulose membrane (Hybond M; Amersham, Arlington Hts., IL) was wetted in ddH_2O for 5 minutes, followed by a 20 minute soak in 10X SSC. The RNA was then transferred to the membrane by capillary action for 12-16 hours. RNA was subsequently cross-linked to the membrane using a UV crosslinker (Stratalinker, Stratagene, La Jolla CA), and stored at -20°C.

The membranes were placed in siliconized glass hybridization tubes and pre-hybridized for 1-6 hours at 42°C in a rotating hybridization incubator (Robbins Scientific, Sunnyvale, CA) The hybridization solution contained 50% formamide, 5X Denhardt's solution (1X Denhardt's solution contains 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (BSA), and 0.02% Ficoll 400), 0.01% sodium dodecyl sulfate (SDS), 10 mM NaCl, 0.1 mM $NaPO_4$ (pH 6.5), 0.001% pyrophosphate, and 0.025 mg/ml salmon sperm DNA (heat-denatured). Labelled cDNA probes (see below) were added to the blots with fresh hybridization solution, and allowed to hybridize 12-16 hours at 42°C. After hybridization, the membranes were washed three times at high stringency (0.2X SSC, 0.5% SDS) with rotation at 65°C, 20 minutes per wash, in order to eliminate background signals. The membranes were then lightly blotted on absorbent paper, wrapped in cellophane and exposed to x-ray film (Hyperfilm M-P, Amersham) at -80°C. The autoradiographs were subsequently developed using an x-ray film developer.

The membranes were capable of being stripped several times and reprobed with different cDNAs without appreciable loss of RNA. This enabled multiple hormone analyses from the same group of animals in a particular experiment. The strip solution was prepared with 0.01% SSPE (1X SSPE contains 150 mM sodium chloride, 10 mM sodium phosphate and 1mM EDTA), and 0.25% SDS. Strip solution was heated to boiling, poured directly onto blots, and incubated at 65°C with shaking for 20 minutes. After three rounds of stripping, the blots were monitored with a geiger counter and then exposed to x-ray film to verify that the radioactive probe had been removed.

K. Labeling of cDNA Probes

Double-stranded cDNA molecules were used as hybridization probes for the Northern blots. The probes were obtained from the following sources:

1) GH cDNA - Dr. John D. Baxter, University of California, San Francisco.

2)PRL cDNA - Dr. Richard Maurer, University of Iowa, Iowa City

3) Pit-1 cDNA - Dr. Holly A. Ingraham (Dr. Michael G. Rosenfeld's laboratory) University of California, San Diego.

4) *c-fos* cDNA - Dr. Tom Curran, Roche Institute of Molecular Biology, Nutley, NJ.

5) 28s rRNA - Dr. Sullivan Reed, University of Missouri, Kansas City.

For probing Northern blots, the cDNA was labelled to high specific activity with (alpha-^{32}P) dCTP (New England Nuclear, Boston, MA) by the random hexamer primer method (Feinberg and Vogelstein, 1983). Approximately 25 ng of template cDNA was brought to a final volume of 30 μl with ddH_2O in a microcentrifuge tube and denatured by boiling for 3 minutes, followed by quick chilling on ice for 5 minutes. The labelling reaction was prepared by adding 10 μl of 5X labelling buffer (250 mM Tris, pH 8.0, 25 mM $mgCl_2$, 10 mM DTT, 1 mM HEPES, pH 6.6, 27 A_{260} U/ml of hexanucleotide primer), 2 μl 1.5 mM dNTPs (dATP, dGTP, dTTP, final concentration 20 μM each), 2 μl acetylated BSA (1 mg/ml, final concentration of 400 μg/ ml), 5 μl alpha-^{32}P dCTP (50 μCi, 3000 Ci/mmole), and 1 μl of DNA polymerase, Klenow fragment (final concentration of 20 mM) in a final volume of 50 μl to the

denatured template (500 ng/ml). The labelling reaction was gently mixed and incubated at room temperature for 45 minutes. To terminate the reaction, 2 μl of 0.5 M EDTA, pH 8.0 (final concentration, 20 mM) and 48 μl of sterile water was added to the tube and was heated to 100°C for 3 minutes, followed by a quick chill on ice for 5 minutes. Removal of unincorporated labelled dCTP was carried out by gel filtration using Sephadex G-50 (Pharmacia, Uppsala, Sweden) spun columns. Sephadex G-50 was prepared in a 20 mM NaOH; 1 mM EDTA solution, autoclaved and stored at room temperature. Spun columns were made from 1 cc tuberculin syringe shafts. The Sephadex was packed by centrifugation at 1000 x g for 5 minutes. The denatured, labelled cDNA probe was then layered on top of the column, and again spun for 5 minutes at 1000 x g. Percent radiolabelled incorporation was determined by comparing the amount of radioactivity left in the column (unincorporated into DNA) to the radioactivity in the column flow-through. The purified probe was then added to hybridization tubes containing fresh hybridization solution, and the pre-hybridized Northern blot.

Densitometric Analysis

Autoradiographs were quantitated using a scanning densitometer (Technology Resources, Nashville TN). A two-dimensional gel analysis program was used to quantify the area under the curve for each band scanned. The area was used for optical density (OD) values, or arbitrary densitometer units (ADU). After analysis for GH,PRL, and Pit-1, the membranes were reprobed with the cDNA for the 28S rRNA

gene or the ribosomal protein gene PO to control for loading differences. Blots were normalized using these loading controls by calculating the ratio of the ADU values for 28S for a particular sample lane to the lane with the greatest ADU for 28S. These values were then used as correction factors by which the hormone mRNA values were multiplied in order to account for differences due to unequal loading of the RNA gel.

M. Reverse Transcription

Three micrograms of total RNA was added to each reaction tube containing 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM $MgCl_2$, 10 mM DTT, 2 mM of each deoxynucleotide triphosphate (dNTP), 10 U RNasin (RNase inhibitor, Promega, Madison, WI), 100 pmol oligo-dT primers (Bethesda Research Laboratories (BRL), Gaithersburg, MD), and RNase-free dd H_2O to a final volume of 19 μl. This mixture was heated for 10 minutes at 65°C, then quenched on ice. Moloney murine leukemia virus RNase H- reverse transcriptase (200 U, Superscript, BRL) in 1 μl were added for a total reaction volume of 20 μl and incubated at room temperature for 10 minutes and then at 42°C for 1 hour. The reaction was terminated by heating at 95°C for 5 minutes and quenching on ice. RNAse-H (2U) was then added to the reaction and incubated for 20 min at 37°C.

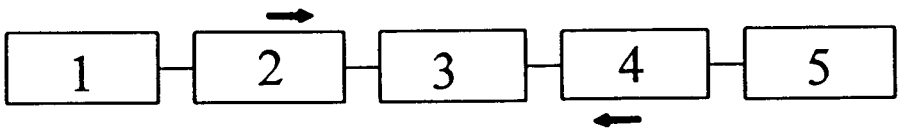
N. PCR Amplification

PCR Primers:

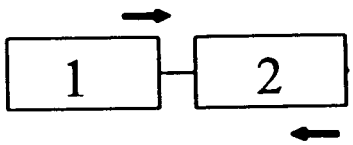
H3.3 primers	5′: 5′-GCAAGAGTGCGCCCTCTACTG-3′
	3′: 5′-GGCCTCACTTGCCTCCTGCAA-3′
GHRH primers	5′: 5′-TGCCCCCCCTCACCTCCCTTC-3′
	3′: 5′-GGCGGTTGAACCTGGATCTT-3′
SRIF primers	5′: 5′-GGGGATCCATGCGCCCTCGGACCCC-3′
	3′: 5′-GGGAATTCACAGGATGTGAATGT-3′

The oligonucleotide primers were designed to span at least one intron in order to detect DNA contamination of RNA preparations. The locations of the primers chosen for amplification are shown diagramatically in figure 2. The expected PCR products using these primers were 213 bp for H3.3, 165 bp for GHRH, and 276 bp for SRIF. Pre- and post-PCR samples were handled in different labs, using separate pipettors and aerosol barrier tips. Five μl of reverse transcription reaction from each sample were diluted into a final volume of 100 μl in 10 mM Tris-HCL (pH 8.3); 50 mM KCL; 1.5 mM $MgCl_2$, 200 μM each of deoxynucleotide triphosphate, 0.01% gelatin, 0.01%Tween-30 and 0.01% Nonidet P-40; 2 μM of each oligonucleotide; P^{32}-dCTP (2 μCi at 3000 Ci/mmol) and 2 U Taq polymerase (BRL). The DNA amplification was carried out using an Eppendorf MicroCycler (Fremont, CA) programmable heating/cooling dry block for 22 cycles of amplification. Each cycle was: 94°C for 30 sec. (template denaturation); 60°C for 1 min. (primer annealing); 72°C for 2 min.(primer elongation).

GHRH:



SRIF:



H 3.3:

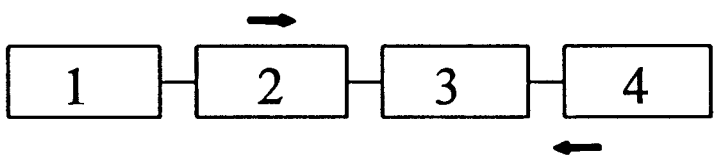


Figure 2. Location of oligonucleotide primers used for PCR amplification. Numbered boxes represent exons, the lines in between the boxes represent introns. Left arrow indicates approximate location of 5′ primer, and the right arrow indicates approximate location of the 3′ primer.

This was followed by one extended elongation step of 10 min. at 72°C. These conditions were established using control DNA substrates. A reaction was performed as described above, but without the addition of reverse transcriptase as a test for the presence of contaminatinng genomic DNA in the RNA from the tissues. As a further control, oligonucleotide primers alone without any RNA were used. Ten μl of the sample was electrophoresed on a 1.5% agarose gel, transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) and exposed to autoradiographic film and analyzed using a scanning densitometer. The GHRH and SRIF signals were normalized to H3.3 to control for sample to sample variation.

N. Nuclear Run-On Transcription Assay

The following nuclear run-on procedure was modified for this study from two previously published methodologies (Kafatos, *et al* 1979; Danerji, *et al* 1984). Binding of plasmids to nitrocellulose was performed as follows: Plasmids containing the target and control cDNAs were linearized by digestion with a restriction enzyme which cuts the plasmid once and outside of the inserted cDNA. The linearized DNA was then denatured in ice cold 10 N NaOH for 30 min. An excess of each plasmid cDNA (20 μg in 500 μl) was spotted onto a nitrocellulose filter using a slot blot apparatus (Minifold II, Schleicher and Schuell, Keene, NH). The slots were rinsed with 500 μl 6X SSC. The filters were baked for 1 hour at 80°C under vacuum, then stored dessicated at -20°C until needed. The run-on labelling of nascent RNAs was carried out as follows (all procedures were carried out on ice unless otherwise indicated):

Three pituitaries from each experimental group were pooled and homogenized using a dounce homogenizer in cell lysis buffer (0.25 M sucrose, 55 mM HEPES pH 7.4, 5 mM $MgCl_2$ and 0.1% Triton X-100. The nuclei were isolated by layering the homogenate over a 0.5 M sucrose cushion and centrifuging at 6,000 rpm for 10 minutes. The pelleted nuclei were resuspended in a synthesis buffer containing 200 μCi ^{32}P-UTP (Amersham, Arlington Heights, IL), 0.5 mM each rGTP, rATP, rCTP, 17% glycerol, 60 mM HEPES pH 7.0, 2 mM DTT, 25 mM ammonium sulfate, 3mM magnesium acetate, 3 mM $MnCl_2$, and 5 mM sodium fluoride. After incubation at 37° C for 30 minutes, the reaction was stopped by adding 600 μl of stop buffer (2% SDS, 7 M urea, 350 mM LiCl, 1 mM EDTA, 10 mM Tris, pH 8.0), 80 μl of 10 mg/ml proteinase K, and 10 μl of 10 mg/ml tRNA and incubating at 42° C for 1 hour. The newly synthesized, labeled RNA was precipitated by addition of 100% TCA, chilling on ice for 20 mins., and centrifuging at 12,000 rpm for 15 minutes. The labeled RNA pellet was then carefully resuspended in 100 μl TE with 0.5% SDS using a pipettor fitted with a wide bore tip. 1 ml of prehybridization solution (50% formamide, 6X SSC, 10X Denhardt's solution, 0.2% SDS) was added to the pellet suspension, gently mixed, and added to a tube containing a nitrocellulose blot of cDNA plasmids which was pre-hybridized for 3 hours at 42°C. Blots were hybridized for 72 hours in sealed tubes at 42°C. After hybridization, the filters were washed with progressively increasing stringency (6X SSC, 0.2% SDS for 20 minutes; 2X SSC, 0.2% SDS for 20 minutes; 0.2% SSC, 0.2% SDS for 20 minutes) all at 65°C. The filters were dried briefly, wrapped in cellophane and exposed to X-ray film (Hyperfilm M-P, Amersham,

Arlington Hts., IL).

To confirm that an equal number of nuclei were collected from saline-injected control and EtOH-treated pituitaries, a 10 μl aliquot of the nuclei suspension was removed and counted manually in quadruplicate using a standard hemocytometer (Hausser Scientific, USA), on a light microscope (Nikon Model TMS-F, Japan). As an additional control, a second aliquot was taken from the labeled nuclei, placed in a scintillation vial containing 5 mls of scintillation cocktail, and counted on a beta counter (Beckman LS 7500). No significant differences between control and EtOH nuclei preparations were found using these procedures.

O. Statistics

Data was calculated as mean $\pm$ standard error of the mean. One-way analysis of variance (ANOVA) with a Bonferroni t-test follow-up was performed as a statistical test for differences between control and ethanol-treated animals on the RIA data and mRNA and semi-quantitative PCR data. When data was expressed as percent of control, statistical analysis was performed on the raw data, which was then converted to percent of control values. Significant differences were reported for $p < 0.05$.

CHAPTER IV

RESULTS

A. "Binge" Ethanol Animal Model

Adult male Sprague Dawley rats were used for these studies. EtOH was administered via a single intraperitoneal injection for the single binge experiments, or two injections, on consecutive days for the double binge experiments as described in the materials and methods chapter. This model allowed each animal to represent an n of 1, since it was possible to measure serum levels of GH andPRL, pituitary GH andPRL content, cAMP content, as well as perform the various molecular analyses from individual animals. The exception to this was the nuclear run-on assays which, due to assay sensitivity, required pooling three pituitaries together for an n of 1. In the earlier single binge experiments, the time points used were 0.5, 1.5, 3.0, and 24 hours. When experiments were expanded to the double binge model, a 6 hour time point was added to better follow trends in the time course during the span between 3 and 24 hours. The time points of 1.5, 3.0, and 24 hours remained constant throughout.

B. Blood Ethanol Concentrations for Single and Double Binge Models

Blood ethanol (EtOH) concentrations were measured on trunk blood collected at

time of sacrifice, using a commercial enzymatic assay kit (see materials and methods for details). Typical blood EtOH concentrations at selected time points after a single i.p. injection of EtOH (3g/kg), (single binge) are shown in Figure 3. Each value represents the mean ± standard error of the mean (SEM) of 5-6 animals. Blood EtOH levels averaged 88 mM 30 minutes after administration, 62 mM at 90 minutes, 56 mM at 180 minutes, 43 mM at 6 hours and undetectable (< 1 mM) at 24 hours. Figure 4 shows blood EtOH concentrations after repeated (double binge) EtOH exposure, the levels at 30 minutes averaged 91 mM, 68 mM at 90 minutes, 62 mM at 180 min, and 50 mM at 6 hours. Again, at 24 hours there was no detectable EtOH present, nor was there any EtOH noted in the saline treated animals at any time point (data not shown).

C. Effects of Single Binge EtOH on GH Protein and mRNA levels

Serum GH levels as measured by radioimmunoassay (RIA) are shown in Figure 5. Large variations in serum GH levels were measured in the control, saline-injected animals and this probably reflects the normal pulsatility in GH secretion noted by others (Tannenbaum *et al* 1979; Jansson, *et al* 1985; Bercu *et al* 1991). Because of this high degree of variability, and for the purpose of clarity, the results in this and other figures in this thesis were expressed as percent of control. Values were calculated as means ± SEM, then converted to percent of control and statistical analyses were then peformed. Despite this highly variable baseline, serum GH levels were significantly depressed at 0.5, 1.5 and 3 hrs. in the single binge EtOH-treated animals compared to values in saline-injected controls. At 0.5 hr. after injection, the levels of serum EtOH

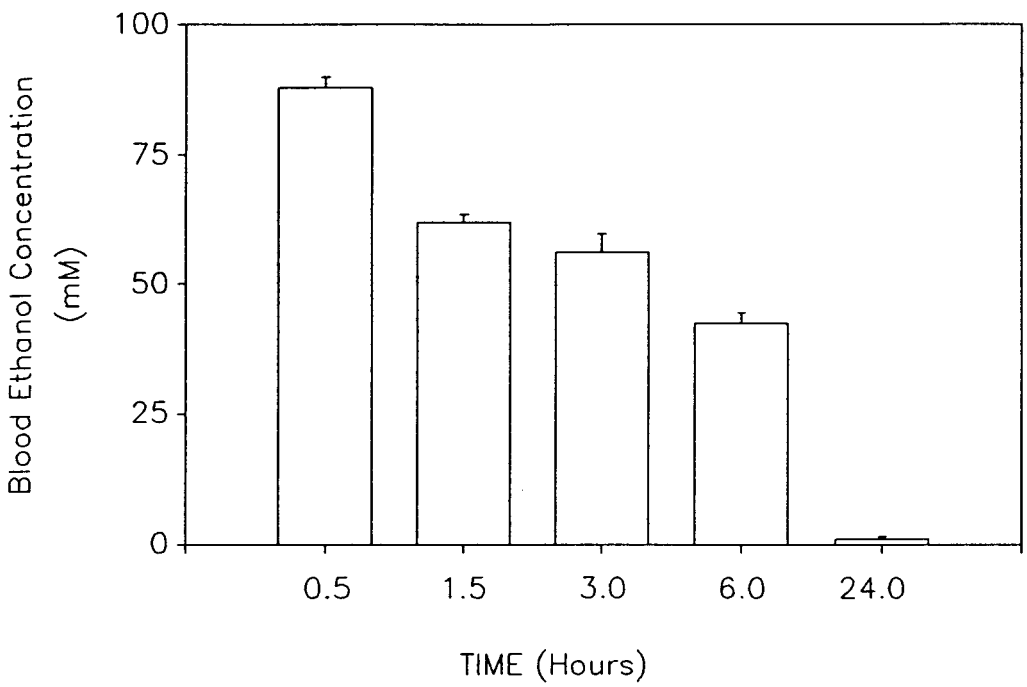


Figure 3. Blood ethanol concentrations after single binge treatment. Serum ethanol concentrations were determined from trunk blood at time of sacrifice using a commercial kit (see Materials and Methods). Animals were treated with a single intraperitoneal (i.p.) injection of 3g/kg EtOH Each value represents mean ethanol concentration $\pm$ SEM of 6-12 animals at each time point.

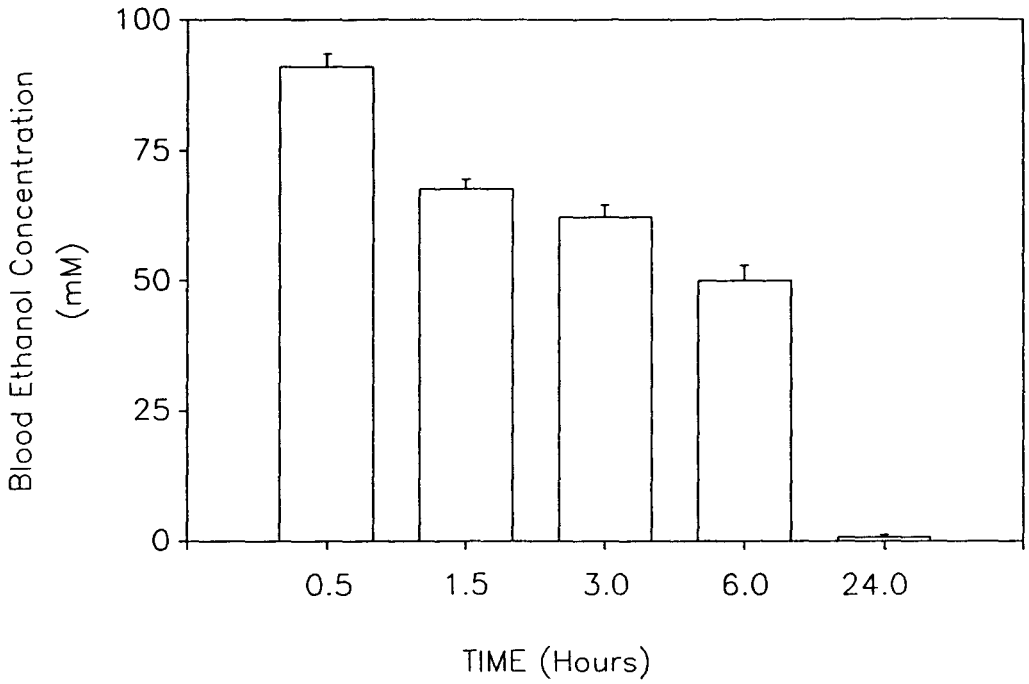


Figure 4. Blood ethanol concentrations after double binge treatment. Serum ethanol concentrations were determined on trunk blood at time of sacrifice using a commercial kit. Blood was taken from animals given two i.p. injections of 3g/kg EtOH on consecutive days. Each value represents mean ethanol concentration ± SEM of 6-12 animals at each time point.

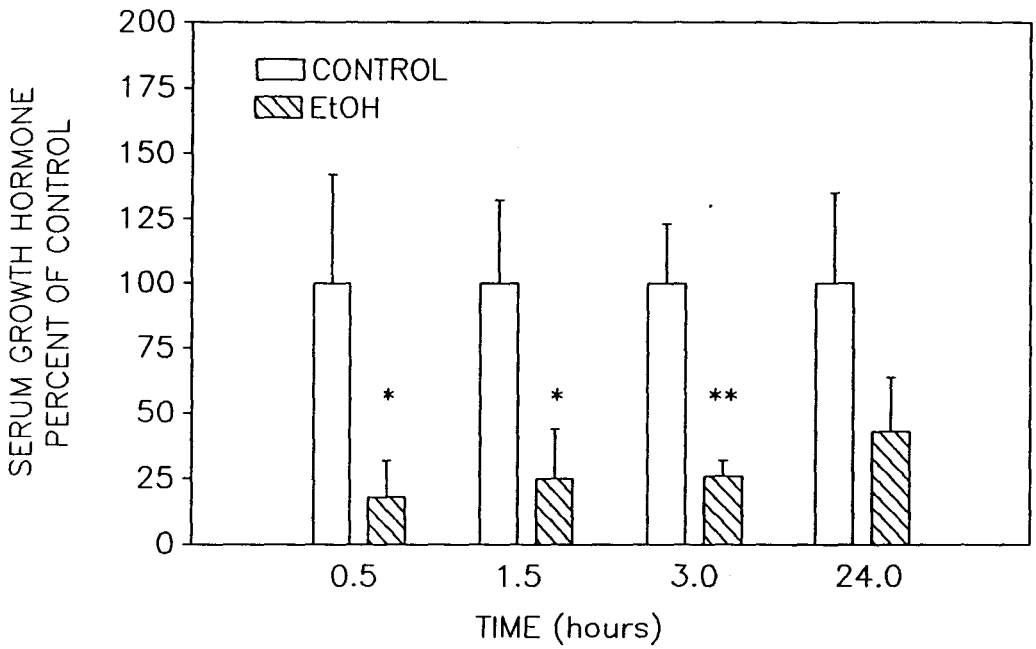


Figure 5. Serum GH levels after single binge EtOH treatment. Trunk blood was obtained at 0.5, 1.5, 3 and 24 hrs after an i.p. injection of EtOH or saline. Serum GH was measured by RIA. GH levels were significantly depressed at the 0.5, 1.5 and 3 hr. time points when compared to saline-injected controls; while the trend downward was still present at 24 hrs., this was not significant. N=5-6/group. (Data is expressed as percent of control (100%). *$p < .05$; ** $p < .01$)

group had fallen to values that were 18% of control animals ($p < 0.05$). At 1.5 hr. the levels in the EtOH group were 25% of control values ($p < 0.05$), and at 3 hrs. the EtOH group had serum GH levels that were 26% of control ($p < 0.01$). Serum GH remained suppressed 24 hrs. after injection in the EtOH group at 43% of controls, but this difference did not achieve statistical significance.

Pituitary GH content was unchanged between the two groups after single binge EtOH treatment at any time point examined. Again, because of the high degree of variability between animals in the control groups, values are expressed as percent of control (Fig. 6). The pituitary GH content of the EtOH treated animals were 72% of control at 0.5 hr.; 80% of control at 1.5 hr after injection; elevated to 206% of control at 3 hrs and 204% of control 24 hrs after injection. While a trend toward increased pituitary GH content was noted at 3 hrs. (30,900 $\pm$ 7200 ng/ml in the control group vs 63,800 $\pm$ 20,500 ng/ml in the EtOH group), this did not achieve statistical significance. At 24 hrs. there was also a tendency for the GH content to be higher in the EtOH group (29,100 $\pm$ 16,900) compared to the EtOH exposed animals (59,400 $\pm$ 29,400 ng/ml) but this was not significant.

The steady-state pituitary GH mRNA levels were measured by Northern blot analysis, and the results are shown graphically in Figure 7. GH mRNA levels were modestly, but significantly decreased by single binge EtOH, as compared to saline-injected controls, at 0.5 and 1.5 hours, time points associated with the fall in serum GH (Fig 5). At 0.5 hours GH mRNA levels were 71% of control, ($p < 0.05$); at 1.5 hours, GH mRNA levels were 67% of control, ($p < 0.05$). By 3 hours post-injection,

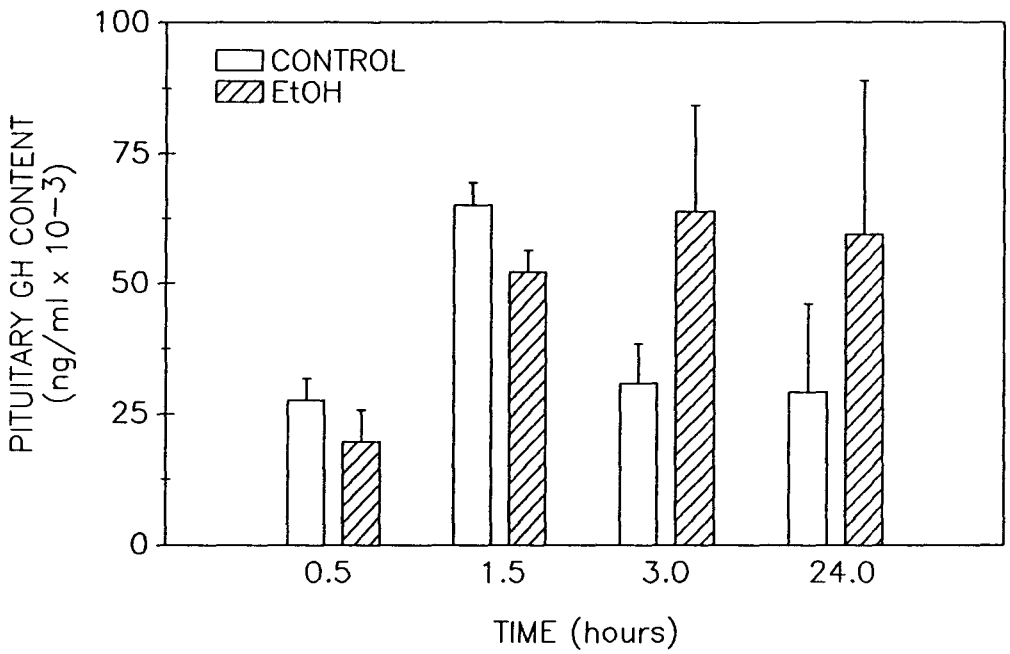


Figure 6. Pituitary GH content after single binge EtOH treatment. Pituitary GH was measured by RIA in animals given a single i.p. injection of EtOH and sacrificed at time points indicated. No significant difference was found at any time point, although there was a trend for EtOH animals to have higher GH content at 3 and 24 hrs. N=5-6/group. (Data is expressed as percent of control.)

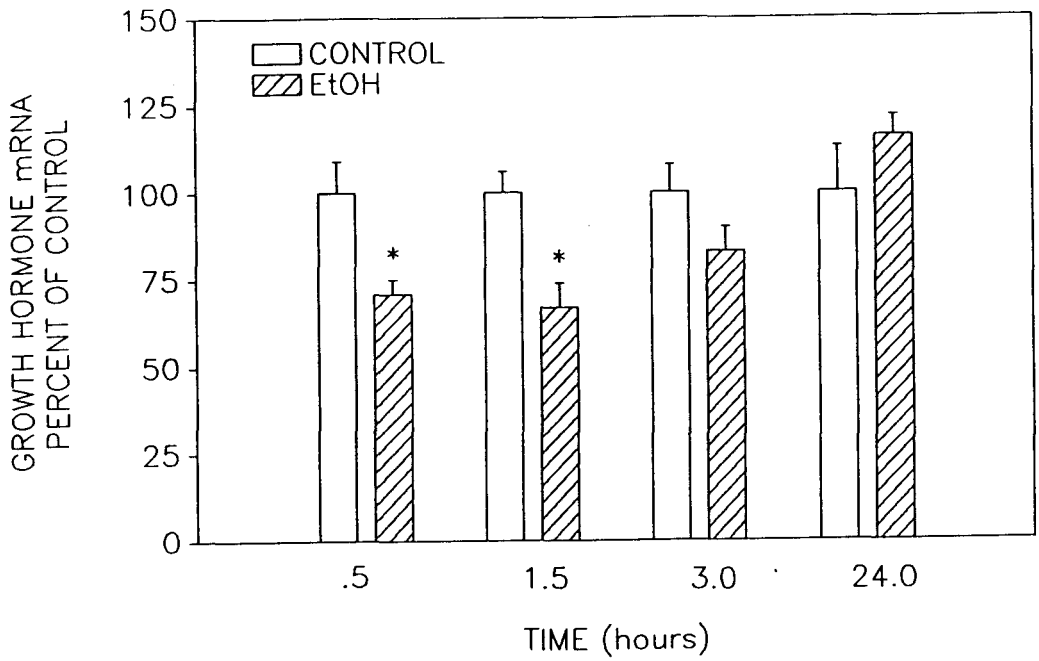


Figure 7. Pituitary GH steady-state mRNA levels after single binge EtOH treatment. GH mRNA levels were assessed by Northern blot analysis on rats given a single i.p. injection of saline of EtOH, and sacrificed at time points indicated. The mRNA for GH was significantly depressed at 0.5 and 1.5 hrs. N=5-6/group. Values were obtained by densitometric scanning of autoradiograms from Northern blot analysis and expressed in arbitrary densitometer units (ADU). The data was corrected for loading as explained in the Materials and Methods section. (Data is expressed as percent of control. * $p < .05$)

the GH mRNA levels in the EtOH group were not significantly different from controls, (83%) as were the animals at 24 hours (116%).

D. Effects of Single Binge EtOH on PRL Protein and mRNA Levels

In contrast to the results for serum GH, serum PRL values were significantly higher in the EtOH animals at 1.5 (11 $\pm$ 2 in the control group vs 23 $\pm$ 5 ng/ml in the EtOH group) and 3 hours (8 $\pm$ 2 vs 22 $\pm$ 3 ng/ml control vs EtOH) (Fig. 8). At the earliest time point, 0.5 hours, serum PRL was virtually identical in both groups, (11.5 $\pm$ 3.5 vs 12 $\pm$ 2.7 ng/ml) By 24 hrs., there was no change between the two groups in serum prolactin levels (10 $\pm$ 1 ng/ml control; 7 $\pm$ 1.5 ng/ml EtOH). Also in contrast to serum GH, there was much less variation among the control groups, as evidenced by the smaller SEMs, even though PRL is also released in a pulsatile fashion (Hadley, 1988).

Pituitary PRL content was not different between control and EtOH groups at any time point (Fig. 9). Although there was an upward trend at 1.5 hrs in the EtOH exposed animals (210% of control level) this was not statistically significant, most likely due to high degree of variability in PRL content between animals. At 3 hrs the values were nearly identical in the two groups, EtOH treated animals were 96% of control nor was there a difference at 24 hours, EtOH treated animals were at 93% of control averages.

PRL mRNA levels were significantly decreased at 1.5 and 3.0 hrs in EtOH treated animals (Fig. 10). Interestingly, these are the time points associated with

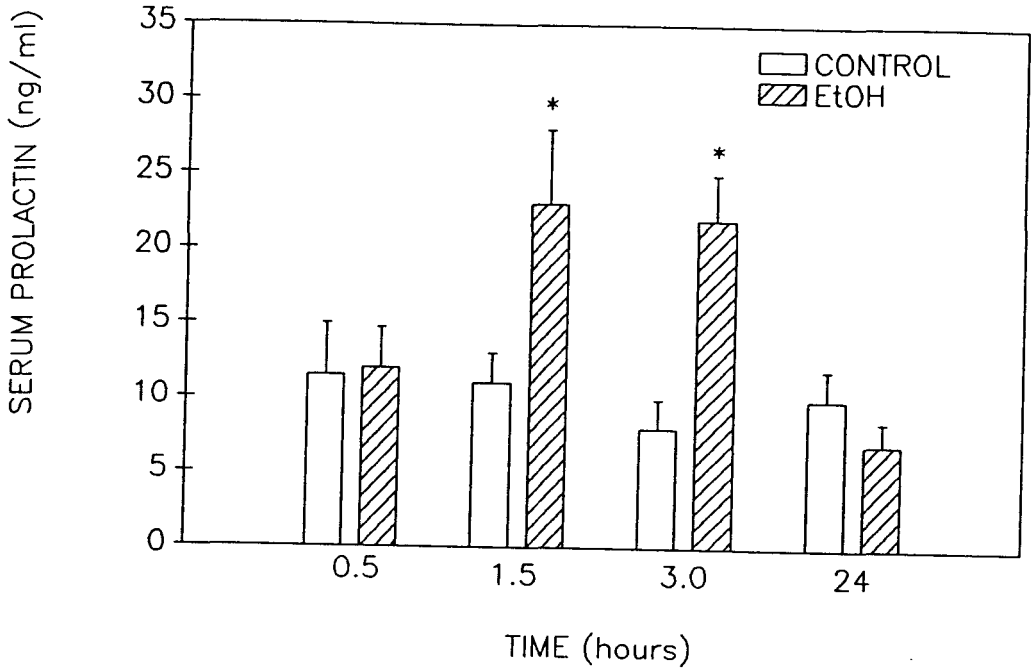


Figure 8. Serum PRL levels after single binge EtOH treatment. Time course effect of a single i.p. injection of saline or EtOH on serum PRL levels 0.5, 1.5, 3 and 24 hrs after injection, as measured by RIA. Prolactin was significantly elevated after EtOH at 1.5 and 3 hrs with return to control values by 24 hrs. n=5-6/group/time point. The data is expressed in ng/ml and each value represents the mean $\pm$ SEM of 5-6 animals at each time point. * $p < .05$).

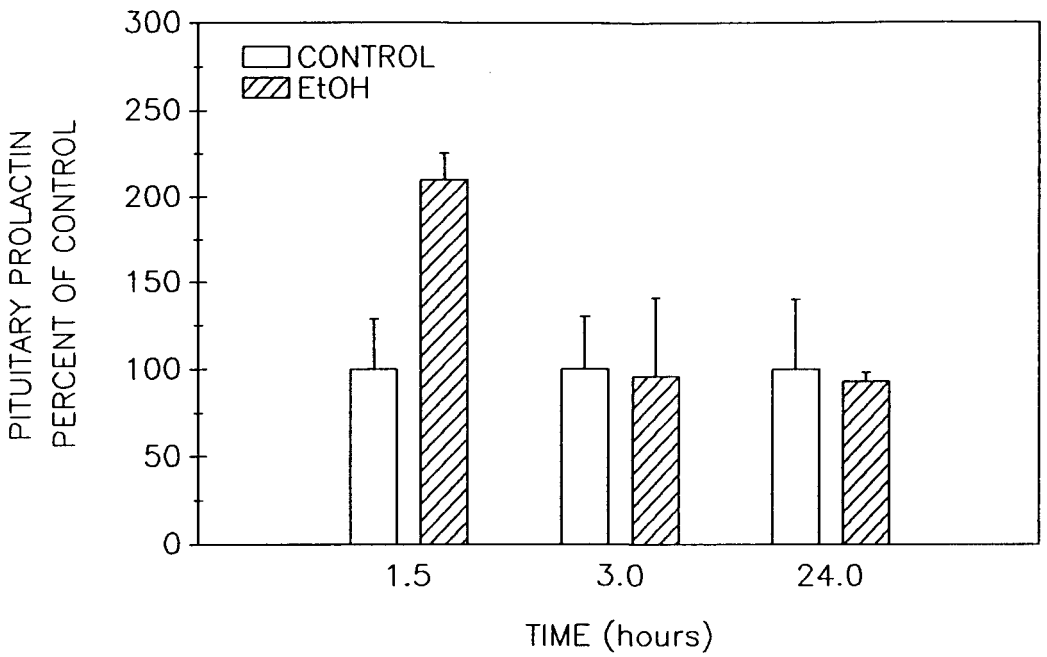


Figure 9. Pituitary PRL content after single binge EtOH treatment. PRL content was measured by RIA at various time points after single binge EtOH or saline. There were no statistical differences between the 2 groups at any time point studied. Data represent mean PRL content ± SEM. n=5-6 per group.

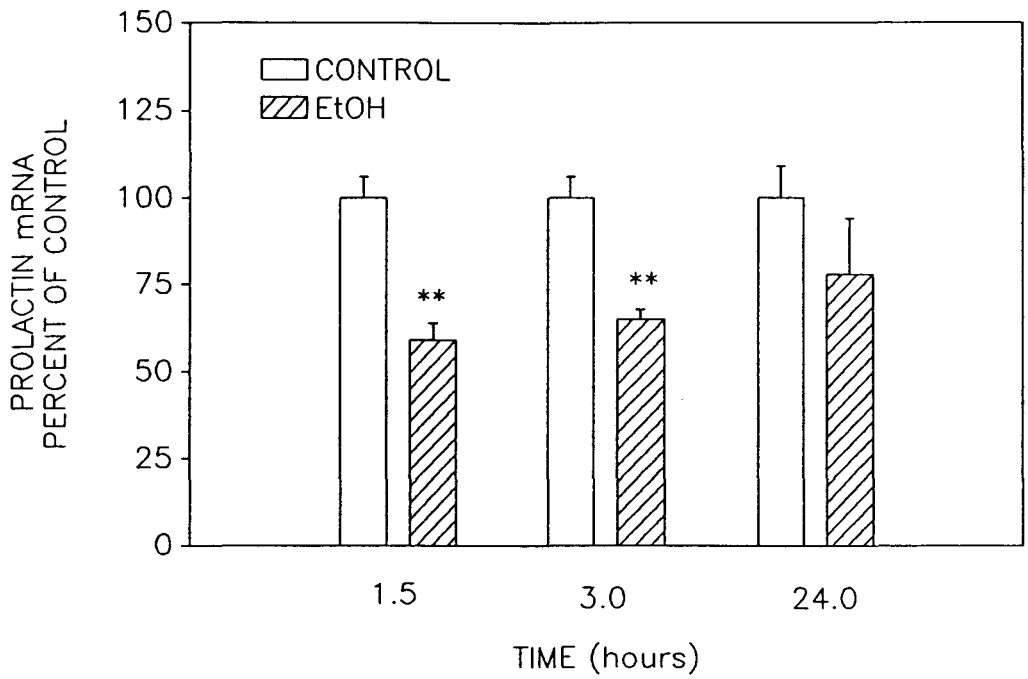


Figure 10. Pituitary PRL steady-state mRNA levels after single binge EtOH treatment. The mRNA levels, as measured by Northern blot analysis, were significantly decreased at 1.5 and 3 hrs. in the EtOH-treated animals, with a return to to control levels by 24 hrs. Data was corrected for loading as explained in the Material and Methods section and expressed in ADU. The results were then converted to percent of control. n=5-8 per group; ** $p < .01$)

elevated serum PRL levels (Fig. 8). At the 1.5 hour time point, PRL mRNA levels are 59% of control ($p < 0.05$), and at 3 hours post-injection PRL mRNA levels are 65% of control ($p < 0.05$). The effect is gone by 24 hours, with an insignificant amount of suppression that is 78% of control levels.

The steady-state mRNA levels for Pit-1, the transcription factor responsible for high level expression of GH and PRL, were also measured by Northern blot to determine if the EtOH-induced effects on GH and PRL mRNA were indirectly related to decreased synthesis of Pit-1. However, there was no difference between the 2 groups at any time point studied (Fig. 11). At 1.5 hours, the values were .27 $\pm$ .1 control vs .3 $\pm$ .01 EtOH, ADU; at 3 hours, .24 $\pm$ .02 vs .25 $\pm$ .03; and at 6 hours, .42 $\pm$ .06 vs .47 $\pm$ .11.

E. Effects of Double Binge EtOH on GH protein and mRNA

To determine if the the GH axis responds in a similar fashion to a repeated challenge with ethanol, a repeat exposure or "double binge" experiment was conducted. Serum GH levels fell dramatically 1.5 and 3 hours after the second EtOH injection compared to levels in saline injected animals (Fig. 12). A similar effect was observed after a single EtOH injection (Fig. 5). At 1.5 hours after injection, serum GH fell to 6% of control value and at 3 hours, 12% of control. The effect dissipated, however, at 6 hours when EtOH treated animals had 85% of control levels of serum GH, and at 24 hours where there was in fact, an insignificant elevation in GH values in the EtOH group to 143% of control levels after the second EtOH injection (Fig. 12). Data are

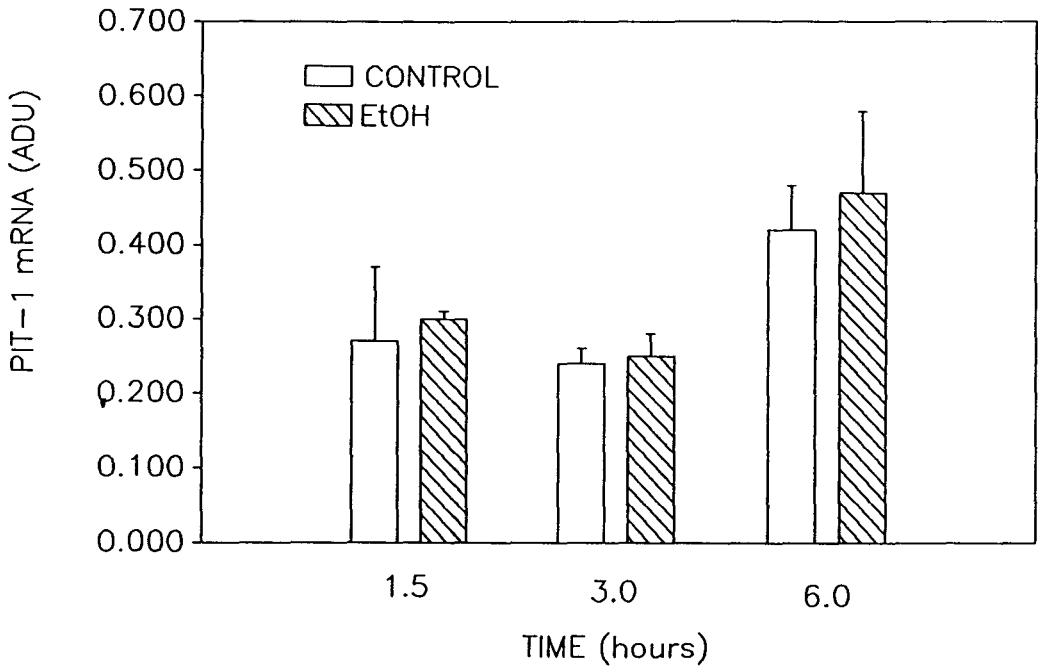


Figure 11. Pit-1 (GHF-1) steady-state mRNA levels after single binge EtOH treatment. The mRNA levels of Pit-1, as measured by Northern blot were found to be unaltered by EtOH exposure at any time point studied. (Data is expressed as percent of control). Values represent mean ADU ± SEMand were corrected for loading as described in the Materials and Methods section. n=5-8 per group.

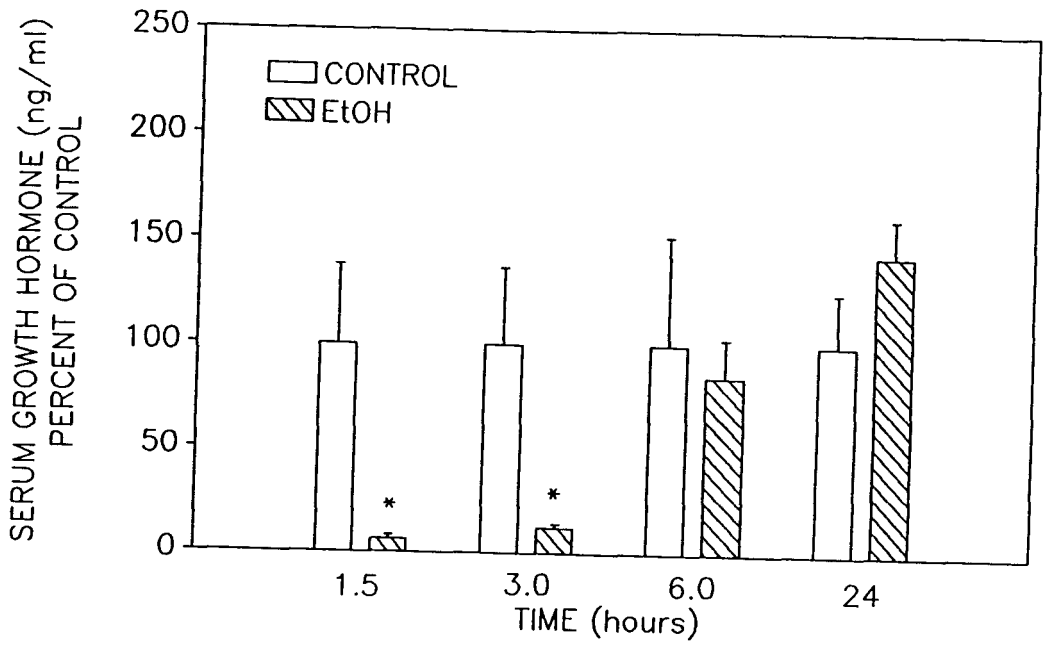


Figure 12. Serum GH levels as measured by RIA obtained from animals exposed to two IP injections of EtOH or saline given 24 hours apart and sacrificed 1.5, 3, 6, and 24 hours after the second injection. GH levels were significantly lower in the EtOH animals at 1.5 and 3 hours after treatment. n = 9 animals per group at each time point. * = $p < .05$.

presented as percent of control due to the high degree of variability of GH values in saline injected animals. Such variability was not, however, seen in the EtOH-injected rats. For example, at 1.5 hours after saline treatment, serum GH values ranged from 13 ng/ml to 537 ng/ml and at 3 hours from 5 ng/ml to 394 ng/ml. However, serum GH levels in EtOH exposed animals were all between 3 and 41 ng/ml.

In contrast to the results of single binge experiments (Fig. 7), steady state levels of GH mRNA, as measured by Northern blot were unchanged by double binge EtOH exposure at any of the measured time points (Fig. 13). At 1.5 hours post-injection, GH mRNA was 120% of control in the EtOH treated animals, at 3 hours, GH mRNA was 101% of control, at 6 hours, GH mRNA was 87% of control and at 24 hours it was 90% of control.

F. Effects of Single and Double Binge EtOH on Hypothalamic Hormones

To determine if acute EtOH exposure has an influence on the release of GHRH from the hypothalamus, and hence, lead to a decrease in steady-state GH mRNA and GH secreted from the pituitary, hypothalamic GHRH content was measured by RIA after single binge EtOH exposure (Fig. 14). The levels of hypothalamic GHRH content were not altered by EtOH at any time points examined, indicating that secretory defects in GHRH neurosecretory cells are most likely not involved in the decreases in serum GH and GH mRNA after single binge EtOH.

Hypothalamic growth hormone-releasing hormone (GHRH) and somatostain (SRIF) mRNA are expressed at low levels in the rat and were beyond the lower limits

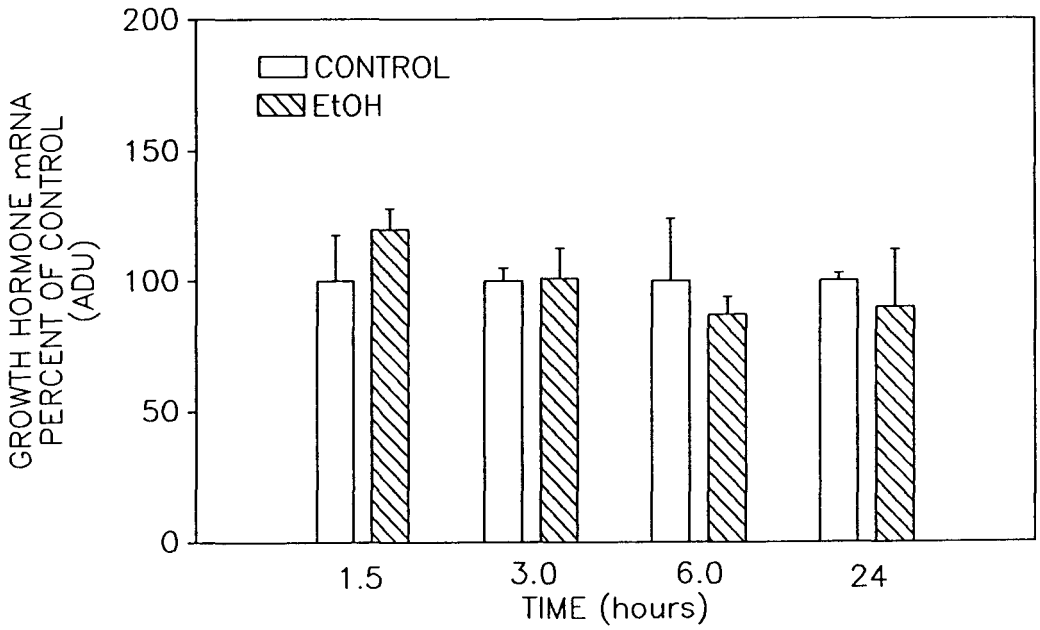


Figure 13. Pituitary GH mRNA levels after double binge EtOH treatment as measured by Northern blot analysis. Data represent mean ± SEM ADU, corrected for loading and converted to percent of control. There were no significant differences noted at any time point studied. n = 5 animals per group at each time point.

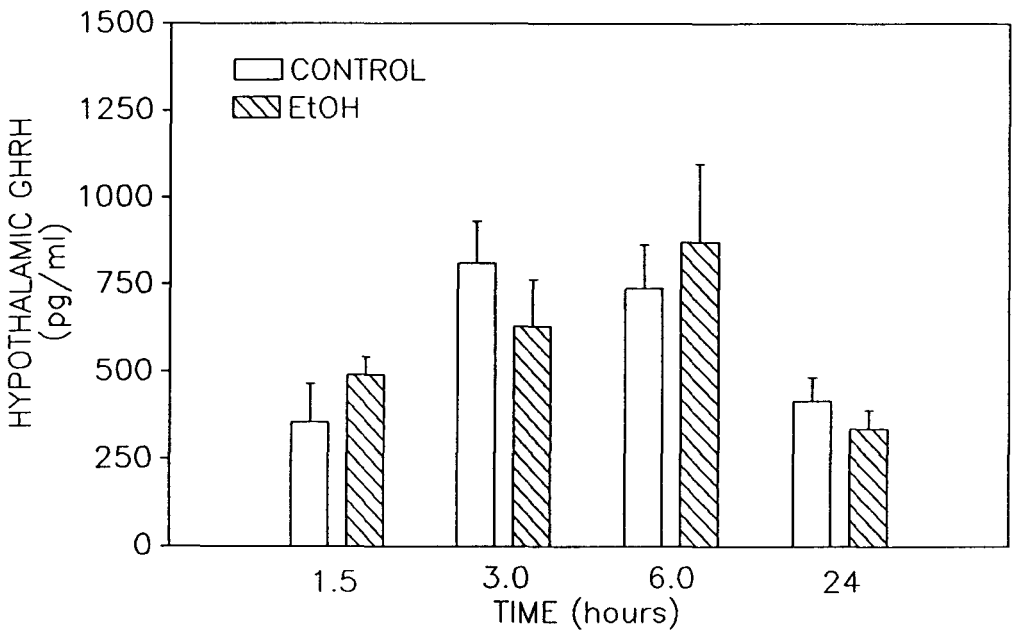


Figure 14. Effects of EtOH on hypothalamic GHRH content. GHRH was measured by RIA in rats exposed to single binge EtOH treatment. Data represents mean ± SEM expressed as pg/ml and corrected for mg protein. There were no significant differences noted at any time point studied. n= 6 animals per group.

of sensitivity of Northern blots performed in our laboratory (data not shown). Therefore, a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique was developed, utilizing histone 3.3 (H3.3) as an internal control. H3.3 was chose as an internal control because it is expressed in a constitutive and cell cycle-independent manner in all cells (Wells, *et al* 1987), and in experiments performed in our laboratory, appears to be unaffected by EtOH exposure (Kelley *et al*, 1993)(data not shown). Furthermore, unlike most members of the histone multigene family, its mRNA product is polyadenylated, allowing oligo dT-primed reverse transcription (Wells, *et al* 1987). As demonstrated previously, and as shown in Figure 15, the amount of PCR product for both GHRH and H3.3 increased in a linear fashion to a plateau with increasing number of PCR cycles (Kelley *et al*, 1993). Aliquots of a standard PCR reaction containing hypothalamic cDNA, GHRH and H3.3 oligonucleotide primers were removed after 20, 25, 30, 35, and 40 cycles. The products were run on an agarose gel, blotted, and the signals scanned densitometrically and plotted as relative amount vs number of PCR cycles as shown in Figure 15. Based on the results of these experiments, the range of linearity was determined to be between 20 and 25 cycles, which was on the ascending portion of the curve. Subsequently, 22 cycles was utilized for these studies (Fig. 15). A linear relationship between the amount of starting RNA and the amount of PCR product was also demonstrated, establishing this as a relatively quantitative tool in our laboratory (Fig 16), (Kelley, *et al* 1993). Subsequent experiments utilized 3 μg of total RNA as starting material.

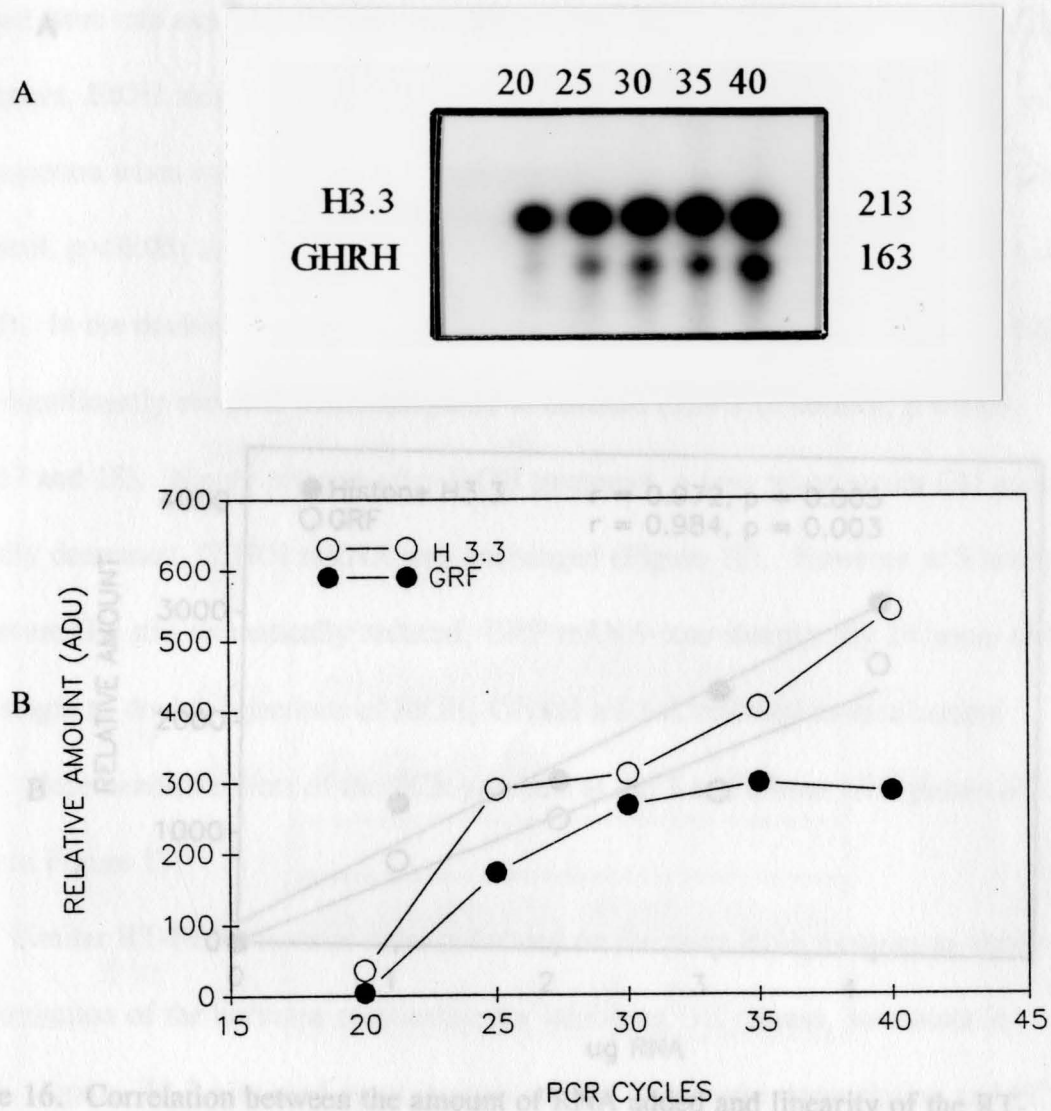


Figure 15. Semi-quantitative PCR standardization. RNA (3 μg) from hypothalamus was subjected to RT-PCR analysis using the GHRH primers and primers for H3.3 (see materials and methods). Aliquots were removed at 20, 25, 30, 35 and 40 cycles. Panel A shows the resulting DNA blot. Panel B is a graphic representation of the gel shown in Panel A. Relative amount refers to arbitrary densitometer units (ADU). The experiment was performed three times with similar results.

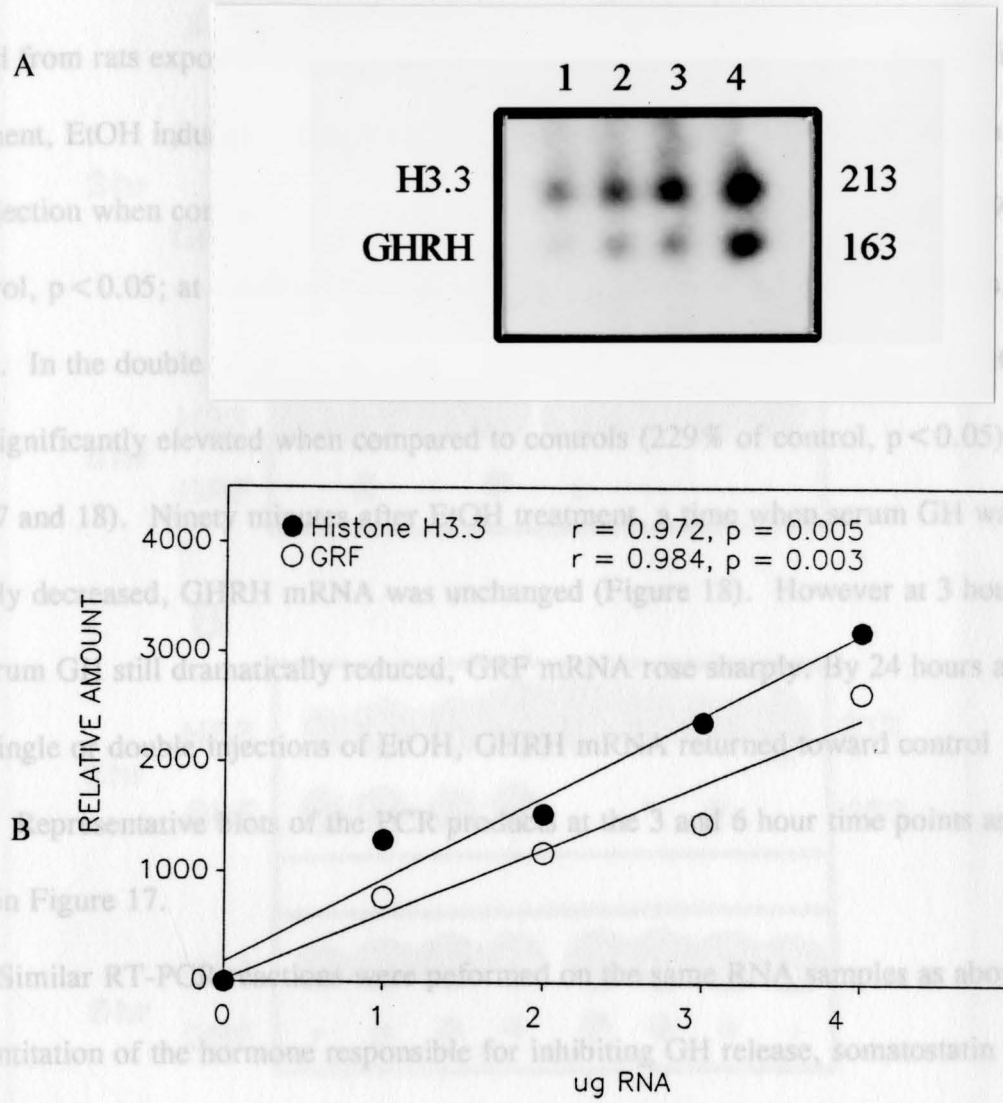


Figure 16. Correlation between the amount of RNA added and linearity of the RT-PCR assay. Different amounts of hypothalmic RNA were used in the RT-PCR assay using primers for GHRH and H3.3. Lanes 1-4 (Panel A) are 1-4 μg of input RNA. Following the RT-PCR reaction, 10 μl was run on an agarose gel, and transferred and exposed to autoradiographic film. The results shown in Panel A were plotted using a regression equation (Panel B). Correlation coefficients and p values are shown along with the fitted line for both GHRH and H3.3.

Relative amounts of GHRH mRNA were quantified from individual hypothalami, obtained from rats exposed to single and double binge experiments. In the single binge experiment, EtOH induced a significant rise in GHRH mRNA levels at 3 and 6 hours after injection when compared to control animals (at 3 hours EtOH values were 379% of control, $p < 0.05$; at 6 hours EtOH values were 232% of control, $p < 0.05$) (Figs. 17 and 18). In the double binge experiments, only at the 3 hour time point was the EtOH group significantly elevated when compared to controls (229% of control, $p < 0.05$) (Figs 17 and 18). Ninety minutes after EtOH treatment, a time when serum GH was markedly decreased, GHRH mRNA was unchanged (Figure 18). However at 3 hours, with serum GH still dramatically reduced, GRF mRNA rose sharply. By 24 hours after either single or double injections of EtOH, GHRH mRNA returned toward control levels. Representative blots of the PCR products at the 3 and 6 hour time points are shown in Figure 17.

Similar RT-PCR reactions were peformed on the same RNA samples as above for quantitation of the hormone responsible for inhibiting GH release, somatostatin (SRIF). Again, H3.3 was used as an internal control for reverse transcription and PCR amplification. Conditions for running the SRIF RT-PCR reactions were determined by performing experiments similar to those described above, using varying amounts of starting RNA and running the reactions for 20, 25, 30, 35 and 40 cycles to find the linear portion of the amplification curve. The results were similar to those for GHRH, that is, 22 cycles of PCR with 3 μg of starting RNA were found to be the optimal conditions (data not shown). Unlike GHRH, however EtOH did not affect SRIF

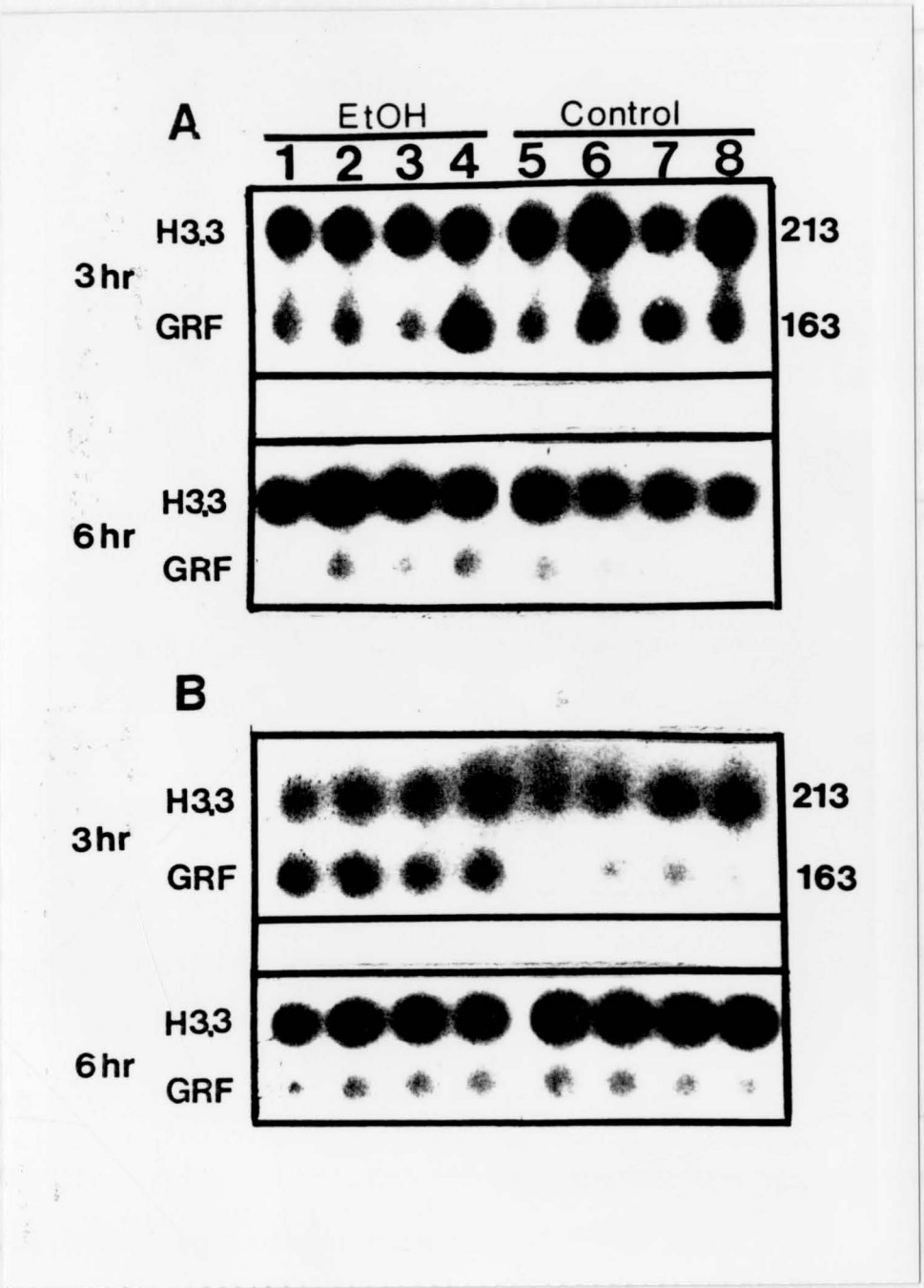
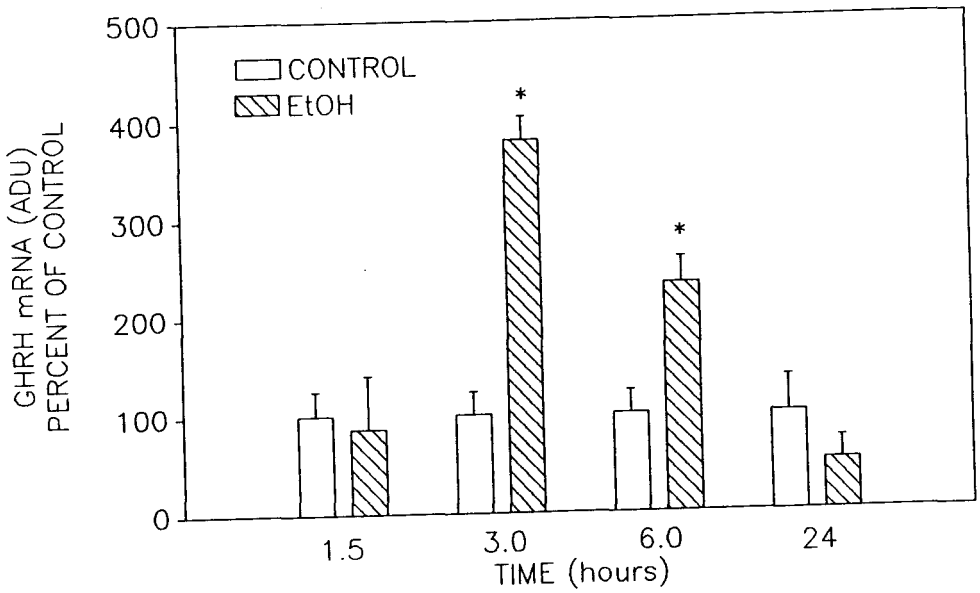


Figure 17. Comparative RT-PCR analysis of GHRH (GRF) mRNA in single (A) or double-binge (B) experiments. Representative time points of 3 and 6 hrs post-treatment are shown. Lanes 1-4 are the EtOH treated rats and lanes 5-8 are the saline treated. Results are normalized to H3.3 levels.

A



B

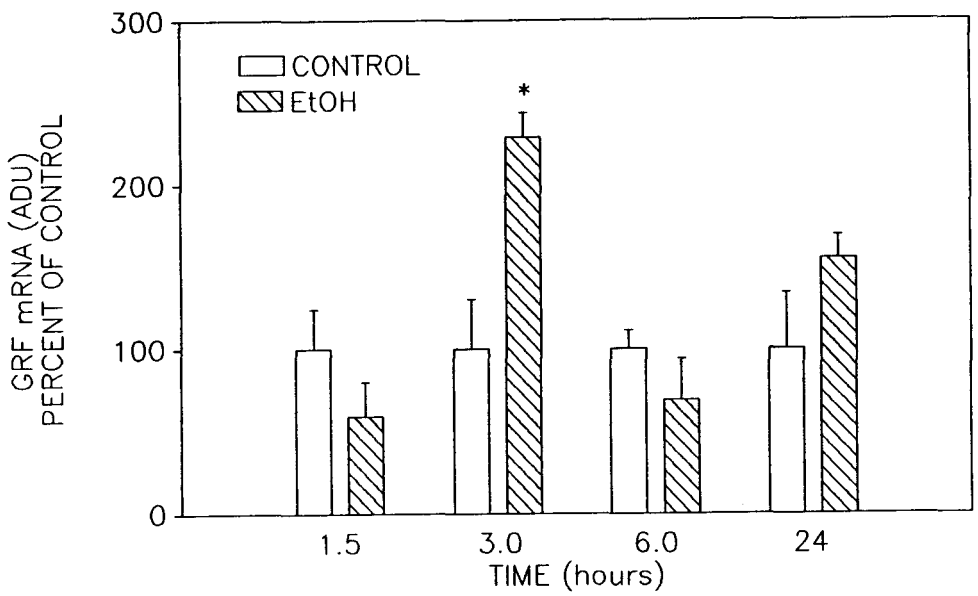


Figure 18. Densitometric analysis of hypothalamic GHRH (GRF) mRNA, as determined by semi-quantitative RT-PCR, in animals treated with a single (Panel A) or double (Panel B) injection of EtOH or saline and sacrificed 1.5, 3, 6 and 24 hours later. GRF mRNA levels were significantly elevated at 3 hours and 6 hours in the single binge experiment in EtOH-treated animals, but only at 3 hours in the EtOH-treated animals exposed to two injections. * $p < .05$.

steady-state mRNA levels, as measured by semi-quantitative PCR, in either single binge or double binge models at any time points measured (Figs. 19 and 20).

G. Effects of Acute EtOH on Hormone Transcription Rates

Nuclear run-on transcription assays were performed to determine if a decrease in the rate of transcription of the GH and PRL genes could account, in part, for the decrease in the steady-state levels of these hormones at various time points after single binge EtOH exposure. The results indicate that EtOH can, in a time-dependent manner, decrease the rate of transcription of the GH, PRL and Pit-1 genes. The most striking decrement was observed at 3 hours post EtOH injection, and a representative autoradiograph and corresponding densitometric plot of this time point is shown in Figure 21. Actin, used as a positive loading control was unchanged by EtOH treatment at this or any other time point studied. PBR 322 is empty bacterial plasmid DNA , used as a negative control for non-specific binding; there is negligible binding of labeled RNAs to PBR 322 in all the blots. Densitometric readings are represented graphically for the time points studied in Figures 22 and 23. Each value represents the mean $\pm$ SEM for the ADU. For each time point, n=2-3, with an n of 1 representing 3 pooled pituitaries. At 1.5 hours, GH, PRL and Pit-1 had reduced transcription rates. GH levels were 1490 $\pm$ 351 control vs 1007 $\pm$ 26 EtOH, ADU;PRL was 522 $\pm$ 62 vs 312 $\pm$ 89; Pit-1 was 344 $\pm$ 24 vs 219 $\pm$ 22 (Fig. 22). At 3 hours, the decrement was greater for all three genes, GH levels were 845 $\pm$ 41 control vs 298 $\pm$ 13 EtOH, ADU; PRL was 195 $\pm$ 13 vs 108 $\pm$ 14; Pit-1 was 110 $\pm$ 16 vs 45 $\pm$ 17.

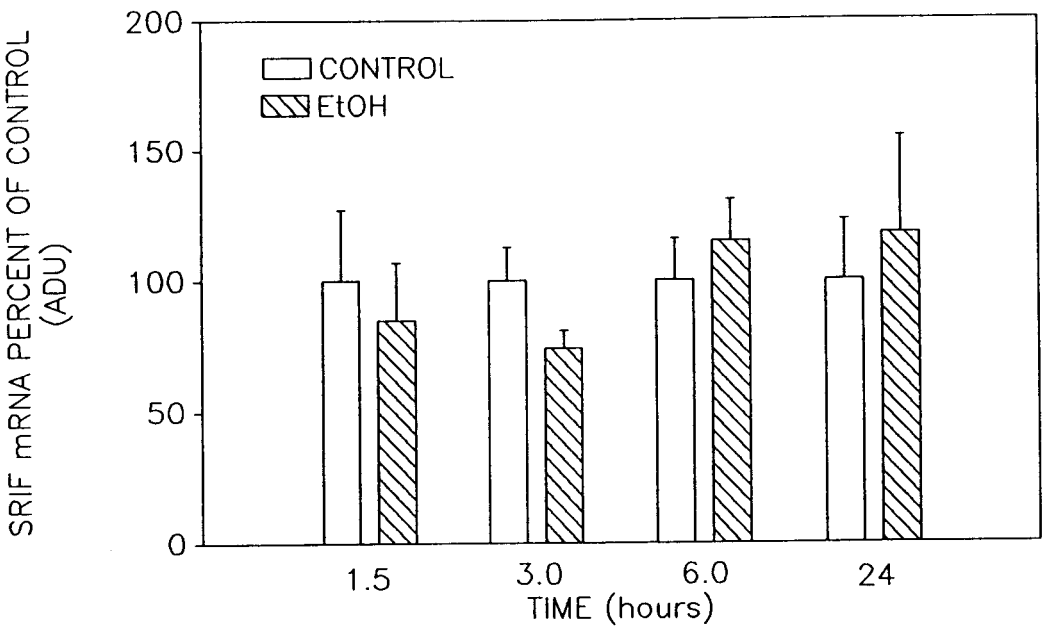


Figure 19. Hypothalamic SRIF levels after single binge EtOH as determined by semi-quantitative RT-PCR. Relative amounts of SRIF mRNA were determined after a single i.p. injection of saline or 3g/kg EtOH, as described in the Materials and Methods section. There were no significant differences between control and EtOH-injected animals at any time point studied. Results are expressed in ADU and were converted to percent of control.

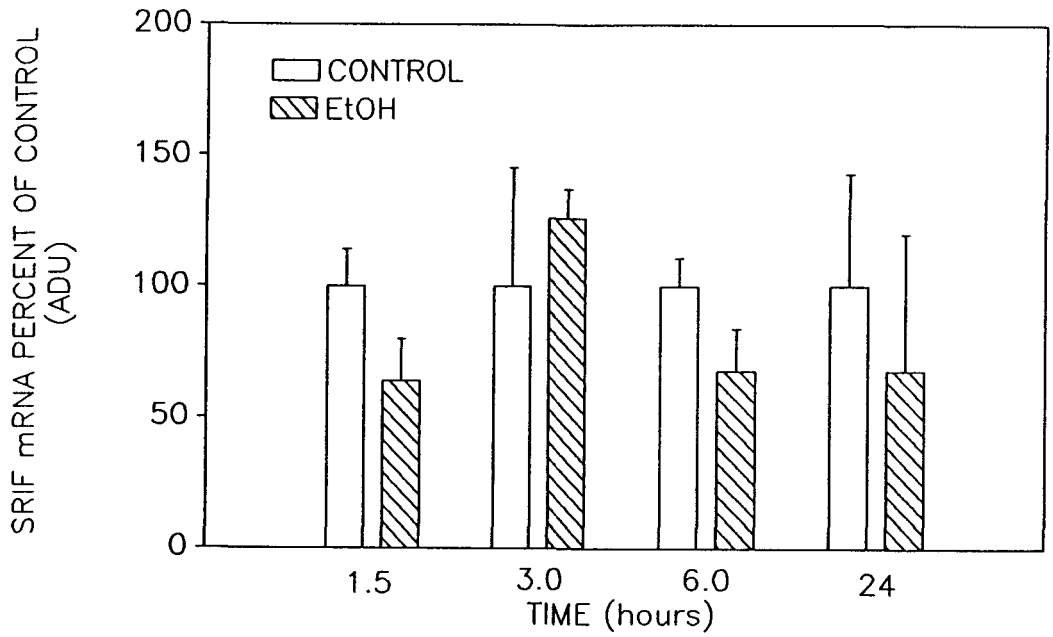


Figure 20. Hypothalamic SRIF mRNA levels after double binge EtOH treatment as measured by semi-quantitative RT-PCR. Relative amounts of SRIF mRNA were determined after two i.p. injections of EtOH or saline. There were no significant differences between the EtOH and saline groups at any time point studied. Results are expressed in ADU and were converted to percent of control.

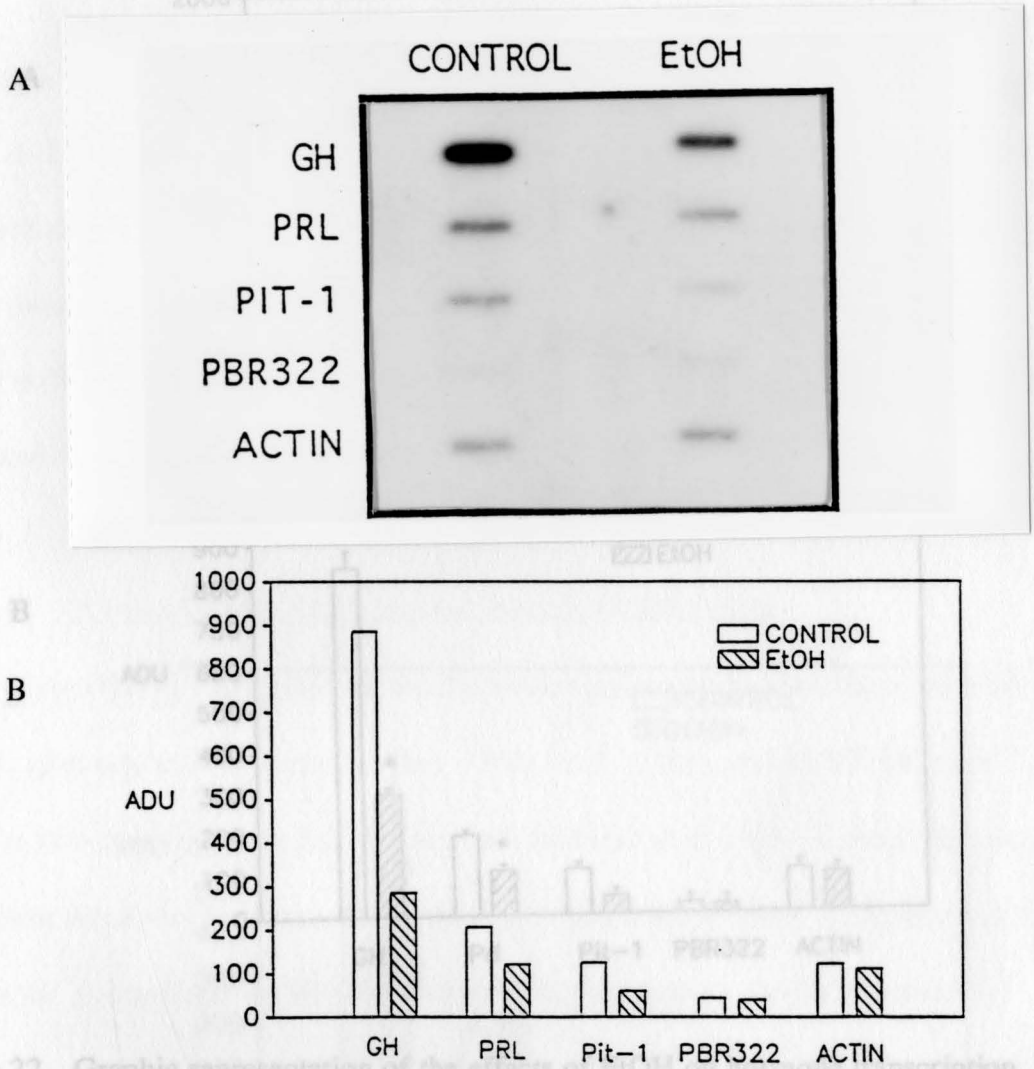


Figure 21. Representative autoradiograph depicting the effects of EtOH treatment on transcription rates of pituitary hormones 3 hours after single binge EtOH treatment as measured by the nuclear run-on transcription assay . See Materials and Methods for details. Panel A depicts typical blot of run-on at 3 hour time point. The blot was scanned and the results are depicted graphically in Panel B. Values are expressed in ADU and are not corrected for background.

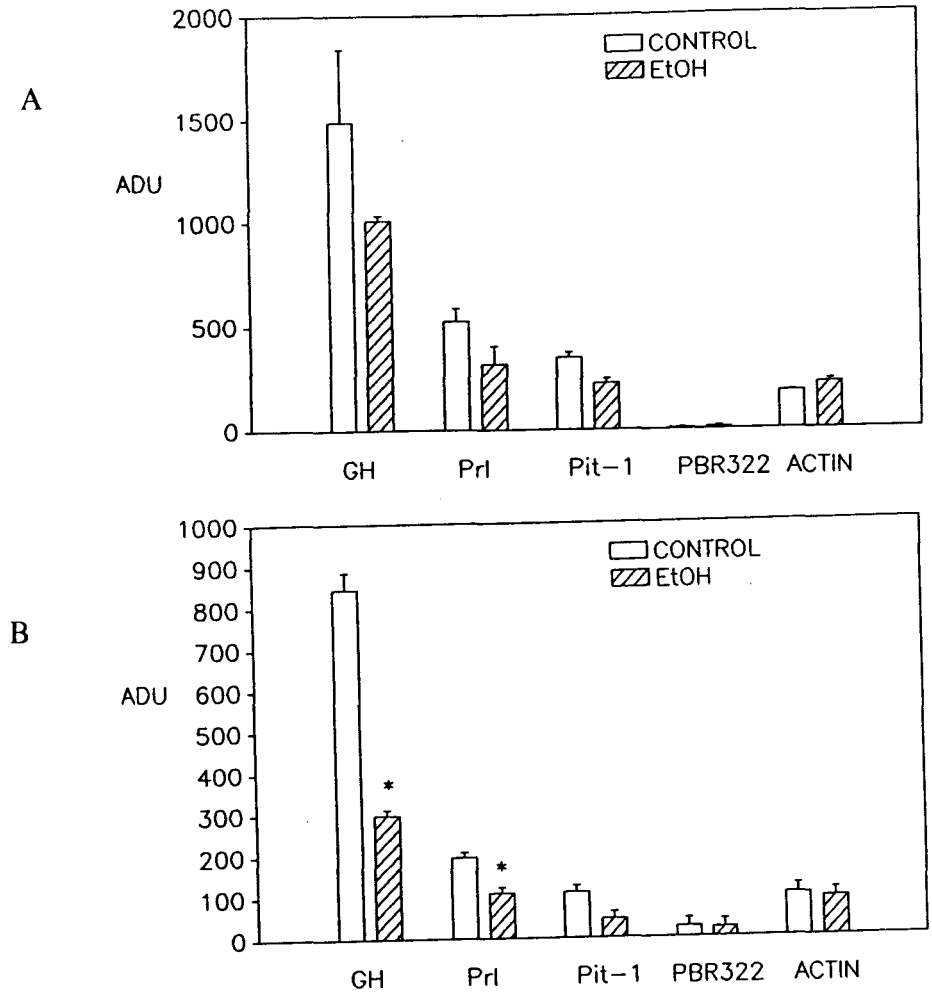


Figure 22. Graphic representation of the effects of EtOH on hormone transcription rates at 1.5 and 3 hours after treatment, as measured by the nuclear run-on transcription assay. Animals were treated with a single injection. Panel A depicts the results after 1.5 hours. GH,PRL and Pit-1 transcription rates are suppressed by EtOH to varying degrees. Panel B depicts the results after 3 hours. This time point was associated with the largest EtOH-induced fall in GH transcription rates. PRL and Pit-1 transcription rates were also suppressed by EtOH treatment at this time. GH andPRL were significantly suppressed at 3 hours ($p < 0.05$). Values are expressed as mean ADU & SEM.

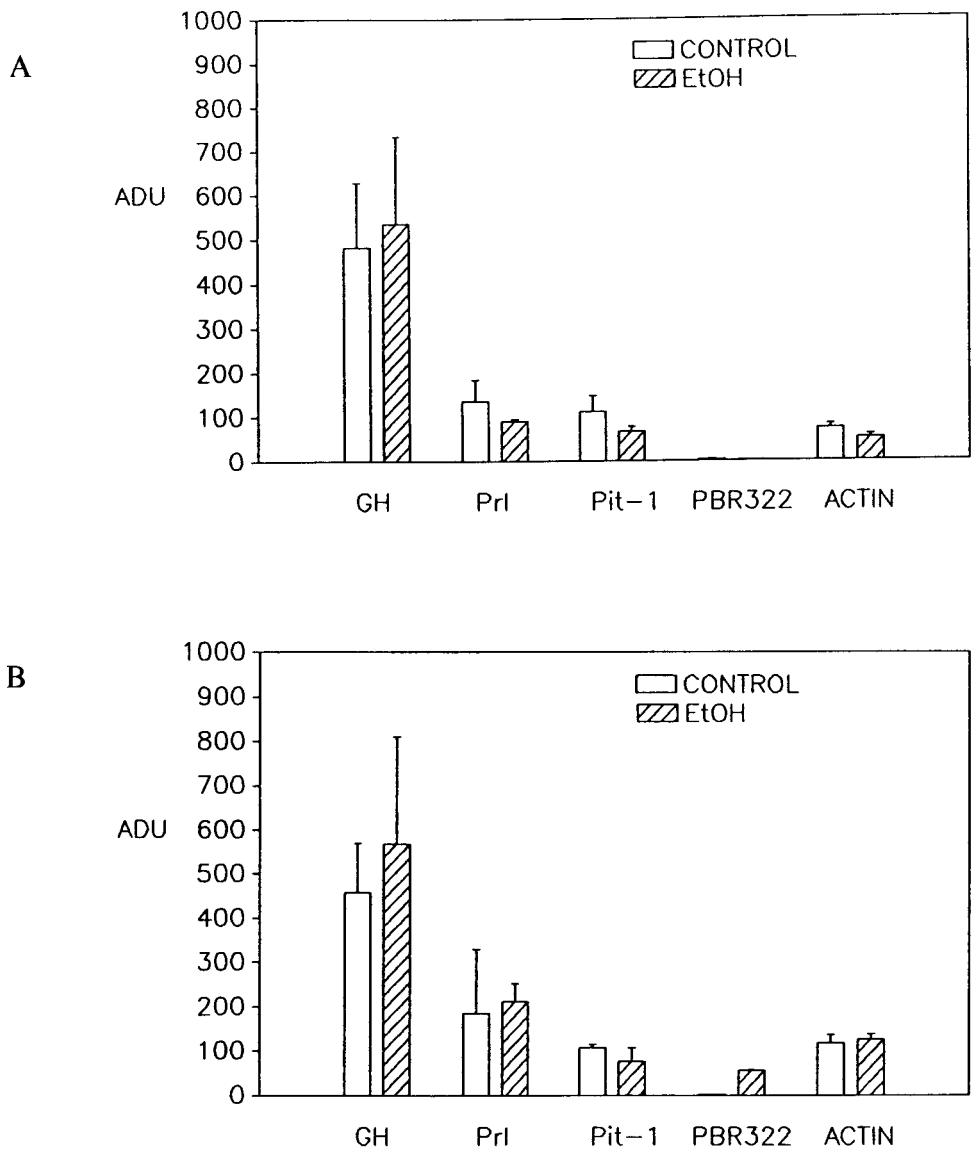


Figure 23. Graphic representation of the effects of EtOH on hormone transcription rates at 6 and 24 hours after treatment as measured by the nuclear run-on transcription assay, after single binge. Panel A depicts the results after 6 hours. Panel B depicts the results after 24 hours. GH,PRL and Pit-1 transcription rates are unchanged from control levels, at either time point, within the range of error. Values expressed as mean ADU $\pm$ SEM.

At 6 hours, GH transcription rate was slightly increased by EtOH, 482 ± 146 control vs 535 ± 199 EtOH, ADU; PRL was 134 ± 48 vs 90 ± 4; and Pit-1 was still decreased almost 50-fold at 112 ± 35 vs 67 ± 10 (Fig. 23). 24 hours after treatment, GH transcription rate was still moderately, but not statistically elevated, 457 ± 112 control vs 567 ± 243 EtOH; PRL rates were virtually unchanged 185 ± 143 vs. 210 ± 41; and Pit-1 was still mildly suppressed, 106 ± 9 vs 76 ± 30 (Fig 23).

H. Effects of Acute EtOH on Intrapituitary cAMP Levels

Since cAMP is an important second messenger molecule involved in both GH andPRL synthesis and secretion (Hadley, 1988; Neill, 1988), and cAMP has been shown to be altered by acute EtOH treatment (Tabakoff *et al* 1988), a short time course experiment measuring intra-pituitary cAMP after single binge EtOH treatment was performed. Intracellular cAMP was measured by RIA and the results, reported as pmol/pituitary are shown in Figure 24. The intrapituitary cAMP levels were elevated significantly at 0.25 hours after single binge treatment in the EtOH animals (9 ± .65 control vs 29.3 ± 3.4 EtOH pmol/pit., $p < .05$), at 0.5 hours, there was a significant ten-fold rise in cAMP in EtOH animals (6.0 ± .78 vs 58.5 ± 7.5 pmol/pit., $p < 0.01$) with a rapid return to control values by 1.5 (18 ± .54 control vs 21 ± .9 EtOH) and 3 hrs. (12.6 ± .6 vs 17.4 ± 2.3) (Fig. 24).

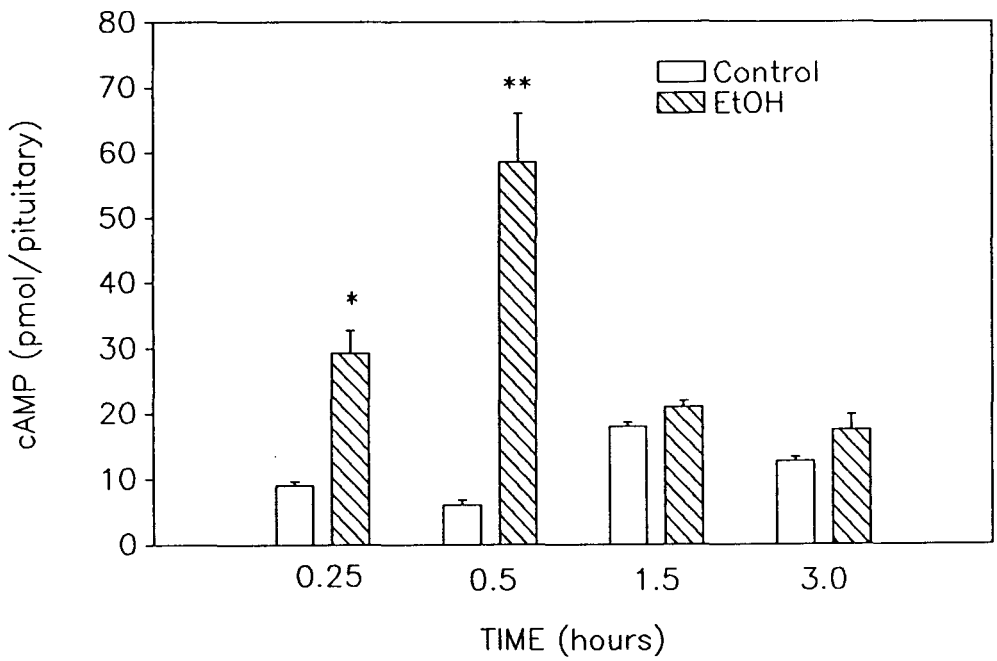


Figure 24. The effects of EtOH on intrapituitary cAMP levels at various time points after EtOH treatment. Animals were treated with a single i.p. injection of 3g/ kg EtOH or saline. The effect of EtOH on pituitary cAMP was statistically significant when compared to controls at 0.25 hrs (3-fold increase) and at 0.5 hrs (10-fold increase), with rapid return to control levels at 1.5 and 3 hrs. Results are expressed as pmol per pituitary. n=5-6 per group; * $p < .05$; ** $p < .01$.

I. Effects of Acute EtOH on *c-fos* Expression

A short time course experiment was performed to measure the response of the *c-fos* gene to acute EtOH exposure. *c-fos* is stress responsive (Kononen *et al*, 1992) and has been correlated with GH and PRL gene expression (Billestrup et al, 1987; Davis, 1990). *c-fos* expression was measured in pituitaries by Northern blot analysis at 15 min., 30 min., 1 hour, 2 hours and 3 hours after a single injection of EtOH (3g/kg) or saline. A representative autoradiograph of a Northern blot is shown in Figure 25. *c-fos* mRNA is rapidly induced 15 minutes after EtOH exposure to levels that are nearly 50-fold greater than control (1.68 ± 0.92 control vs 24.39 ± 2.7 EtOH, $p < 0.05$). This effect persists to a lesser degree after 30 minutes, when EtOH treated animals had an almost 10-fold increase in induction of *c-fos* than controls ($2.95 \pm .13$ control vs 20.99 ± 10.6 EtOH). The induction quickly abated, which is typical of auto-regulated genes such as *c-fos*, and from 1 hour to 3 hours there were unmeasurable levels of *c-fos* mRNA in either control or EtOH treated pituitaries by Northern blot analysis (Fig. 25).

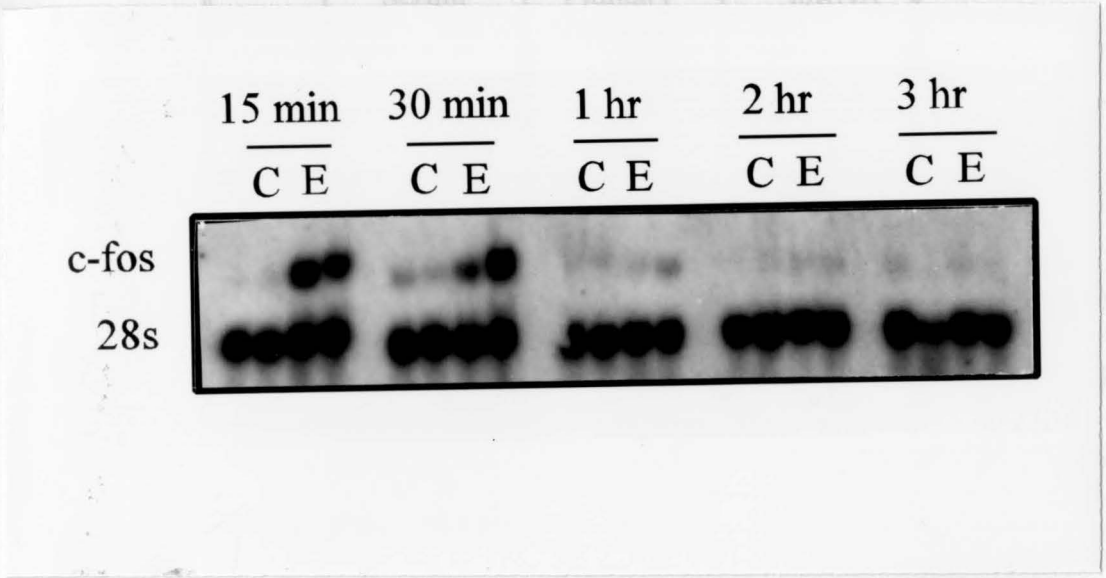


Figure 25. Time course effects of single binge EtOH treatment on pituitary *c-fos* mRNA levels. Steady-state mRNA levels of *c-fos* were measured by Northern blot analysis. A representative autoradiograph is depicted. At each time point, there were 2 control samples and 2 EtOH samples. The blot was simultaneously probed with the cDNA for 28s rRNA to control for loading differences. *c-fos* mRNA is rapidly induced (50-fold) 15 min after EtOH treatment when compared to saline-injected controls. At 30 min after EtOH treatment, *c-fos* mRNA levels were still elevated over control, but to a lesser degree (10-fold). This effect quickly dissipated, and there is no difference between EtOH and control groups at any remaining time points examined. This experiment was performed three times with similar results.

SUMMARY OF RESULTS

Table I. Pituitary

"SINGLE BINGE"

	Serum	Pituitary Content	mRNA
GH	a,b,c	NC	a,b
Prl	b,c	NC	b,c

"DOUBLE BINGE"

	Serum	Pituitary Content	mRNA
GH	b,c	--	NC

Table II. Hypothalamus

"SINGLE BINGE"

	HT Content	mRNA
GHRH	NC	c,d
SRIF	--	NC

"DOUBLE BINGE"

	HT Content	mRNA
GHRH	--	c
SRIF	--	NC

a) 0.5 hours after ethanol injection
b) 1.5 hours after ethanol injection
c) 3.0 hours after ethanol injection
d) 6.0 hours after ethanol injection

NC: no change
--: not measured

CHAPTER V

DISCUSSION

The endocrine system, like the central nervous system, is adversely affected by alcohol abuse. The pituitary hormones in particular seem vulnerable to EtOH's effects, potentially leading to a wide range of endocrine dysfunctions, since anterior pituitary hormones are responsible for a variety of physiologic and biochemical functions including growth, reproduction, immune regulation, stress response and carbohydrate and protein metabolism (Hadley, 1988). The overall goal of the present study was to further characterize the adverse effects of acute EtOH exposure on the GH and PRL gene expression, pituitary hormones of vital importance, in the male rat. In addition, efforts were made to elucidate some of the possible mechanisms underlying the EtOH-induced disruption of normal GH and PRL regulation. This included measurements of gene expression in response to acute EtOH exposure and alterations in regulatory hypothalamic releasing and inhibiting hormones as well as effects on second messenger systems. As has been observed in other hormone systems, results of the present study indicate that EtOH acts at multiple levels within the GH and PRL axes and that no single site of action is solely responsible for the overall effects of the drug.

A. The Effects of Ethanol on Growth Hormone

Many studies have demonstrated that alcohol exerts a negative effect on the GH axis in male rats (Redmond 1980; Mannisto, *et al* 1987; Dees, *et al* 1988; Emanuele, *et al* 1992; Sontag and Boyd 1989). In the present acute study, this observation was confirmed. A single dose of EtOH at 3g/kg ("single binge" model) was sufficient to cause a rapid and profound decrease in serum levels of GH. As early as 30 minutes after ethanol administration, serum GH levels fell significantly, and remained suppressed through the 3 hour time point. After repeated alcohol exposure, as shown in "double binge" experiments described here, the pattern is essentially the same, with significant suppression at 1.5 and 3 hours post EtOH. Since pituitary GH content remained statistically unchanged after EtOH treatment, there is no direct evidence that a defect in secretion is the cause of this suppression. It may be, however that secretion is affected to some degree, but this is not appreciable due to the vast store of GH present in the pituitary. GH content did increase in this study, at 3 and 24 hours after EtOH treatment, but the effect did not reach statistical significance, most likely due to a high degree of variability between animals. Bercu *et al* (1991) observed that sex differences in GH secretory patterns of rats was due to differential responsiveness to growth hormone-releasing hormone (GHRH). EtOH could lead to intracellular perturbations that cause diminished response to GHRH and produce a lowered GH secretory pattern. Interestingly, there was a very large range in measured immunoreactive serum GH in control, saline injected rats, which was not seen in the

EtOH treated animals. This large variation was anticipated in light of the long recognized episodic nature of GH secretion and similar observations by others of wide swings in GH levels when individual animals were sampled only once, as in the present study (Emanuele, 1992; Zeitler, 1991; Frohman, 1986; Tannenbaum, 1991). In the EtOH animals there was no such variation suggesting that the normal surges of GH secretion were abolished by EtOH. In studies of animals sampled repeatedly, EtOH has, in fact, been shown to do this (Redmond, 1980).

An alternative explanation for the decrease in serum GH is that EtOH somehow leads to increased metabolic clearance of the hormone from the bloodstream. In this model, alcohol could induce liver enzymes that would speed up GH protein turnover. However this might also be expected to result in increased clearance of PRL as well, and in light of increased serum PRL, this does not seem to be the case.

Finally, the possibility exists that the decrement in serum GH is due to a stress effect of handling and injections or of ethanol itself, since GH secretion is suppressed in rats by stress (Bercu, *et al* 1991). However, control animals are handled in an identical manner as EtOH animals and given an injection of an equal volume of saline, without showing diminished GH levels. The "stress of intoxication" in these alcohol naive rats may play a role in the GH suppression, but the fact that EtOH can suppress GH secretion from pituitaries *in vitro* argues against this (Emanuele, *et al* 1989).

The EtOH-induced suppression of GH mRNA suggests that EtOH may have an impact not only on GH secretion but on GH synthesis as well. The results described here have also shown a decrease in steady-state levels ofPRL mRNA but not in the

levels of the transcription factor Pit-1 (GHF-1). Pit-1 is a POU/homeodomain transcription factor shown to be important in regulation of both GH and PRL (as well as thyrotropin) transcription (Mangalam, *et al* 1989). The observation of no change in Pit-1 mRNA levels does not rule out the possibility that other alterations in this protein may have taken place as a result of EtOH exposure. For example, changes in the phosphorylation state of Pit-1 can affect the DNA binding capabilities and transactivation potential of this factor (Mangalam, *et al* 1989). Further studies are required to determine if this is the case. The results observed in this study of decrements in GH and PRL mRNA, but not Pit-1 message indicate that the effects of EtOH on pituitary hormone mRNAs are not global. This is in agreement with recent reports that intraperitoneal EtOH administered to castrated male rats resulted in a dramatic 80% reduction in LHβ mRNA, but no change in FSHβ mRNA or the mRNA for the common α-subunit for these hormones (Emanuele, *et al* 1991). This change was coupled with significant falls in serum levels of both LH and FSH. The biological significance of the modest 30% decrease in GH and PRL mRNA is difficult to determine. However, any disruption in gene expression has the potential for far-reaching effects given the importance of these hormones. The findings of no change in pituitary GH mRNA in the double binge model reported here is at variance with the single binge data, showing a modest but statistically significant fall in GH mRNA after a single binge. The reasons for this discrepancy are unclear, but in a recent study examining GH mRNA levels in animals exposed to EtOH for 6 days a similar lack of change was noted (Soszynski, 1992b), indicating that repeated or long-term exposure

may lead to compensatory mechanisms being activated.

Since steady-state mRNA levels in a cell represent a balance between the intracellular processes of synthesis (transcription) and degradation (targeted nuclease digestion) (Peltz, et al 1991), attempts were made to discern whether the changes observed were due to alterations in the balance of these processes. Nuclear run-on transcription assays were performed to determine if acute EtOH had the ability to alter the rate of transcription of the GH and PRL gene, leading to a decrease in the steady-state mRNA levels. The results of these experiments indicate that alcohol can influence the rate of transcription of GH and other genes in a time-dependent fashion. At 3 hours after injection, the time point associated with the largest fall in serum GH and steady-state GH mRNA levels, the amount of nascent GH mRNA was approximately (30)% of control levels. Transcription rates were also repressed for the PRL and for the Pit-1 gene (50% of control levels), indicating that the effects of EtOH are pervasive, and that hormone gene expression in the pituitary is affected at multiple levels.

However, it should be noted that the decrease in the transcription rate of the GH gene in response to EtOH cannot fully explain the decreases in serum GH and steady-state GH mRNA levels since the published half-life of GH mRNA is in the range of 12-24 hours (Gertz, *et al* 1987; Yaffee and Samuels 1984). Also, no change in Pit-1 steady-state mRNA levels was observed at times when Pit-1 transcription rates were diminished. An alternative possibility or contributing factor is an EtOH-induced decrease in the stability of the GH mRNA molecules leading to an increase in the

turnover rate of this message and hence, less mRNA translated into protein. GH mRNA half-life can be dramatically lengthened or shortened through changes in stability by various agents including other hormones (Carter *et al* 1993). Diamond and Goodman (1985) reported that in the absence of thyroid hormone, the GH mRNA half-life in culture was reduced from 20 hours to 2 hours.

It is also possible that EtOH is affecting some other hormone such as thyroid hormone, and indirectly destabilizing the GH message. While this is an attractive possibility, the current techniques to measure half-lives are not feasible in an *in vivo* system. The application of transcriptional inhibitors such as actinomycin D or thiolutin to cells in culture is a well-accepted method for measuring rates of mRNA turnover in response to various agents (Parker *et al* 1991). While this type of measurement in pituitary cells cultured in the presence or absence of EtOH could yield valuable data on alcohol's effects on pituitary hormone half-lives, the results may not be relevant to the present study due to differences inherent in *in vitro* versus *in vivo* systems (physical separation from hypothalamic influences *in vitro,* for example). Additionally, transcriptional inhibitors administered to animals would effectively halt all transcription, including genes which may affect GH regulation in other ways, masking or confusing the effects of the alcohol alone.

Another factor which has been suggested to be associated with changes in mRNA stability is poly-A tail length (Peltz, *et al* 1991). Hormones, growth factors and pharmacologic agents are known to affect poly-A tail length of a number of

mRNAs including those of GH and PRL (Murphy *et al*, 1992; Carter *et al*, 1993). Attempts were made to determine if acute EtOH exposure in this study led to changes in GH or PRL transcript size. The results showed a slight, but most likely insignificant, decrease in GH transcript size in EtOH-treated animals when run on high percentage agarose gels (data not shown). More sensitive techniques are required to determine if this effect is real or artifactual.

B. The Effects of Ethanol on Prolactin

As has been previously described by several laboratories (Chapin, *et al* 1980; Bertello, *et al* 1983; Salonen and Huhtaniemi, 1990), acute EtOH treatment in this study led to a significant increase in the levels of serum PRL in male rats. This effect is rapid, with PRL levels raised significantly over controls at 1.5 and 3 hours after single binge EtOH treatment. The action of ethanol on serum PRL levels could result from a stimulatory effect on the secretion of a residual PRL reserve in the pituitary or be secondary to a direct effect on DA levels in the hypothalamus. Though there was a higher average PRL content in pituitaries of alcohol-treated rat 1.5 hours after injection, this was not significantly different from controls, nor were there any significant differences in PRL content between alcohol and control groups at any time point examined. Other investigators have found pituitary prolactin content decreased after chronic alcohol exposure (Salonen and Huhtaniemi, 1990). This finding argues in favor of increased secretion of PRL in response to alcohol and neatly explains the raised serum PRL levels, but this result could not be duplicated in the present, acute

exposure study. This may be due to one of several situations. First, it may well be that the actual amount of extra secreted PRL is small compared to the total pituitary PRL pool and thus any pituitary differences might be so low as not to be easily detected. Indeed, the average rise in serum PRL between EtOH and control groups of 12-14 ng/ml, reflects an increased total secretion of only 120-140 ng, assuming distribution through a 10 ml volume of rat blood. Such an amount would be small when compared to the approximately 25,000 ng total content of pituitary PRL seen in these studies. The rise in serum PRL after acute EtOH exposure reported here has also been demonstrated in *in vitro* studies of male rat pituitary cells (Emanuele, *et al* 1987) and by others in male rat (Seilicovich, *et al* 1982) and has been attributed to the "stress" effect of drinking on this stress-responsive hormone. Similarily, the ability of ethanol to increase the activity of the hypothalamic-pituitary-adrenal axis, another stress-responsive hormonal system, has been documented in both acute and chronic studies (Rivier, *et al* 1984). An EtOH induced increased rate of CRF biosynthesis was noted (Rivier, *et al* 1990) in addition to an altered ability of CRF or stress to stimulate ACTH secretion. The mechanisms through which these stress changes take place, however remains speculative. Numerous changes in the brain levels and/or rate of turnover of neurotransmitters such as dopamine, acetylcholine and norepinephrine have been reported after EtOH exposure (Hunt, *et al* 1979), in addition to effects on thyrotropin releasing hormone (Rudeen and Zoeller 1991) which is known to regulate PRL release. The fact that ethanol alters the secretion of a brain neurotransmitter, however, only suggests a potential role for this mediator in EtOH-induced hormone changes, without

explaining the mechanisms involved in this change.

As previously mentioned, PRL mRNA was significantly supressed at 1.5 and 3 hours after acute EtOH administration. It is difficult to reconcile this observation with the significantly increased levels of PRL protein in the serum at these same time points. However, this result serves to demonstrate that the processes of gene regulation and hormone secretion are not inextricably linked. It also demonstrates once again that the effects of acute EtOH on endocrine cells is selective and not global, as some systems within the same cell are increased, while other are decreased.

C. The Effects of EtOH on Hypothalamic Regulatory Hormones

Another goal of the studies reported here was to determine the impact of EtOH in this single and double binge model on the synthesis of GHRH, and somatostatin (SRIF) the two major hypothalamic hormones controlling pituitary GH (Frohman, 1986; Tannenbaum, 1991). As previously discussed, serum GH is suppressed after both single and double binge alcohol exposure, at 1.5 and 3 hours after treatment. While hypothalamic GHRH content was unchanged, single binge alcohol significantly increased the steady-state mRNA for this hormone, at 3 and 6 hours post treatment. The increase in GHRH mRNA also occurred at the 3 hour time point in the double binge experiment, a time when serum GH levels were still markedly depressed. Since GH itself has been shown to have a negative feedback effect on GHRH mRNA (Zeitler, 1991; Frohman, 1986; Tannenbaum, 1991), this rise in GHRH mRNA, at a time when GH was sharply reduced, becomes all the more significant. GHRH mRNA levels then

gradually returned to control levels by 24 hours.

Taken together, these data suggest an interesting model. The drop in serum GH and the subsequent rise in GHRH mRNA represents the hypothalamus reacting to the continued fall in serum GH. At later time points, as EtOH metabolites are cleared and GH mRNA levels and serum GH normalize (in response to elevated GHRH), GHRH itself falls toward control levels. After prior exposure to alcohol, the response may be more rapid, with no alterations in GH mRNA levels. It thus appears that the hypothalamic-arcuate nucleus neuronal system is rapidly responsive to perturbations in ambient levels of GH. Since somatostatin mRNA levels are unaffected by EtOH, it appears that the control point is at the level of GHRH.

D. Effects of Acute EtOH on Second Messenger Systems in the Pituitary

The sharp ten-fold rise in pituitary cAMP levels after acute EtOH treatment is intriguing. The data presented here reflects total pituitary cAMP and, thus, it cannot be determined whether EtOH's effect is seen in all the different cell populations of pituitary (e.g. lactotropes, somatotropes, gonadotropes, etc.) or is restricted to a few. The former possibility is more likely the case since data from several other laboratories have consistently demonstrated that EtOH acutely causes enhanced cAMP accumulation in a diverse variety of cells including hepatocytes, (Hoek, *et al* 1981), NIE-115 neuroblastoma cells (Gayer and Gordon), PC12 cells (Rabe and Weight, 1988) and platelets (Gordon, *et al* 1986). Thus, it seems reasonable to suppose that the cAMP response to EtOH in pituitary cells is a generalized cellular phenomenon, and our

findings of increased total pituitary cAMP reflect rises in each of the cellular subtypes. It is relevant that cAMP has been shown to stimulate transcription and steady-state levels of the, GH and PRL genes as well as secretion of both of these hormones. The elevated cAMP levels seen in our EtOH treated rats compared to control rats might then be expected to result in substantial increases in steady-state levels of GH and PRL mRNA's. Thus, the 30% decrement in GH andPRL messages in EtOH treated rats and the lack of apparent change in Pit-1 message might be inappropriate in light of the sharp cAMP rise. There are several potential explanations for this. It may be that EtOH did not raise cAMP levels in somatotrope or lactotrope populations in the pituitary, leaving the possibility open for decreased GH and PRL gene transcription, and/or diminished stability of these messages. This seems unlikely in light of evidence that EtOH enhances intracellular cAMP accumulation in response to GRH, indicating that somatotropes may be targeted by EtOH (Soszynski and Frohman 1992a). Alternatively, EtOH could be inducing GRF and TRH receptor down regulation via cAMP elevation. In other cell systems, cAMP is known to mediate receptor down-regulation via phosphorylation after cAMP and protein kinase A have been activated (Collins, *et al* 1991). Also, the discrepancies may be explained by desensitization of cAMP-dependent pathways of intracellular signalling by high cAMP levels (Hausdorff, *et al* 1990). Therefore, in the system described here, the decrease in GH and PRL mRNA levels might be attributed to diminished GRF and/or TRH receptor number on pituitary cells secondary to EtOH-induced cAMP elevation. A final potential explanation for the elevated cAMP levels could be the known increase in membrane

fluidity that occurs in response to EtOH exposure (Hoek, *et al* 1988). Most investigators, however, agree that bulk membrane lipid pertubations do not totally account for the deleterious actions of EtOH, and there is unanimity of opinion that specific targets within the cell membrane exist for EtOH (Tabakoff, *et al* 1988). Further studies involving membrane fluidity measurements in pituitary cells exposed to EtOH are required to determine the degree of this potential influence. Although the cAMP response to EtOH is short-lived, it should be noted that a short-term activation of signal transduction systems may result in the phosphorylation of a wide range of intracellular target proteins, effects that may persist for a considerable period of time after the actual exposure to ethanol has subsided.

The results showing a rapid induction of the *c-fos* gene with acute EtOH treatment raises some interesting possibilities. The induction may be part of a stress response to alcohol, but not handling or injection stress since control animals were not induced to the same level (fig 25). Alternatively, it represent a non-specific perturbation of the pituitary membrane and a subsequent activation of protein kinase pathways. Studies have shown that cAMP is a positive regulator of *c-fos* mRNA levels in BALB/c 3T3 fibroblasts, provided the cells recieve a simultaneous signal, probably calcium influx (Ran, *et al* 1986). Therefore, a third possibility is the c-fos induction seen in the present study may be a secondary response to cAMP elevation, since both events occur very rapidly after exposure to EtOH and in the same general time frame (compare figs. 24 and 25) and, EtOH added to pituitary cells in culture has been shown

to result in a calcium influx into these cells (Sato, *et al* 1990). Correlations have been made between *c-fos* induction and GH and PRL gene expression (Billestrup, *et al* 1987; Davis, 1990). However, as is the case for the increase in cAMP, it is difficult to ascertain which systems may be affected by the transient increase in *c-fos*, since measurements were made in whole pituitaries, which are composed of many cell types. There may be at least a functional relationship between the PRL gene and that of *c-fos*. Although *c-fos* has a very general role in cell function, it shares marked calcium dependence with the PRL gene (Bandyopadhyay and Bancroft, 1989), and *c-fos* mRNA levels are regulated similarly to prolactin secretion (Gourdji, *et al* 1989). Moreover, one report suggests that the *c-fos* gene was amplified in cells from a human prolactin-secreting tumor (U, *et al* 1988). In this study elevated serum PRL is seen 1.5 hours after EtOH exposure fitting into a time frame which may account for *c-fos* stimulation. This fails to explain however the fall in serum GH at the same time point. A functional link between *c-fos* and the GH and PRL genes remains speculative, but it may be of interest in that even transient elevations of this transcriptional activator could have long-lasting effects on cell function.

In summary, the studies reported here provide further insight into the molecular and physiological mechanisms by which EtOH disrupts pituitary GH and PRL secretion. These and other studies suggest that EtOH acts both at the brain and anterior pituitary levels. Further, this study indicates that within these loci, EtOH has multiple effects including alterations in steady-state mRNA levels, gene transcription rates,

secondary messenger systems and hormone secretory dynamics. The effects of EtOH are selective, and not global in nature. Some hormone systems are affected at many levels, while others remain unchanged by the drug. Additionally, these studies indicate that the GHRH-GH system, though initially affected by EtOH, is able to compensate for these disturbances and operates rapidly to restore GH homeostasis, especially when subjected to repeated ethanol exposure.

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VITA

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In August, 1979, Mr. Tentler entered Marquette University in Milwaukee, Wisconsin. He received the degree of Bachelor of Science in Biology from Marquette in December, 1983. The following year, 1984, John joined the Biochemical Endocrine Research Laboratory at Hines V.A. Hospital, Hines Il. as a research assistant under the direction of Drs. Ann M. Lawrence, Nicholas Emanuele, and Mary Ann Emanuele. In August, 1990, he enrolled in the Department of Molecular and Cellular Biochemistry at Loyola University of Chicago, Maywood, IL. He joined the laboratory Dr. Mary Ann Emanuele and Dr. Mark R. Kelley where he continued research on the effects of ethanol on pituitary hormones in the male rat. In 1992, he recieved a National Institutes of Health Predoctoral Fellowship. He is a student member of the Endocrine Society, The National Association for the Advancement of Science, and The Research Society on Alcoholism.

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The final copies have been examined by the director of the Dissertation committee and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the Dissertation is now given final approval by the Committee with reference to content and form.

The Dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Ph.D.

11/7/94
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