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Ethanol regulates tyrosine hydroxylase gene expression

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ETHANOL REGULATES TYROSINE HYDROXYLASE GENE EXPRESSION

by

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DISSERTATION

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To my parents,

Harold R. Gayer
Angeline E. Gayer

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ETHANOL REGULATES TYROSINE HYDROXYLASE GENE EXPRESSION

ABSTRACT

Catecholaminergic neurotransmission is important in the development of tolerance to ethanol. Tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine biosynthesis, is regulated by second messenger systems that are modulated by ethanol. Therefore, ethanol-induced changes in TH gene expression may be central to tolerance development. N1E-115 neuroblastoma cells were used in this study. Northern and Western blot analyses showed that treatment with 25-200 mM ethanol for 3 days caused a dose-dependent increase in TH-mRNA and protein levels. To determine ethanol-induced changes in TH promoter activity, N1E-115 cells were transfected with pTH5'CAT and stable clones were isolated. Subclones expressing pTH5'CAT showed ethanol-induced increases in chloramphenicol acetyltransferase (CAT) activity, suggesting that ethanol modulates TH gene transcription. Simultaneous treatment of transfected cells with 100 mM ethanol and either prostaglandin E_1 (PGE$_1$), (-)-N^6-(R-phenylisopropyl)-adenosine, or 8-bromo-cAMP, potentiated TH-promoter activity when compared to treatment with the respective agents alone. N1E-115 cells were also transfected with a plasmid containing the minimal cAMP responsive portion of the human vasoactive intestinal peptide gene fused to the CAT gene, and stable clones were isolated. In CRE-CAT containing clones ethanol did not stimulate basal CAT activity. Therefore, mechanisms other than cAMP-mediated processes may also be involved in ethanol regulation of basal TH expression.

Coincubation of CRE-CAT-clones with ethanol and PGE_1, however, resulted in increased CAT activity compared to cells incubated with PGE_1 alone. These data suggest that chronic ethanol exposure has a prominent effect on both basal and cAMP-regulated TH expression. Using deletion analysis of the cis-acting regulatory sequences of the TH-promoter, the regions responsible for both basal and cAMP-mediated ethanol induction were characterized.

Ethanol-induced increases in TH expression may help to explain how chronic ethanol consumption alters catecholamine neurotransmission. Furthermore, because the catecholaminergic system participates in the development of tolerance, increases in TH expression may be central to tolerance development. Finally, insights gained in how TH expression is altered by ethanol may lead to a better understanding of how ethanol alters gene expression in general.

Adrienne Gordon, Ph.D.

Thesis Committee Chairman

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Chapter I

RATIONALE

1.1   Catecholamines and Alcoholism

1.1.1   *Alcoholism*- Alcohol abuse and dependence (alcoholism) affect about 10 percent of adult Americans and constitute one of the world's largest public health problems. In the United States alone, the cost of alcohol abuse and dependence was estimated at $116.9 billion in 1983, of which 61 percent was attributed to losses in work productivity and employment and 13 percent to health care costs [1]. Unfortunately, the price paid is more than monetary. A minimum of 3 out of 100 deaths are due to chronic ethanol exposure related causes, such as, cirrhosis, cancer, heart disease, suicide, homicide, and highway fatalities [1]. In addition, chronic ethanol consumption results in several neurodegenerative diseases, including, Wernicke's encephalopathy, central pontine myelinolysis, and Marchiafava- Bignami [2]. Contributing to the severity of these medical complications and possibly the maladaptive drinking patterns common to alcohol abuse is the development of tolerance. Tolerance development allows individuals to consume greater amounts of ethanol which, in turn, increases the risk and severity of ethanol-induced pathology. Many behavioral and pharmacological studies have, therefore, focused on understanding the neuronal pathways important in the development of tolerance. However, the biochemical and molecular events altered by chronic ethanol consumption that lead to the development of tolerance are unclear and therefore are the focus of this thesis.

The following sections discuss the types of tolerance important in the adaptation of the central nervous systems (CNS) to chronic ethanol

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consumption. The role of catecholamine neurotransmission and the possible role of tyrosine hydroxylase in the development of tolerance is also discussed.

1.1.2 *Types of tolerance* - Tolerance is defined as an acquired resistance to the effects of ethanol. This resistance is produced by two major types of tolerance, dispositional (pharmacokinetic) and functional (pharmacodynamic) [3]. Dispositional tolerance refers to an alteration in the rate of metabolism, absorption, distribution, or secretion. Functional tolerance has been variously suggested to be produced by compensatory adaptation in membrane fluidity [4], neurotransmitter synthesis [5], neuroreceptor number or sensitivity [6, 7], second messenger generation [8, 9, 10] and ion channel number and activity [11, 12]. It is clear that changes in disposition rates of ethanol in the body, although they occur, are not sufficient to account for the tolerance observed in humans. For example, metabolism and excretion of ethanol are at most increased about two-fold after chronic ethanol consumption [13]. However, alcoholics have been reported to survive or even remain sober with blood ethanol levels above 100 mM [14]. This concentration of ethanol is usually associated with severe sedation or death and is approximately 7 times the legal limit of blood ethanol for operating motor vehicles. These data indicate that alterations in brain function must play an important role in the adaptation to chronic ethanol.

1.1.3 *Catecholaminergic neurons and tolerance-* Several studies demonstrate that catecholaminergic (dopamine, norepinephrine, and epinephrine) neurotransmission is necessary for the development of functional tolerance. For example, blocking catecholamine synthesis [15] or receptor activity [16] prevented the development of tolerance in mice. Tabakoff et. al.,

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observed that specific ablation of catecholaminergic neurons with 6-hydroxydopamine (6-OHDA) before chronic ethanol exposure prevented the development of tolerance to the hypnotic and hypothermic effects of ethanol as well as cross-tolerance to barbiturates in mice that consume the same volume of ethanol [17, 18]. Treatment with 6-OHDA did not effect the behavioral signs of acute intoxication or the severity of withdrawal symptoms after stopping ethanol administration [17, 18]. In addition, the development of tolerance to morphine was not effected by 6-OHDA treatment [19]. Thus, the effects of 6-OHDA treatment were selective in inhibiting ethanol tolerance and not a general effect on neural function. These findings, however, do not distinguish whether ethanol-induced changes in catecholaminergic function are a direct effect of ethanol or are secondary to ethanol-induced changes in other neurotransmitter systems. It is possible that alterations in catecholamine neurotransmission represent the accumulation of ethanol's effect on several other neuronal cascades. Indeed, compensatory responses occur in many neuronal messenger systems following chronic ethanol exposure (see section 1.3). More defined model systems without heterosynaptic inputs are needed to distinguish between the direct and indirect effects of chronic ethanol on catecholaminergic activity. Regardless of how ethanol is altering catecholaminergic function, these studies indicate that intact catecholamine neurotransmission is necessary for the final expression of tolerance.

Ablation of noradrenergic neurons also prevents the effects of other hormonal and neurotransmitter systems that influence the maintenance or rate of tolerance development to ethanol. For example, serotonergic neurotransmission has been observed to alter the rate of tolerance development to the hypothermic and motor impairment effects of ethanol. This effect has been demonstrated by treatment of rats with either the

serotonin precursor, l-tryptophan, or inhibitors of serotonergic function that accelerate and retard the development of tolerance, respectively [20]. Complete blockade of tolerance development was only demonstrated when both noradrenergic and serotonergic neurons were ablated [21]. Similarly, arginine vasopressin (AVP) administration maintains tolerance to the hypnotic effects of ethanol even in the absence of further ethanol consumption [22]. In mice, administration of 6-OHDA prevented this effect of AVP [23]. These findings suggest that the chronic ethanol-induced changes in seratonin and AVP transmitter pathways are mediated through catecholaminergic neurones. These studies further support the role of catecholamine neurotransmission in the development of tolerance.

1.1.4 *Genetic animal models* - Selective breeding of rats leads to genetic differences between rat lines and is used to identify the processes underlying ethanol-induced alterations in behavior, such as the development of tolerance. For example, ethanol sensitive Alcohol Nontolerant (ANT) and ethanol insensitive Alcohol Tolerant (AT) rat lines were selectively outbred for differences in ethanol-induced motor impairment [24]. Subsequently, AT rats were shown to develop tolerance to the hypnotic effects of ethanol more readily than ANT rats. Studies suggest that catecholaminergic neurotransmission is involved in the differences between the two lines. For example, the amount of dopamine per gram of tissue was significantly higher in the striatum and limbic forebrain of ANT rats compared to AT rats [25]. ANT rats are also more sensitive than AT rats to ethanol-induced decreases in dopamine levels following α-methyl-tyrosine pretreatment [25]. α-Methyl-tyrosine is a specific inhibitor of the rate limiting enzyme for catecholamine biosynthesis, tyrosine hydroxylase (TH). Thus, ANT rats turnover dopamine pools in the limbic

forebrain and striatum faster in ANT rats than AT rats. These findings demonstrate that rat lines differing in their ability to develope ethanol tolerance have dissimilar dopaminergic levels and turnover. Furthermore, these studies provide additional evidence for the correlation between the development of tolerance and changes in catecholamine neurotransmission. The differences between these two rat lines, however, are not restricted to the catecholaminergic system. For example, GABA turnover has been reported to be increased in ANT compared to AT rats [26]. Thus, other neurotransmitter systems may also contribute to the differences in ethanol sensitivity observed between the two rat lines.

Differences in catecholaminergic function has also been demonstrated in long sleep (LS) and short sleep (SS) mice. LS and SS mice were genetically selected and differ in the duration of the loss of the righting reflex following administration of ethanol [27]. Acute ethanol exposure increased adrenal TH activity in LS mice and decreased activity in SS mice [28]. Baizer et. al. also found that hypothalamic TH activity was significantly increased at 25 minutes in the SS mice and 125 minutes in the LS mice following acute ethanol exposure [29]. The duration of 25 and 125 minutes directly corresponds with the time it takes to regain the righting reflex in SS and LS mice, respectively. Basal TH activity was identical between the two mouse lines in all brain region studied. Furthermore, no increase in TH activity was seen in the hypothalamus when animals were sleep-induced by pentobarbital treatment. These controls suggest that the increases in TH activity were a result of and specific to ethanol administration. The results described above suggest that TH is involved in the behavioral differences seen in these mice. This data does not rule out the involvement of other neuronal systems in the behavioral differences seen in these mice lines but does provide another example that

selection based on differences in behavioral sensitivity to ethanol leads to measurable changes in catecholamine function.

1.1.5 *Catecholamine levels and turnover* - Taken together, these studies suggest that catecholaminergic mechanisms are involved in the development of tolerance. Ethanol-induced alterations in the catecholamine system could be produced by increased synthesis or release of catecholamines. Therefore, many researchers have attempted to measure changes in the levels and turnover of catecholamines. However, the data measuring catecholamine levels in animal models is not conclusive. Effects of ethanol on brain catecholamine levels vary both in direction and magnitude depending on ethanol dose, region of measurement, time and method of ethanol administration. The clearest evidence for the acute effects of ethanol on catecholamine levels was performed by Di Chiara and Imperato. These researchers used transcerebral dialysis in freely moving rats to demonstrate increased dopamine release in the nucleus accumbens and the dorsal caudate nucleus following acute ethanol administration [30]. Transcerebral dialysis avoids the complications that result from decapitation and dissection, the standard method of measuring catecholamine levels. Handling stress and decapitation are known to alter catecholamine levels. Studies using standard methods have reported decreases [31], or no change [32] in both dopamine and norepinephrine levels following acute ethanol administration. Catecholamine levels have also been reported to increase [33], decrease [34] or remain unaltered [28] in response to chronic ethanol treatment or withdrawal. These studies did not use transcerebral dialysis to measure catecholamine levels.

There is, however, general agreement that chronic ethanol exposure and withdrawal from ethanol increase norepinephrine turnover. Neurotransmitter

turnover reflects the activity of neurons using a given transmitter. Turnover is measured by either observing the depletion rate of catecholamines after inhibiting the rate limiting biosynthetic enzyme, the conversion of [^{3}H]-tyrosine into [^{3}H]-catecholamines, or the conversion of [^{3}H]-norepinephrine to [^{3}H]-metabolites. Chronic ethanol treatment increased norepinephrine turnover when measured by all of the above methods [32, 35, 36]. Taken together, these data suggest that chronic ethanol exposure results in increased catecholaminergic neurotransmission.

1.1.6 *Tyrosine hydroxylase (TH) and ethanol-* TH is a mixed function monooxygenase that catalyzes the initial, rate-limiting step in catecholamine synthesis by hydroxylating tyrosine (1 in Fig. 1) to form dihydroxyphenylalanine (L-DOPA) [37]. Tetrahydrobiopterin, a required cofactor, is oxidized during the course of the reaction [38]. TH containing nerve cell bodies are located mainly in nuclei of the brain stem, midbrain, olfactory epithelium, and adrenal glands [39]. DOPA is next decarboxylated by DOPA-decarboxylase (2 in Fig. 1) to form dopamine. The third enzyme, dopamine β-hydroxylase (3 in Fig. 1), converts dopamine to norepinephrine. The final enzyme in the pathway is phenylethanolamine-N-methyltransferase, which is predominantly expressed in the adrenal medulla (4 in Fig. 1). This enzyme methylates norepinephrine to form epinephrine.

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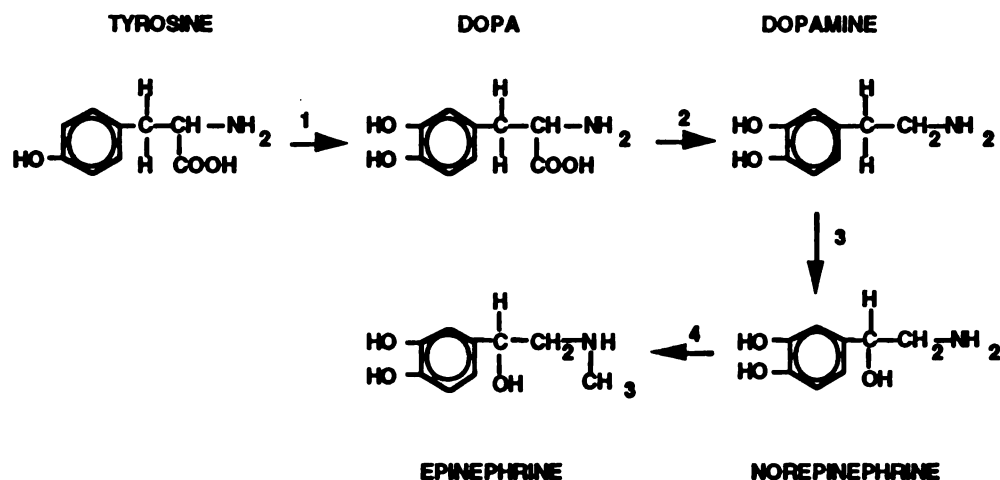
FIG. 1 **Catecholamine biosynthetic cascade.** Tyrosine is hydroxylated by tyrosine hydroxylase (1) to form dihydroxyphenylalanine or DOPA. DOPA is then converted to dopamine by DOPA decarboxylate (2). Dopamine is then hydroxylated by dopamine-β-hydroxylase (3) to form norepinephrine. Finally, norepinephrine is methylated by phenylethanolamine-n-methyl transferase (4) to form epinephrine.

Studies involving direct measurements of TH suggest that ethanol exposure to animal models increases TH enzyme activity. For example, acute ethanol treatment increases TH enzymatic activity in the locus coeruleus, striatum, frontal cortex [28], medulla oblongata [40], and adrenal glands [41] of rats. The effects of chronic ethanol on TH enzyme activity, however, are less clear. Increases in TH activity in the brainstem and adrenal glands of rats following chronic ethanol intake has been found [35, 42]. No change in TH enzyme activity measured in the same tissues as above has also been reported [43]. These disparate results may be due to differences in the duration and/or route of ethanol administration.

The above studies taken together indicate that catecholamine pathways are important for the development of tolerance. These studies also demonstrate that increases in catecholamine turnover are involved in the chronic effects of ethanol. Increased catecholamine turnover is produced by an

increases in synthesis, release or metabolism. Some reports have demonstrated chronic ethanol-induced increases in TH activity. This suggest that ethanol-induced increases in catecholamine synthesis may occur. Therefore, TH, the rate limiting enzyme for catecholamine synthesis, is a likely candidate for mediating at least some aspects of CNS adaptation to long term ethanol consumption. Further evidence supporting a possible involvement of TH expression in the chronic effects of ethanol comes from the study of second messenger systems. Perhaps due to its central role in catecholamine biosynthesis, TH is highly regulated by several second messenger cascades. As discussed below, ethanol exposure has been shown to alter the same second messenger cascades that regulate TH expression.

1.2   Regulation of Tyrosine Hydroxylase

In the short term (minutes), TH function is modulated by phosphorylation, which decreases the K_m for the required pteridine cofactor and increases the K_i for feedback inhibitors. In the long term (hours to days), activity is regulated by increased synthesis of new protein. Physiologic stress (cold or insulin), non-physiologic stress (electric shock or immobilization) [44, 45, 46], reserpine treatment [47], transynaptic nerve stimulation mediated by trophic factors [48], cholinergic [49], peptidergic [50], adrenergic [51], purinergic [52], and prostaglandin E_1 receptors [53], increase activity of preexisting enzyme. Long term exposure to these stimuli causes increased amounts of TH mRNA and protein [54, 55, 56, 57, 58, 59, 60].

Recent evidence suggest that these changes in TH expression are due to transcriptional and post-transcriptional regulation by second messengers. For example, *in vivo* studies show that second messenger   activation of

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Ca^{2+}/calmodulin-dependent protein kinase (CAM-K), Ca^{2+}/phospholipid-dependent protein kinase (PKC), cAMP-dependent protein kinase (PKA), or cGMP-dependent protein kinase (PKG) leads to the phosphorylation of TH and subsequent increase in TH enzyme activity [61, 62, 63, 64]. These kinases are activated by increases in their respective second messengers, Ca^{2+}, diacylglycerol, cAMP, and cGMP. *In vitro,* TH is phosphorylated by all of these kinases at serine residue 40 (Fig. 2) [65]. PKA and PKG phosphorylate TH at serine residue 143 as well [65, 66]. CAM-K also phosphorylates TH at serine residues 19 and 40 [65]. Interestingly, activation by CAM-K differs from that due to PKA because a 70 kDa protein is required for activation of TH by CAM-K [67].
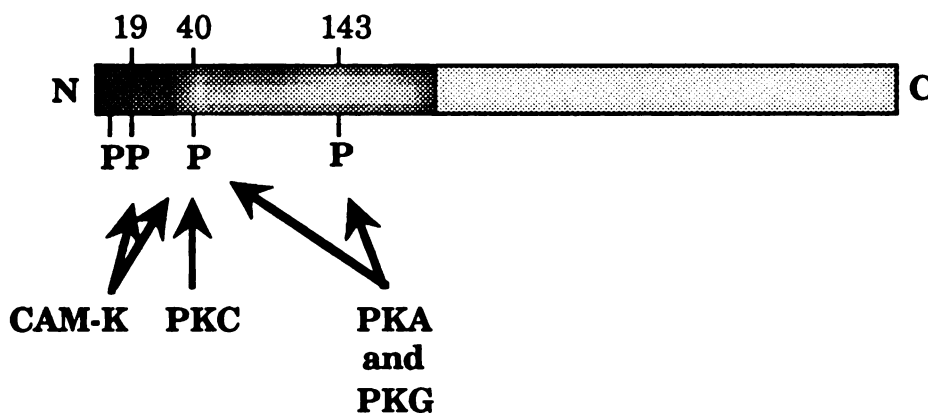
FIG. 2 Phosphorylation sites of tyrosine hydroxylase. TH is phosphorylated on serine residues 40 and 143 by PKA and PKG. PKC also phosphorylates at serine 40. CAM-K can phosphorylate TH on serine residues 19 and 40. TH is also phosphorylated at serine residue 8 by a TH specific kinase [65].

Nerve growth factor (NGF)-induced phosphorylation may be mediated through either PKA or PKC, since treatment of PC12 cells leads to phosphorylation of TH at the same serine residues as PKA and PKC [48].

These same second messengers and their respective kinases also increase transcription of the TH gene. For example, activation of PKA by elevating intracellular cAMP or activation of PKC by treatment with phorbol esters increases TH gene transcription in PC12 cells and cultured adrenal medullary cells [68, 69, 70]. Incubation with Ca^{2+} ionophore A23187 also increased TH mRNA levels in cultured adrenal medullary cells, suggesting a role for calcium signaling in TH expression [70]. These kinases increase TH gene expression by phosphorylating DNA-binding factors which when bound to specific nucleotide sequences in the TH promoter induce transcription of the TH gene. The mechanism of transcriptional activation mediated by PKA, PKC, and CAM-K is discussed in more detail in sections 1.4 and 5.1.

Other agents or conditions that increase TH mRNA levels include, epidermal growth factor [59], increases in cell contact [71], and differentiation [72]. The second messengers underlying these latter inductions have not yet been identified. It has, however, been shown that NGF-induction of TH promoter-directed chloramphenicol acetyl transferase (CAT) activity in PC12 cells is mediated through c-fos and c-jun binding to the AP-1 site in the TH promoter [73]. Since TH is regulated both transcriptionally and postranslationally by PKA, PKC and possibly CAM-K, it is likely that drugs, such as ethanol, which alter second messenger systems and subsequent activation of these kinases, will regulate TH expression.

1.3   Ethanol and Second Messengers

Ethanol is a small amphipathic molecule that, similar to other anesthetics, appears to interact physically with neural membranes [74, 75]. It is thought that by inserting into the lipid bilayer, ethanol alters the mobility

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of lipids or the interactions of lipids with the hydrophobic portions of proteins residing in the membrane, thus altering their function. Indeed, ethanol-induced changes in the function of many integral and peripheral membrane proteins important for signal transduction have been observed. These include GTP-binding proteins [8, 10], ion channels [8, 10, 11], and neurotransmitter receptors [6]. Examples of recent studies which demonstrate that ethanol alters the production of the second messengers cAMP, inositol-1,4,5-trisphosphate (IP3), diacylglycerol (DAG) and calcium are described below.

1.3.1 *Cyclic-AMP-* Intracellular cAMP is generated by a cascade of events initiated by the occupation of receptor by agonist. This promotes the nucleotide exchange of GDP for GTP on the α subunit of the heterotrimeric GTP-binding protein, Gs, located on the intracellular face of the plasma membrane. With GTP bound, the α subunit dissociates from the β and γ subunits of the G-protein complex. GTP-bound Gαs then activates adenylyl cyclase resulting in cAMP production.

In general, acute ethanol treatment has little effect on basal, but increases receptor-stimulated cAMP production . This effect of ethanol was shown in mouse striatal preparations [76], cultured neural cells [9, 77], and human lymphocytes [78]. These studies suggest that the acute effects of ethanol on cAMP are not due to changes in agonist binding to receptors but rather result from increased coupling of Gαs to adenylyl cyclase [79, 80, 81]. For example, ethanol treatment of mouse striatal preparations increased dopamine-stimulated adenylyl cyclase activity without affecting dopamine binding to its receptors [76]. Furthermore, Luthian and Tabakoff demonstrated that activation of adenylyl cyclase in mouse striatal membrane preparations by treatment with Gpp(NH)p or cholera toxin could be further

increased by ethanol co-treatment [81]. These results suggest that ethanol treatment increased the ability of GTP-bound Gαs to interact with adenylyl cyclase.

In the cell culture models mentioned above, chronic ethanol exposure leads to a decrease in receptor-mediated cAMP production. The response to chronic ethanol exposure involves adaptive changes in receptor number, receptor affinity [6, 7], and amounts of Gs and/or Gi, the GTP-binding protein which inhibits adenylyl cyclase activity. For example, Mochly-Rosen, et al. [10] have shown that ethanol-induced heterologous desensitization of receptor-stimulated cAMP production in NG108-15 cells is due to ethanol-induced decreases in Gαs mRNA and protein. In N1E-115 and other neural cell lines receptor desensitization may result from ethanol-induced increases in Gαi [8].

1.3.2 *Inositol-1,4,5-trisphosphate and Diacylglycerol-* Acute ethanol exposure has also been shown to produce changes in phosphatidylinositol (PI) hydrolysis [82, 83, 84]. Hydrolysis of PI by phospholipase C produces the second messengers IP$_3$ and DAG. IP$_3$ acts as an intracellular second messenger to mobilize intracellular Ca^{2+}, and DAG is a required component for the activation of PKC [85]. Acute ethanol exposure has been shown to increase both basal and receptor-mediated PI hydrolysis. For example, serotonin-induced phospholipase C-activation in astrocytes is increased with ethanol co-incubation [84]. Ethanol treatment of isolated hepatocytes also rapidly and transiently increases basal phospholipase C activation leading to IP$_3$ formation and intracellular Ca^{2+} mobilization [83]. Inhibition of PI turnover, however, has also been reported. Addition of ethanol to rat cortical slices inhibits both carbachol- and norepinephrine-induced PI turnover [82]. The later finding may be due to methodology because IP$_3$ levels were measured

only after long reaction times. The chronic effects of ethanol on PI hydrolysis have not been studied.

1.3.3 *Calcium*- Due to the role of Ca^{2+} as a second messenger, the storage in intracellular organelles, as well as efflux and influx of this ion through the plasma membrane, is tightly controlled. Interestingly, acute ethanol treatment has been shown to differentially affect several aspects of calcium homeostasis resulting in a net increase in intracellular Ca^{2+} levels. For example, measurements of Ca^{2+} by fluorescent indicator dyes showed that ethanol treatment of brain synaptosomes or PC12 cells increased levels of intracellular Ca^{2+} [86, 87]. Shah and Pant, using brain microsomes, demonstrated that the ethanol-induced rise in intracellular Ca^{2+} could be due, in part, to release from the endoplasmic reticulum [88]. In addition to storage, efflux of Ca^{2+} through the Na^+/Ca^{2+} exchanger [89, 90], or by active transport through a Ca^{2+}-ATPase are inhibited and increased, respectively, by acute ethanol exposure. Chronic exposure to ethanol results in adaptive or opposite effects of acute ethanol exposure on the Na^+/Ca^{2+} exchanger [91] . For example, in rats fed ethanol for 3 weeks, increased Na^+-dependent Ca^{2+} transport activity was observed [91].

Influx of Ca^{2+} through voltage-dependent Ca^{2+} channels and the NMDA subtype of glutamate receptors is also altered by ethanol exposure. Voltage-dependent Ca^{2+} currents are reduced with acute ethanol treatment and chronic treatment leads to the opposite effect. Potassium-stimulated [45]Ca^{2+} uptake into rat brain synaptosomes or into cultured PC12 cells is inhibited by acute ethanol treatment [11, 92]. Chronic ethanol administered to PC12 cells increases [45]Ca^{2+} uptake [11]. This adaptive effect of chronic

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ethanol exposure was due to PKC-mediated increases in L-type Ca^{2+} channel expression [11, 93].

Influx of $[45]Ca^{2+}$ through the NMDA subtype of glutamate receptors is also inhibited by ethanol treatment of primary cultures of cerebellar granule cells, at concentrations as low as 10 mM [94]. Ethanol-induced inhibition of NMDA responses has created much excitement since this effect of ethanol occurs at concentrations associated with mild intoxication. Furthermore, it is the most potent effect of ethanol studied thus far.

Chronic ethanol treatment has been reported to increase NMDA receptor number [95]. The electrophysiological consequences of ethanol-induced increases in NMDA receptor number are not known. However, it has been demonstrated that NMDA receptor antagonists decreased the seizure incidence during ethanol withdrawal [96] and administration of NMDA exacerbated the withdrawal syndrome in rats [95]. These results suggest that NMDA responses may be important in both the acute intoxicating effects of ethanol and in the withdrawal syndrome seen with chronic ethanol use followed by cessation of ethanol intake .

1.4 Regulation of Gene Expression by Second Messengers and Ethanol

Increases in the concentration of second messengers (cAMP, Ca^{2+}, IP3, and DAG) generated by hormone-receptor interactions results in activation of their respective protein kinases (PKA, CAM-K, and PKC) leading to important biological consequences including changes in neurotransmitter synthesis, receptor function and ion channel activity. In addition to such post-translational modifications, increases in second messenger concentrations lead

to a cascade of events which culminate with longer-lasting changes in mRNA levels, and thus production of, many peptide hormones and metabolic enzymes. The cascade of events that leads to gene transcription induction by second messengers is discussed below.

1.4.1 *CRE-* Induction of eukaryotic gene transcription by cAMP is well documented; P-enolpyruvate carboxykinase, tyrosine hydroxylase, c-fos, somatostatin, proenkephalin, proopiomelanocortin, α-chorionic-gonadotropin, lactate dehydrogenase, and vasoactive intestinal peptide are all regulated by cAMP at the level of gene transcription (for reviews see [97, 98]). The common feature of the genes coding for these proteins is an 8 base pair palindromic sequence (T(G/T)ACGTCA) which is highly conserved in the promoter-regulatory region. This cis-acting cAMP regulatory element (CRE) lies within the first 150 base pairs of the 5'-flanking region of these genes [97, 98, 99]. Furthermore, the CRE displays properties of a classical enhancer sequence, such as, stimulating transcription at a distance and in an orientation-independent manner [99]. Cylic-AMP mediated activation of PKA leads to the phosphorylation of a CRE-binding factor (CREB), at serine residue 133 [100, 101]. Phosphorylation of CREB promotes homodimerization which results in increased transcription [100, 101]. In addition to phosphorylation by PKA, PKC and CaM-kinase II also phosphorylate CREB [100, 102]. CaM-kinase phosphorylates CREB on serine-133 as well and leads to increased CRE-dependent transcription [102, 103]. CREB is a member of the nuclear factor family that contains leucine-zipper dimerization domains and an adjacent basic DNA-binding domain [104]. A growing number of CREB-like proteins have been cloned which bind to the CRE in a homo and heterodimeric manner [105, 106, 107, 108, 109]. The role of these CREB-related proteins in the

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regulation of CRE-mediated transcription is unclear but their existence suggests complex regulatory interactions.

1.4.2 *AP-1-* Several second messenger cascades increase transcription of genes containing AP-1 (TGA(C/G)TCAT) enhancer elements by inducing the synthesis and possibly phosphorylation of AP-1 binding proteins. For example, elevations of intracellular Ca^{2+} [110], cAMP [111, 112], or activation of PKC [113], as well as binding of growth factors, such as NGF [114], PDGF [115], EGF [112], and FGF [116] to their respective receptors will induce c-fos expression in a variety of cell systems. The protein c-Fos is a member of the AP-1 DNA-binding protein family, that includes: c-Jun, JunB, JunD, FosB, Fra-1, Fra-2 and perhaps IP-1 [117, 118]. Transcriptional activation, mediated by elevation of c-Fos or c-Jun levels, is stimulated when homo-(Jun:Jun) and hetero- (Jun:Fos or Fra) dimers bind to AP-1 elements [117].

Like CREB, AP-1 proteins are members of a leucine repeat-basic region DNA binding protein family [104]. The similarities between the two families in protein structure and DNA sequence specificity suggest that cross-interactions at both protein and DNA binding levels are possible. Indeed, recent reports demonstrate heterodimer formation between CREB and c-Jun, mediated by the leucine zipper motif in each protein [119]. Recent studies, also, demonstrate that Fos and Jun can bind and activate CRE promoter elements [120]. Cross-interactions of the components for PKA- and PKC-activated gene expression provide additional regulatory mechanisms in controlling AP-1- and CRE-mediated gene expression.

1.4.3 *Other elements-* Phosphorylation is a common motif for regulating other transcription factors as well. For example, both PKA and PKC will

activate the AP-2 DNA-binding protein, which when bound to the AP-2 DNA-enhancer region (CCCCAGGC) increases transcription of the human metallothionein II_A promoter [121]. Sp1, which binds to the GC-box motif, was recently shown to be phosphorylated in a DNA-dependent manner [122]. The functional consequence of Sp1 phosphorylation is unknown. Phosphorylation of regions of the octamer binding transcription factor Oct-2 may be required for the mRNA gene-specific transcriptional activity of this protein [123]. Finally, phosphorylation of the yeast heat shock factor (HSF) leads to increased transcription when HSF is already bound to DNA [124].

1.4.4   *Ethanol and gene expression-* The above data indicate that second messenger cascades serve a critical function by regulating the gene expression of many important neuronal proteins. Because ethanol alters these second messenger cascades, it is likely to have consequent effects on gene expression. Indeed, ethanol has been shown to alter gene expression of many neural proteins. Miles et. al., used high resolution, two-dimensional (2-D) gel electrophoresis to quantitate products translated *in vitro* from mRNA isolated from NG108-15 neuroblastoma cells treated with 50-200 mM ethanol for 48 hours. They found significant ethanol-induced increases and decreases in specific mRNA abundance as compared to control products [125]. One protein whose abundance was shown to be increased by 2-D gel analysis was Hsc70. This protein is a member of the 70 kDa stress protein family, a group of proteins important in protein folding and trafficking [126]. Recently, it was demonstrated that 48 hour treatment of NG108-15 cells with 200 mM ethanol increased Hsc70 mRNA and protein levels by approximately 2-fold when compared to mock-treated control cells [127]. Nuclear run-off experiments showed that the ethanol-induced increases in Hsc70 expression were due to

transcriptional induction by ethanol [127]. In addition, ethanol increased CAT activity in cells that were stably transfected with an Hsc70 promoter-CAT plasmid, further suggesting that cis-acting elements in the Hsc70 promoter mediate ethanol induction [127].

Similarly, expression of the major histocompatibility antigen type I (MHCI) antigen is increased by approximately 2-fold in cultured L cells exposed to 125 mM ethanol for 72 hours [128]. Increased MHCI expression is also found in human peripheral blood lymphocytes isolated from acutely intoxicated patients admitted to the emergency room [129]. Like Hsc70, treatment of L cells, stably transfected with an MHCI gene promoter-CAT plasmid, with 125 mM ethanol for 72 hours increased CAT activity by approximately 2-fold compared to control cells [128]. The role of ethanol-induced increases in MHCI expression is unknown but may be important in autoimmune reactions associated with chronic ethanol use, since the MHCI antigen participates in a receptor for self-antigens [130].

Increases in the protein and mRNA levels for Gαi have also been reported in neuroblastoma cells exposed to chronic ethanol [8]. Finally, the protein levels of enzymes responsible for metabolizing ethanol, such as, alcohol dehydrogenase and ethanol-inducible cytochrome P450, are increased in Drosophila melanogaster larvae and rat liver, respectively, by chronic ethanol exposure [131, 132]. Unlike Hsc70, MHCI and alcohol dehydrogenase, ethanol-induced increases in cytochrome P450 protein is not associated with an increases in mRNA [132].

In addition to ethanol-induced increases in gene expression, the expression of several proteins is decreased by chronic ethanol treatment. Mochly-Rosen et. al., showed that NG108-15 cells exposed to 100 mM for 48 hours had 30% less Gαs mRNA and protein levels than mock-treated cells

[10]. Ethanol-induced changes in Gαs may be important in controlling proopiomelanocortin (POMC) expression since the cAMP cascade is involved in the expression of the POMC gene. For example, rats exposed to ethanol for 1, 7, and 14 days produced a time-dependent decrease in POMC mRNA levels in the pituitary when compared to mock-treated rats [133]. Decreased POMC mRNA was associated with a 24% decrease in corticotropin releasing factor-stimulated adenylyl cyclase activity in the anterior pituitary [133]. The GABA$_A$ and acetylcholine receptor α subunit mRNA levels are decreased by 43 and 39%, respectively, in rats exposed to chronic ethanol [134, 135].

The increases and decreases in gene expression described above may represent compensatory adaptive changes that occur during chronic ethanol exposure. Furthermore, these changes in gene expression may be important in the development of dispositional and functional tolerance. The mechanisms underlying the ethanol-induced changes in expression of the genes described above are unknown. It is possible that common regulatory mechanisms are involved in ethanol-induced changes in mRNA and protein levels.

1.5 Significance and Specific Aims

Second messengers are important in the regulation of the amount and activity of many proteins. Since ethanol has a well-established influence on second messenger cascades, the effects of ethanol on neuronal function may be produced, in part, by changes in the expression of specific genes and their protein products mediated by changes in second messengers. Gene expression changes result in lasting effects on cellular function. These long term changes may be important for adaptive changes seen when tolerance to ethanol develops in the CNS.

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Tyrosine hydroxylase is regulated both transcriptionally and post-transcriptionally by cAMP, Ca^{2+} and possibly diacylglycerol, all of which are affected by ethanol. TH occupies a critical role in catecholamine neurotransmission, which in turn, has been implicated in the development of tolerance to ethanol. Thus, ethanol-induced changes in the expression of TH may help to explain how ethanol modifies CNS function.

The specific aims of this project were to determine the changes in basal and agonist-stimulated TH gene expression due to chronic ethanol treatment and the mechanism underlying such changes. To do this, I first selected a suitable cell line, the N1E-115 neuroblastoma (Chapter II). A cell culture model avoids complicating factors such as routes of administration, and secondary effects of ethanol on the stress state of animals. Changes in TH protein, mRNA and promoter activity were then measured (Chapter III). In addition the ethanol-induced changes in cAMP-stimulated TH promoter activity were measured (Chapter IV). Finally, deletion analysis of the TH promoter was performed in order to characterize the cis-acting regulatory sequences for TH that are responsive to ethanol (Chapter V).

Chapter II

CELL CULTURE MODEL

2.1 INTRODUCTION

Ethanol researchers have used animal models extensively to characterize the neurophysiologic changes and neurotransmitter pathways associated with the development of tolerance. However, recent studies using animal models to identify the cellular mechanisms underlying the development of ethanol tolerance have produced conflicting results (see rationale). These variable results have been attributed to differences in dose, time, and route of ethanol administration, brain region studied, and secondary effects of environmental stress [136]. Thus, efforts have shifted to simpler and more definable cell culture model systems. Cell culture models have been successfully used to characterize ethanol-induced changes in second messenger systems [6, 8, 9, 10, 77, 78, 137], adenosine transport [138], ion channels [11, 93] and gene expression [10, 125, 127].

The use of neuronal cells in culture has particular advantages when studying ethanol-induced changes in catecholamine biosynthetic enzymes, such as TH. For example, catecholamine activity is easily influenced by secondary factors such as health and stress state of the animal. Furthermore, TH is highly regulated by a variety of influences such as trans-synaptic activity and circulating levels of glucocorticoids. Neuronal cultures avoid these complications. However, neuronal cultures are limited since they are not subjected to the multiple regulatory influences that are present *in vivo*. Therefore, *in vivo* regulatory mechanism may influence cellular pathways

differently than the regulatory mechanism present in cell cultures. The final goal is to understand ethanol-induced changes in TH expression in the brain. In order to relate the underlying mechanism of ethanol-induced changes in TH expression seen in culture to corresponding mechanisms in the brain, it is important that the neuronal cultures used be as faithful to *in vivo* regulation of TH as possible.

Therefore, to study ethanol-induced changes in TH expression, I decided to choose a cell line that would meet the following requirements. First, endogenous TH should be present. Second, neurotransmitters, neuromodulators, glucocorticoids, peptide hormones, and trophic factors should induce and activate TH, similar to *in vivo* regulation. Third, since regulation of TH by cAMP is the most predominant form of *in vivo* regulation (see section 4.1), the cell line should have a functional receptor coupled adenylyl cyclase system. Furthermore, since ethanol-induced changes in cAMP-stimulated TH expression are a possible mechanism for altering TH expression, ethanol-induced changes in the adenylyl cyclase system should be similar to those in other cell culture systems [9, 77, 137] and in the brain [76]. Finally, cells should be able to grow under defined conditions. Serum containing media contains glucocorticoids and growth factors which are known to alter TH expression (section 1.2).

N1E-115 mouse neuroblastoma cells and PC12 rat pheochromocytoma cells meet many of these criteria. For example, N1E-115 cells, a subclone of C-1300 neuroblastoma cells, were found to express TH activity [139]. Adenylyl cyclase can be stimulated via purinergic A2 and prostaglandin E1 (PGE1) receptors in this cell line [137]. TH activity is induced by cAMP analogous in these cells [140]. Finally, acute ethanol exposure potentiated PGE1-mediated cAMP formation [137], while chronic ethanol treatment

resulted in a diminished response to PGE_1 [77]. In addition, other second messenger systems are present in these cells, such as, bradykinin [103], muscarinic [141], histamine [142], and neurotensin [143] receptors which are coupled to PI turnover and cGMP generation.

The PC12 cell line was established from a transplantable rat adrenal pheochromocytoma [144]. TH protein levels are increased and preexisting TH protein is activated in PC12 cells by nicotinic agonists [49], vasoactive intestinal peptide [60], secretin [50], adenosine and adenosine analogs [52], glucocorticoids [68], NGF [73], and EGF [59]. Incubation of PC12 cells with VIP or adenosine results in increased intracellular cAMP [52, 60]. Ethanol-induced changes in receptor-mediated cAMP generation appear to depend on the source of the cells. For example, Rabe et. al. demonstrated that acute ethanol exposure inhibited 2-chloroadenosine-mediated cAMP generation in high-activity (HA) PC12 variants but stimulated adenosine agonist-mediated cAMP generation in low-activity (LA) PC12 variants [145]. LA and HA cells were obtained from separate laboratories and are reported to differ in their basal and adenosine-stimulated cAMP levels [145]. Another PC12 variant line, JW, is of interest because a mutant cell line was developed from the parent (JW PC12) which lacks type II cAMP-dependent protein kinase [146]. Cell lines that lack PKA would be useful to determine the role of PKA in ethanol-induced changes in TH expression. Ethanol-induced changes in receptor-mediated activation of adenylyl cyclase in the later subclone are not known.

In order to evaluate whether PC12 (HA, LA, or JW) or N1E-115 cells would be more suitable for this study, defined growth conditions were determined for each cell line. Also, all three PC12 variants were assayed to determine if ethanol exposure increased stimulation of adenosine-mediated

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cAMP generation. Finally, to confirm that the TH gene in N1E-115 is functionally regulated by cAMP, similar to other systems, I characterized the response of TH mRNA to stimulation by PGE_1 in these cells.

2.2 METHODS

2.2.1 *Materials* - Nylon (.45 μm) membranes were purchased from Schleicher and Schuell. All reagents for RNA isolation and Northern blot analysis were from BRL. Oligonucleotide mixed hexamers and deoxynucleotides were from Pharmacia. Radioisotopes were obtained either from Amersham or Du Pont. Organic solvents were from Fisher or Aldrich. All other reagents were of analytical grade from Sigma Chemical Company.

2.2.2 *Cell Cultures* - Stock cultures of N1E-115 neuroblastoma cells, passage 18 to 25, were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS), and 2 mM glutamine in 75 cm^2 and 175 cm^2 flasks (Falcon). N1E-115 cells used for experiments were subcultured into 75 cm^2 flasks or 60 mm multiwell plates with DMEM supplemented with 10% NuSerum (Hyclone). For N1E-115 cells, defined media contained either DMEM: Ham's F-12 (3:1), 10 μg of oleic acid complexed to fatty acid-free albumin, 25 μg/ml of insulin, 50 μg/ml transferrin, 2 mM glutamine, 0.5 nM Mn, 0.5 nM Mo, 5 nM Cd, 15 nM Se, 0.25 nM Ni, 0.25 nM Sn, 0.25 nM Si, and 25 mM Hepes, pH 7.6 (S media) [147], or DMEM:Ham's F12 (1:1), 5 μg/ml of insulin, 50 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 2 mM glutamine, and 25 mM Hepes, pH 7.6 (N2 media) [148]. PC12 cells were grown in DMEM supplemented with 10% horse serum and 5% FCS and 2 mM glutamine in 75 cm^2 and 175 cm^2 flasks.

PC12 cells used for experiments were subcultured into 75 cm^2 flasks or 60 mm multiwell plates, that had been pretreated with poly-d-lysine [149], with DMEM supplemented with 10% horse serum and 5% FCS. Defined media for PC12 cells was N2 media except only DMEM was used. N1E-115 and PC12 cells used for Northern analysis and adenylyl cyclase activity analysis were subcultured in 10% NuSerum then switched to defined media after 16 hours and maintained under defined conditions for at least 4 days. All cultures were grown at 37°C in 100% humidity, 10% CO_2 and 90% air. Cells were fluid changed every day except the day after subculture or switching to defined media. Cell viability was measured by trypan-blue exclusion [150].

2.2.3 *RNA Isolation and Northern Blot Analysis* - Total cellular RNA was isolated from N1E-115 cells as described by Cathala et. al [151]. RNA was quantitated spectrophotometrically. Duplicate samples of RNA (10 µg) were analyzed by RNA Northern blot analysis [127]. RNA blots were hybridized as described previously [152] using the TH, Eco RI-Bam H1 fragment of pSPTH3. The plasmid pSPTH-3 was the generous gift of K. Vrana (W. Virg. Univ. H. Sci. Cent.). Probes were labeled by the random hexamer oligonucleotide method of Feinberg and Vogelstein [153].

2.2.4 *Adenylyl Cyclase Activity*- After cells were maintained for 4 days in defined media cAMP levels were measured by radioimmunoassay, as described previously [9].

2.3 RESULTS

2.3.1 *Defined Media-* PC12 cells were reported to grow well in N1 media without Ham's F12 [148]. However, HA PC12 cells did not grow well or adhere to the wells in this media (data not shown). This result is not unexpected. PC12 cells have been observed in various laboratories to require different growth conditions, presumably due to clonal variations between cultures. The growth rate and viability of HA PC12 cells in N3 defined media was, however, comparable to the growth rate of parallel cultures grown in serum containing media (Fig. 3A). Similar responses to growth conditions were observed in LA and JW PC12 cells (data not shown). N1 and N3 media differ in the concentration of transferrin used (5 and 50 µg/ml, respectively) [148].

N1E-115 cells have been reported to grow in Bottenstein's N1 defined media [148]. Related clones derived from C1300 neuroblastoma cells grow in a defined media developed by Sato et. al. (S-media) [147]. I found that N1E cells had similar growth rates in S media and media containing 10% FCS (Fig. 3B). In contrast, N1E cells grown in N1 media had a slower growth rate (Fig. 3B). Furthermore, the viability, measured by trypan-blue exclusion, was greater than 90% in S media or 10% FCS media, but was only 60-70% in N1 media (data not shown).
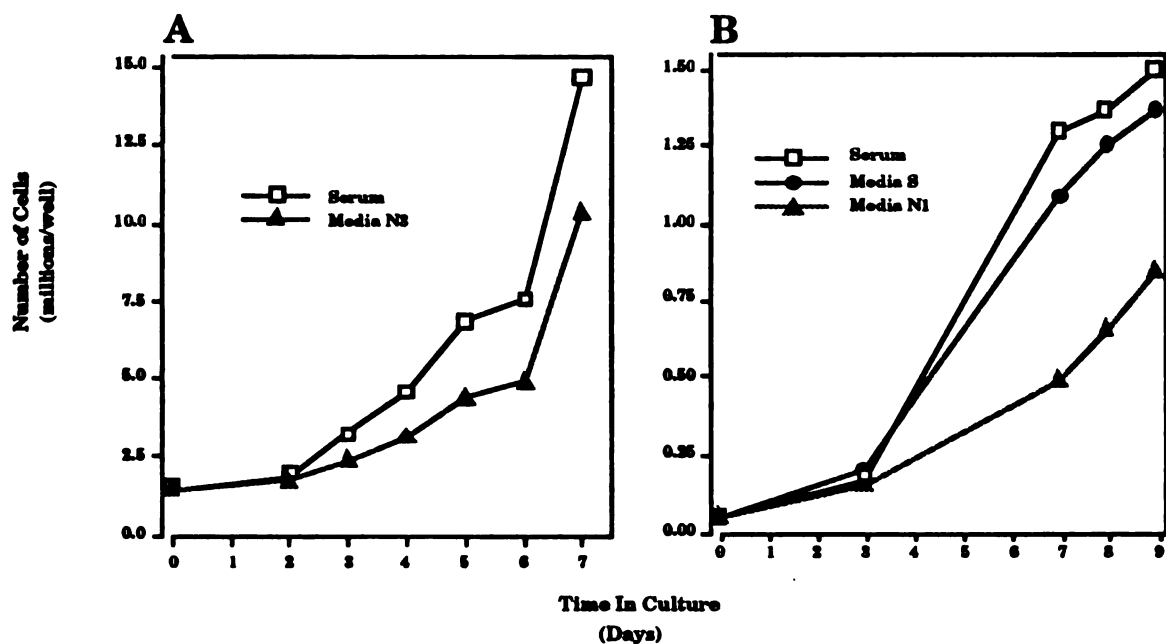
FIG. 3. Growth rates of PC12 pheochromocytoma and N1E-115 neuroblastoma cells in defined media. Panel A, PC12 cells, HA variant, were grown in 10% FCS and 5% HS media and parallel cultures were grown in N3 defined media as described in "Experimental Procedures." Panel B, N1E-115 cells were grown in 10% FCS, N1 and S defined media. Data represent average of triplicate values of a representative experiment.

2.3.2 *Ethanol and adenylyl cyclase-* N1E-115 cells were obtained from E. Richelson, who has demonstrated that acute ethanol potentiated and chronic ethanol inhibited PGE_1-mediated stimulation of adenylyl cyclase activity [77, 137]. These findings have been corroborated using the same cells in our laboratory (M.Charness. personal communication and also see section 4.3). Results differ only in the relative magnitude of acute ethanol-induced potentiation of PGE_1-mediated increases in intracellular cAMP.
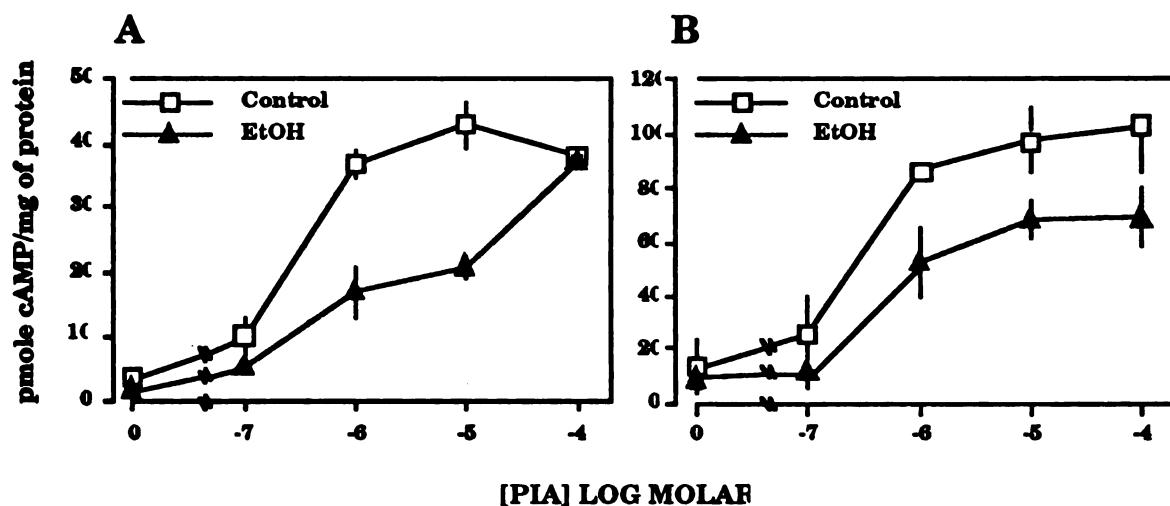
FIG. 4. Acute ethanol-induced changes in PIA-stimulated cAMP levels in JW and LA PC12 subclones. Panel A, LA cells, and panel B JW PC12 cells were grown under defined conditions and incubated with various concentrations of PIA with and without coincubation of 200 mM ethanol for 15 min. Cyclic-AMP levels were measure as described in "Experimental Procedures". Data from representative experiment repeated at least three times. Error bars represent standard deviation of triplicate data points.

Rabe et. al. demonstrated that adenosine-agonist stimulation of adenylyl cyclase in LA PC12 subclone was increased by acute ethanol exposure [145]. To confirm this, LA PC12 cells were treated with various concentrations of the adenosine receptor agonist, $(-)-N^6-(R\text{-phenylisopropyl})$-adenosine (PIA) with and without 200 mM ethanol (Fig. 4A). However, instead of the expected potentiation by ethanol, a 50-60% reduction in cAMP levels was seen with 10^{-6} and 10^{-5} M PIA, respectively. This result was also observed with 50 mM ethanol was used (data not shown). Similarly, a 30-40% decrease was obtained when JW PC12 cells were treated with PIA and ethanol (Fig, 4B). HA PC12 stimulated with 10^{-6} M PIA and various concentrations of ethanol showed little change in PIA stimulated intracellular cAMP levels (Fig. 5). In contrast to previously reported data, acute ethanol treatment to all PC12 cell variants results in decreased PIA-stimulated cAMP levels.

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[EtOH] mM	0	0	100	200	400
PGE$_1$	-	+	+	+	+
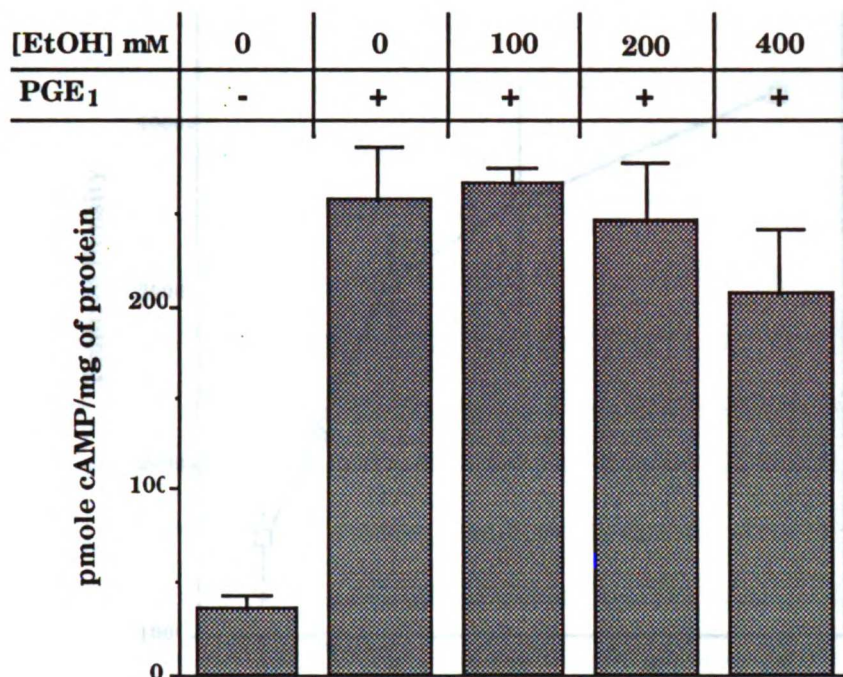
FIG. 5. Acute ethanol-induced changes in PIA-stimulated cAMP levels in HA PC12 cells. HA PC12 cells grown under defined conditions were treated with and without 10^{-6} M PIA and various concentrations of ethanol for 15 min. Data from representative experiment. Error bars represent standard deviation of triplicate determinations.

2.3.3 *Cyclic-AMP induction of TH mRNA-* To determine if cAMP induced TH gene expression in N1E cells, cultures were treated with various concentrations of PGE$_1$ for 8 hours, total RNA extracted and TH mRNA quantitated by a Northern blot analysis. TH mRNA levels were increased by PGE$_1$ in a concentration-dependent manner (Fig. 6).

30
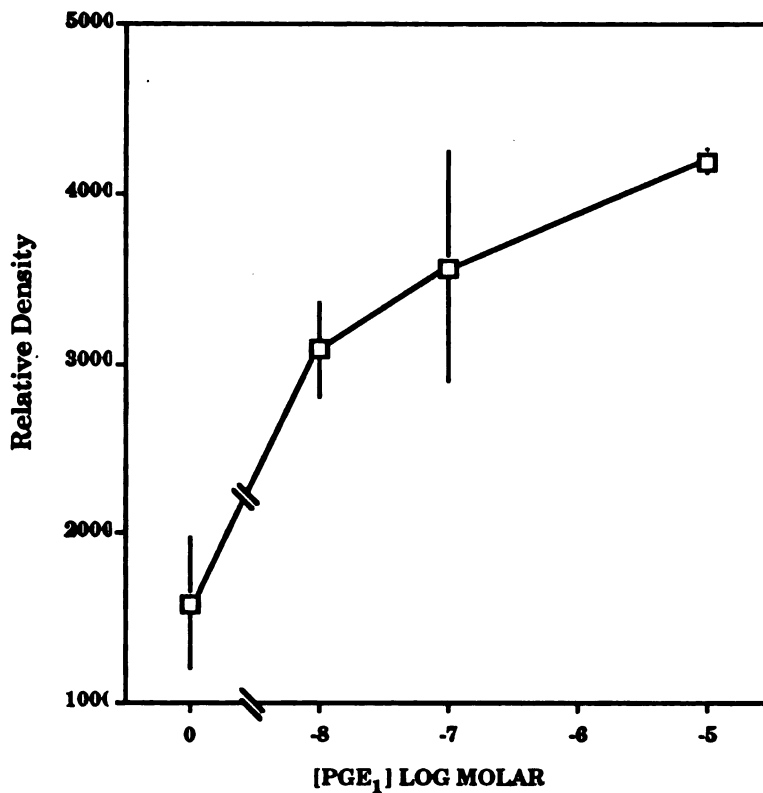
FIG. 6. **Cyclic AMP-responsive TH mRNA levels.** N1E cells were incubated for 8 hours with various concentrations of PGE_1. Northern analysis was performed as described in "Experimental Procedure". Data represents the mean ± S.D. from duplicate lanes representing duplicate cultures for each data point. Results are from a representative experiment.

2.4 DISCUSSION

N1E-115 neuroblastoma and PC12 pheochromocytoma cell lines were evaluated for use in studies of ethanol-induced changes in TH gene expression. Selection of a cell line was based on growth in defined media, acute ethanol-induced increases in receptor-mediated cAMP generation, and cAMP regulation of TH expression.

Defined media- To evaluate which cell line would be used the first objective was to determine defined growth conditions. Defined growth conditions are important because TH gene expression is increased by components in serum-containing growth media which may interfere with ethanol-induced changes in TH expression [154]. In addition to raising background expression of TH, serum also shows batch to batch variability of the levels of growth factors and hormones. I found that PC12 cells grew best in N3 defined media (Fig. 3A). Furthermore, growth rates and viability's of PC12 cells were similar when grown in N3 defined media and serum-containing media, suggesting that N3 media contains all necessary components for growth (Fig. 3A). I also found that media S contains all the necessary components for growth of N1E-115 cells, since the same growth rate and viability in media S and 10% FCS media were observed (Fig 3B). Growth in serum media was used as a standard because N1E-115 and PC12 cells grow best under these conditions.

The time necessary to reduce basal levels of TH mRNA to their lowest levels after removal of serum containing media was not determined. However, it has been reported that PC18 cells grown under serum-free conditions for 2 days had a 4-fold decrease in TH activity compared to parallel cultures grown in serum-containing media [154]. No further decrease in TH activity was seen after longer incubation times suggesting that serum-related factors were no longer increasing TH expression [154]. Therefore, cells used for my experiments were grown in defined media for at least 4 days to minimize that the influences of serum factors on TH expression.

Ethanol-induced changes in cAMP production- Both N1E-115 cells and the PC12 cell variants grow in defined media. Furthermore, both cell lines also

express TH which is induced and activated by similar second messenger cascades that induce TH expression *in vivo*. Thus both cell types meet many of the requirements stated earlier. However, differences were found in ethanol-induced changes in receptor-mediated stimulation of adenylyl cyclase activity between these cell types. For example, ethanol-induced changes in adenylyl cyclase activity in N1E-115 cells are similar to those found in other neural cell cultures and in brain. However, acute ethanol had the opposite effect in all PC12 cell variants tested (Fig. 4 & 5). For example, ethanol decreased adenosine-stimulated intracellular cAMP generation in both JW and LA PC12 cells (Fig. 4). No effect of ethanol on adenylyl cyclase activity in HA PC12 cells was observed (Fig. 5). The reason for these disparate observations are unclear. Perhaps they stem from the type of adenosine analog used. In this study, PIA was used, while 2-chloroadenosine (2-ClA) was used previously [145]. The EC_{50} for stimulating adenylyl cyclase activity for PIA and 2-ClA in HA PC12 cells is similar (980 and 460 nM, respectively) [155]. It seems unlikely that differences in Kd could account for our observation since ethanol decreased cAMP accumulation at every concentration of PIA used (Fig. 4). Alternatively, our results could be attributed to differences in growth conditions (defined vs. serum) or variations in the concentrations of the adenosine A1 receptor subtype. A1 receptors when bound to adenosine inhibit adenylyl cyclase via the inhibitory G-protein, $G\alpha i$ [155].

TH induction by cAMP in N1E-115 cells- TH gene expression has been shown to be increased by cAMP in the rat PC12 cell line [156]. Cyclic-AMP induction of TH mRNA levels is produced by transcriptional activation, mediated by the CRE element in the rat TH promoter [68]. This CRE element is identical in both the rat and human TH promoters [157], suggesting the

importance of cAMP in the regulation of TH expression. Therefore, it seems likely that there is a similar CRE in the mouse TH promoter. However, this has not been previously demonstrated. To determine if the TH gene in mouse N1E-115 cells is also induced by cAMP, I measured the effects of PGE_1 generated cAMP on TH mRNA levels in this cell line. Figure 6 demonstrates that TH mRNA levels are increased in a dose dependent manner with PGE_1 stimulation, suggesting that the TH gene is functionally regulated by cAMP in N1E-115 cells. These data further suggest that cAMP-mediated induction of TH expression is conserved in various species.

Conclusion- Regardless of the explanation, the response to ethanol is not consistent between PC12 variants and is not consistent with previously reported results for PC12 cell variants [145]. Thus, PC12 cells have unknown variables that make them a less desirable choice for the study of ethanol-induced changes in cAMP-stimulated TH expression. In contrast, N1E-115 cells met all of the stated requirements. Therefore, the effects of ethanol on TH gene expression were studied in N1E-115 cells, using S-media defined culture conditions.

Chapter III

ETHANOL-INDUCED CHANGES IN TH GENE EXPRESSION

3.1   INTRODUCTION

Regulation of TH expression occurs at two levels: increased activity of preexisting protein and increased amount of TH protein. Many agents that increase TH activity with short term exposure also increase the levels of TH mRNA and protein after long term exposure (section 1.2).

As discussed in the rationale (section 1.1), acute ethanol treatment increases catecholamine levels [30] and TH enzyme activity in rat brain nuclei [40]. Furthermore, some studies demonstrate increased TH activity [35] and turnover [32, 35, 36] in rats and mice exposed to chronic ethanol treatment. It is therefore possible that chronic ethanol-induced changes in catecholaminergic function observed *in vivo* could be explained by increased TH mRNA and protein levels.

I have therefore used N1E-115 neuroblastoma cells, grown under defined conditions, to study ethanol-induced changes in TH protein, mRNA and promoter activity. The results suggest that chronic exposure of N1E-115 neuroblastoma cells to ethanol increases basal TH gene expression. Furthermore, I show that ethanol increases TH expression, at least in part, through changes in gene transcription mediated by cis-acting elements in the TH promoter.

3.2   METHODS

3.2.1   *Materials* - Nitrocellulose (.45 μm) and nylon (.45 μm) membranes were purchased from Schleicher and Schuell. Monoclonal anti-tyrosine hydroxylase antibody was obtained from Boehringer Mannheim. Chloramphenicol acetyltransferase, n-butyryl coenzyme A, oligonucleotide mixed hexamers, and deoxynucleotides were from Pharmacia.

3.2.2   *Western Blot Analysis* - Cells were seeded at 0.5 x 10^6 cells/75 cm^2 flask. Duplicate flasks of control and ethanol-treated cells were tightly capped to avoid evaporation of ethanol. For analysis, cells were washed twice with 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) and resuspended in 10 ml of PBS/flask. Aliquots were removed for protein determination [158], cell count [150], and Northern blot analysis. Remaining cells (1 ml) were pelleted at 500 x g and resuspended in 1 ml of sample buffer containing 2% (w/v) sodium dodecyl sulfate (SDS), 62.5 mM Tris, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 20 μg/ml each of leupeptin, aprotinin and soybean trypsin inhibitor. Protein aliquots of 25, 50 and 75 μg were then analyzed by electrophoresis on a 10% SDS-polyacrylamide gel [159]. Three different protein concentrations were used to insure linearity of the quantitative method. Proteins were electrophoretically transferred to a .45 μm nitrocellulose membrane [160] and processed as described previously [127] except that TH immunoreactive protein was detected using a monoclonal anti-TH antibody (40 μg/ml; Boehringer Mannheim) followed by [125I] protein A labeling. Membranes were placed for autoradiography at -70°C for 8-16 hours. Autoradiograms were quantitated by computerized densitometry using a

Microscan 1000 gel analyzer (Technology Resources Inc.). Multiple exposures were done to insure linearity of the film response.

3.2.3 *RNA Isolation and Slot Blot Analysis* - Samples of RNA (1, 2.5, and 5 μg) from duplicate flasks of cells were analyzed by RNA slot-blot analysis [152]. RNA slot-blots were hybridized as described previously [127] using the following probes: for TH, the Eco RI-Bam H1 fragment of pSPTH3 was used, for β-tubulin, the 1.2 kbp Bam H1 fragment of pHFβT-1 was used, for lactate dehydrogenase M-type subunit (LDH), the 250 and 450 bp Pst I fragments of pRLD42 was used, and for actin, the 1.2 kbp Bgl I fragment of pRBA-1 was used. Plasmids pRBA-1 and pHFβT were obtained from L. Kedes (Stanford Univ.). Probes were labeled by the random hexamer oligonucleotide method of Feinberg and Volgelstein [153]. Polythymidylate probe was labeled exactly as described by Hollander et al. [161].

3.2.4 *Stable Transfection* - Stably-transfected N1E-115 cells were isolated by co-transfection of cells with pSV3neo [162] and either pMSVCAT or pTH5'CAT. The pSV3neo plasmid contains 2 copies of the SV40 early promoter controlling expression for aminoglycoside phosphotransferase 3' (I) that inactivates aminoglycoside antibiotics [162], while pMSVCAT contains the murine Maloney sarcoma virus tandem repeat fused upstream from the reporter enzyme chloramphenicol acetyltransferase (CAT) coding sequence [163]. The pTH5'CAT plasmid was a generous gift of E. Lewis (Oregon Health Science Univ.) and contains the proximal 773 bp of the tyrosine hydroxylase promoter fused upstream to the CAT coding sequence [68].

N1E-115 cells were co-transfected with either pMSVCAT or pTH5'CAT and pSV3neo by the calcium phosphate transfection technique as described

previously [127]. After 14 days, viable colonies were picked, expanded, and screened for CAT activity. Five clones from both the pTH5'CAT and the pMSVCAT transfection were obtained and analyzed.

3.2.5 *CAT Assay* - Cells were lysed by freeze-thawing in 0.25 M Tris, pH 8.0. CAT assays were performed as described by Seed and Sheen [164], except 5 μM cold chloramphenicol was included during the assay.

3.3 RESULTS

3.3.1 *Chronic Ethanol Increases Basal Levels of TH mRNA and Protein* - We first measured TH protein levels in N1E-115 cells exposed to various concentrations of ethanol for 3 days to determine whether ethanol causes changes in TH protein. Quantitative Western blot analysis was performed using a TH-specific monoclonal antibody [165], followed by labeling with [^{125}I] protein A and autoradiography. Fig. 7A shows a representative experiment, illustrating that the TH monoclonal antibody labeled a single 60 kDa band, consistent with the known molecular weight of TH [165]. Ethanol-treated cells showed dose-dependent increases in TH immunoreactivity. The cumulative data for 50 and 100 mM EtOH, is shown in Fig.7B; TH protein was 142% and 172% of control, respectively. Total protein levels per cell did not change (Table 1) suggesting that ethanol-induced changes in TH protein were not a result of global changes.

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TABLE 1

	control	25 mM	50 mM	100 mM	200 mM
protein	1.8 ± .2	1.7 ± .3	1.7 ± .1	1.9 ± .2	1.9 ± .2
RNA	32 ± 2	34 ± 4	32 ± 3	33 ± 2	34 ± 2

Table 1. **Quantification of total RNA and protein levels per cell exposed to various concentration of ethanol.** N1E-115 cells were grown under defined conditions and exposed to various concentration of ethanol for 3 days. Protein values are nanograms/cell and RNA values are picograms/cell. Quantification of cell number, protein and RNA levels was performed as described in "Experimental Procedures." Values are the mean ± S.E.M. of experiments repeated four to six times.

To determine whether ethanol-induced increases in TH protein were due to increased TH mRNA, we used slot blot analysis to quantitate mRNA levels. Labeled [^{32}P] cDNA probes for β-tubulin, actin, and LDH were used as controls. Additionally, we quantitated total poly(A) RNA levels by hybridizing blots with [^{35}S]-polythymidylate [161]. Northern blot analysis showed that probes for TH, LDH, and actin hybridized to single species of mRNA, while the β-tubulin probe hybridized to the characteristic 1.8 and 2.6 kilobase forms of β-tubulin mRNA (data not shown) [166]. Figure 8A shows a representative RNA slot blot with membranes hybridized to either [^{32}P]-TH, β-tubulin, actin, or LDH DNA probes. These data show that 100 mM ethanol caused an increase in TH mRNA when compared to mRNA from control cells. Furthermore, ethanol-induced increases in TH mRNA were specific. Actin and LDH mRNA (Fig. 8) as well as total RNA (Table 1) levels did not change with ethanol treatment and β-tubulin mRNA levels
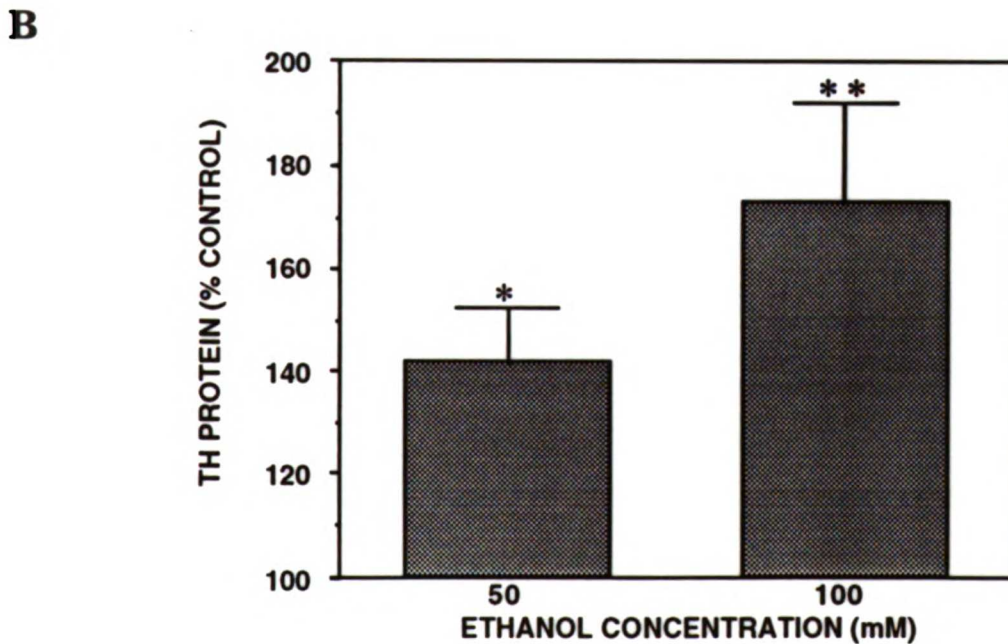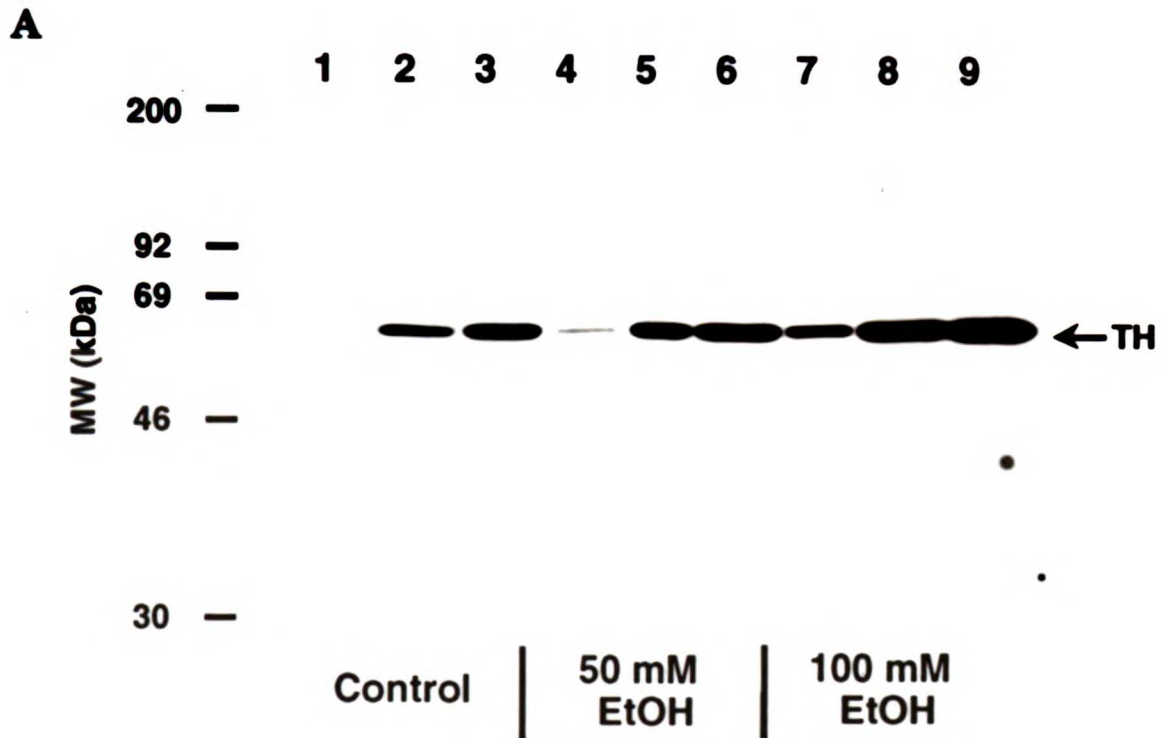
FIG. 7. **Western blot analysis of ethanol-induced changes in TH protein.** Panel A, N1E-115 cells were grown in defined medium and 25 µg (lanes 1, 4, and 7), 50 µg (lanes 2, 5, and 8), or 100 µg (lanes 3, 6, and 9) of cell lysates were subjected to Western blot analysis using a monoclonal antibody

directed against tyrosine hydroxylase as described in "Experimental Procedures." Protein lysates from control cells (lanes 1-3) and cells treated with 50 mM (lanes 4-6), or 100 mM ethanol (lanes 7-9) for 3 days are shown. Molecular weights of standard proteins are indicated on the left and the position of TH is identified on the right. Panel B, Western blots of control cells and cells exposed to ethanol for 3 days, at the indicated concentrations, were quantitated for TH protein by computerized densitometry. Data are expressed as a percent of control (untreated). Values are the mean of experiments repeated five to seven times. Error bars represent standard error of the means (* indicates $p < .02$, n=7, and ** indicates $p < .006$, n=5; single group, two tailed t -test).

appeared to decrease slightly. Quantitative analysis of RNA slot-blots from cells treated for 24 hours with 25-200 mM ethanol is shown in Fig. 8B. Total poly(A) RNA content did not change appreciably over the indicated concentration range and was used to normalize data from TH, LDH, actin, and β-tubulin hybridizations. TH-mRNA abundance was increased in ethanol-treated cells to 200, 238 and 240% of control (mock-treated) at 50, 100, and 200 mM ethanol, respectively.

A

TH

tubulin

LDH

actin

poly(A)

[ethanol]
(mM)   cont   25   50   100   200
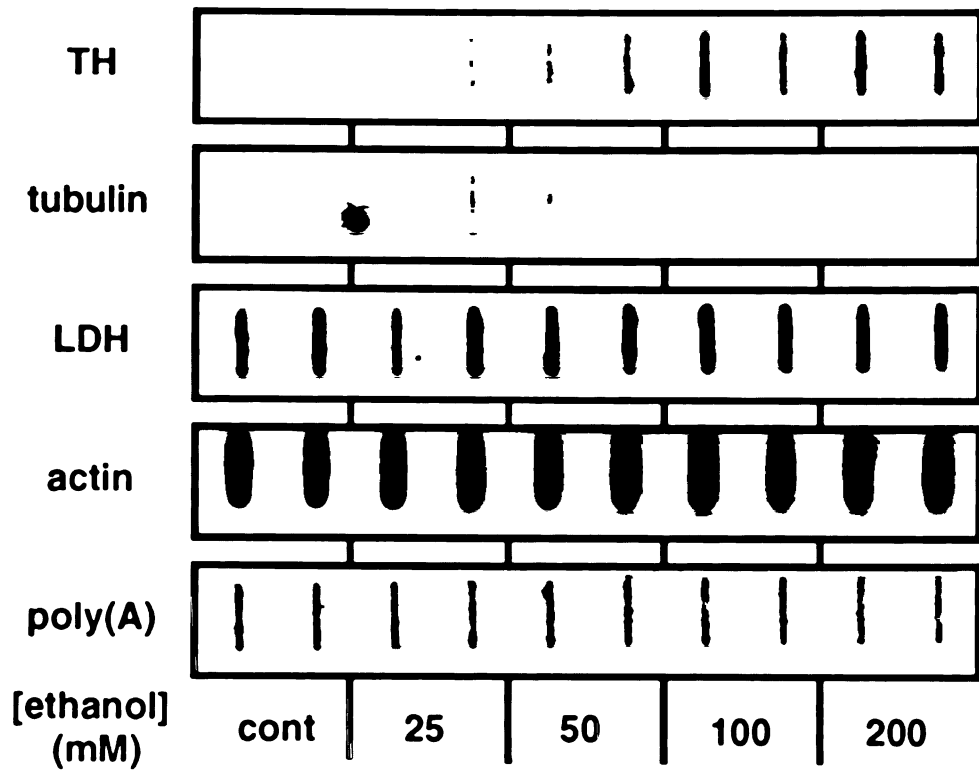
B
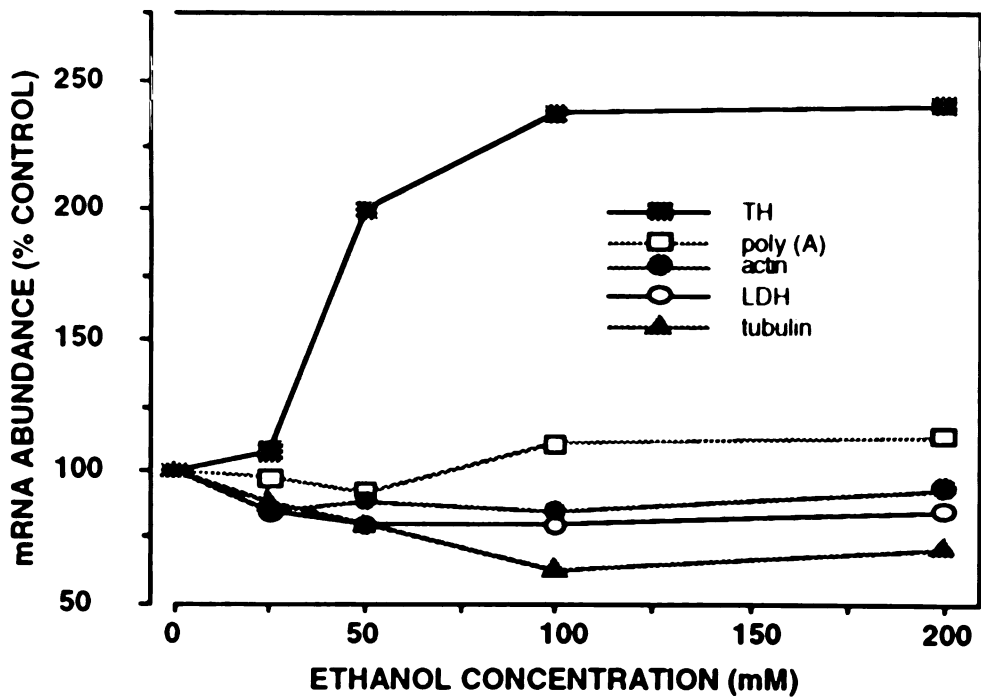
FIG. 8. **Slot blot analysis of ethanol-induced changes in TH mRNA levels in N1E-115 cells.** Panel A, Total cellular RNA was extracted from control cells and cells treated for three days with 25, 50, 100, or 200 mM ethanol. TH, β-tubulin, LDH, actin, and poly(A) mRNAs were analyzed by RNA-slot blot analysis. Identical amounts (2.5 μg) of total cellular RNA were loaded on each slot. Duplicate flasks were used for each condition. Panel B, Levels of TH, actin, LDH, and β-tubulin mRNAs from control cells and cells exposed to ethanol at the indicated concentration for 3 days were quantitated by slot blot hybridization and computerized densitometry. Data are expressed as a percent of control (untreated) and are normalized to poly(A) RNA content. The values for poly(A) content are included and show no significant change with ethanol over this concentration range. Results are representative of experiments repeated at least three times.

3.3.2 *Stable Transfection Analysis: Chronic Ethanol Increases TH Promoter Activity* - Ethanol-induced increases in TH mRNA could be due to an increase in the rate of TH gene transcription or a change in TH mRNA processing or degradation. I therefore used stable transfection analysis to study whether ethanol alters TH promoter-activity. I isolated clones of N1E-115 cells that were transfected with a plasmid containing 773 base pairs of the TH promoter fused to the CAT reporter gene. This promoter portion of the TH gene is expressed in a tissue specific manner [167], and CAT activity is induced by elevated levels of intracellular cAMP [68], as well as calcium [70], phorbol esters [70], NGF [73], EGF [59], and increases in cell density [68]. When a stably-transfected clone (N1E-C3) was incubated with 50 or 100 mM ethanol for three days, CAT activity was 138% and 170% of control, respectively (Fig. 9A). Four other independently isolated clones showed similar responses to ethanol (Table 2). Ethanol-induced increases in CAT activity were seen as early as 8 hours and reached a plateau by 24 hours (Fig 9B).

A
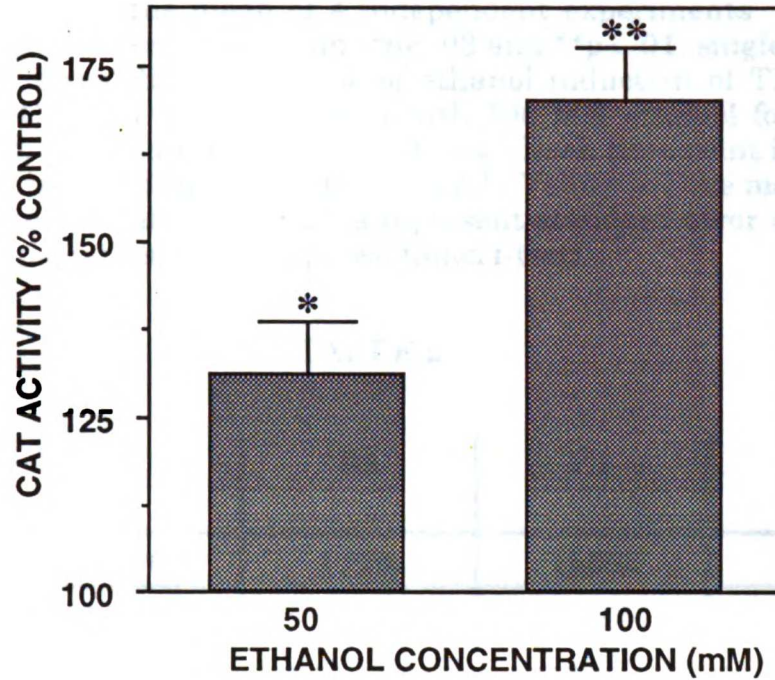
B
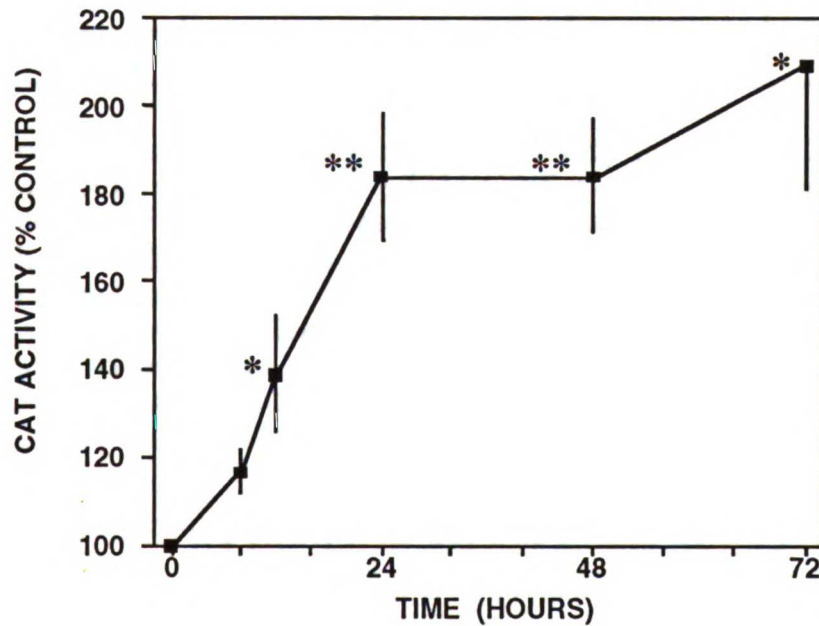
FIG. 9. **Stable transfection analysis: ethanol increases TH promoter activity.** Stable transfection of N1E-115 cells with a 773 bp TH promoter fragment fused to a CAT reporter gene was done as in "Experimental Procedures." Panel A, cells of a stable clone (N1E-C3) were incubated with the indicated concentration of ethanol for three days. Cell lysates (15 μg) **were**

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analyzed for CAT activity. Data are expressed as a percent of control (mock treated). Values are the mean of 4 independent experiments. Error bars represent standard error of the mean (*p< .03 and **p< .01; single group, two tailed t test). Panel B, time course of ethanol induction of TH promoter activity. N1E-C3 cells were incubated with 100 mM ethanol for the times indicated, lysed, and analyzed for CAT activity. Each time point is expressed as a percent of its paired (mock treated) control. Values are the mean of 4 to 6 independent experiments. Error bars represent standard error of the mean (*p< .03 and ** p< .001; single group, two-tailed t-test).

TABLE 2

A

TH Subclones	A1	B3	F1	J2
Ethanol	143%	177%	140%	110%
PGE$_1$	287%	191%	135%	162%

B

MSV Subclones	A4	A8	B8	D6	D11
Ethanol	89%	88%	98%	98%	90%

Table 2. **Ethanol-induced changes in CAT activity from N1E-115 clones stably transfected with either pTH5'CAT plasmid or pMSVCAT plasmid.** Table A, individually isolated subclones containing pTH5'CAT were incubated with either buffer, 100 mM ethanol or 10^{-6} M PGE$_1$ for 48 hours. Lysates from control, ethanol, or PGE$_1$ treated cells were analyzed for CAT activity. Table B, individually isolated subclones containing pMSVCAT were treated with and without 100 mM ethanol for 48 hours. Data represents percent difference of mock-treated control cells compared to treated cells (n=1-3).

The stably transfected clone, N1E-C3, was similar to the parent N1E-115 cells, when analyzed for growth rate, morphology, and induction of TH expression by increased cell density or cAMP (data not shown) [68, 71]. In addition, ethanol-induced changes in receptor coupling to adenylyl cyclase were similar to previously reported observations [77, 137]. For example, exposure of

N1E-C3 cells to 50 and 100 mM of ethanol for 3 days resulted in a 22% and 30% inhibition, respectively, of PGE$_1$-stimulated cAMP production (Fig. 10).

Stable cell lines constructed with pMSVCAT were used to control for possible effects of ethanol on CAT enzyme activity and stability. In a pMSVCAT transfected N1E-115 clone (B8) exposed to 100 mM ethanol for 48 hours, CAT activity in lysates from ethanol-treated cells was 95% ± 8 (n=3) of lysates from control cells not exposed to ethanol. Similar results were obtained in 4 other independent clonal isolates containing pMSVCAT (Table 2).
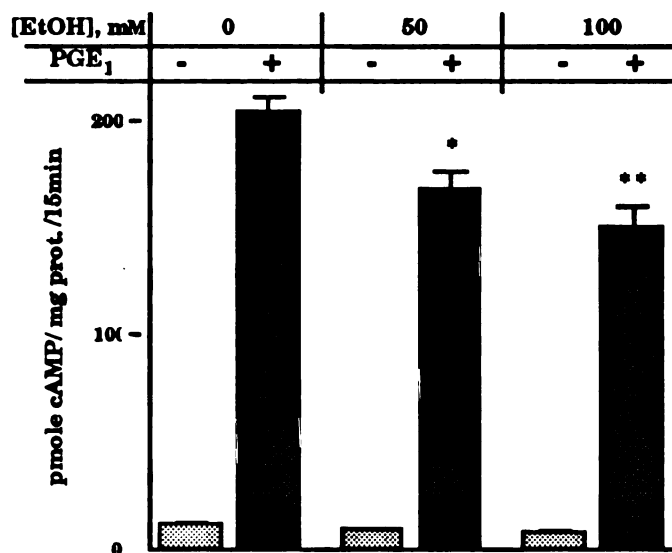
Fig. 10. **Chronic ethanol-induced inhibition of PGE1-stimulated cAMP generation in N1E-C3 cells.** N1E-C3 cells (parallel cultures of the cells used for experiment shown in Fig. 9A) were incubated with indicated concentrations of ethanol for 3 days. Cells were then incubated with ZK-62711, a phosphodiesterase inhibitor, with or without 1 μM PGE$_1$ for 15 min. Cyclic-AMP levels were measured as described in methods (Chapter 2). Values are the mean of 5 independent experiments. Error bars represent standard error of the mean (*indicates p< .02 and ** indicates p< .003; single group, two-tailed t-test).

3.4   DISCUSSION

I have used a neural cell line, N1E-115, to study the regulation of TH gene expression by ethanol. I found that chronic exposure to concentrations of ethanol observed in sober alcoholics [14] causes a significant increase in TH protein and mRNA (Figs 7 & 8). Furthermore, my studies using N1E-115 cells stably transfected with a TH-promoter CAT construct (Fig. 9), suggest that ethanol increases TH mRNA by activating gene transcription through an ethanol-responsive cis-acting sequence(s) contained in the proximal 773 base pairs of the TH promoter.

Ethanol-induced increases in TH expression are specific and not due to global changes in mRNA abundance. Poly(A), actin, and LDH mRNA levels did not change and small decreases in β-tubulin mRNA were observed with ethanol treatment (Fig. 8B). The magnitude, time course, and concentration dependence of TH induction by ethanol are similar to those for Hsc70 and MHCI and for other ethanol-induced proteins (section 1.4.4). The time course and ethanol concentrations used here also correlate well with animal models of alcoholic tolerance and dependence [168, 169]. Taken together these data suggest a relationship between ethanol-induced changes in TH gene expression and alcoholic tolerance and dependence.

Increases in TH mRNA may result from increased synthesis or decreased degradation. We have found that TH-promoter directed CAT activity is increased as early as 8 hours after ethanol exposure (Fig 9B). Furthermore, ethanol-induced increases in CAT activity were not observed in stable cell lines containing the control plasmid pMSVCAT. This suggests that the ethanol-induced increase in TH-promoter directed CAT activity is not due

to CAT mRNA or protein stabilization. Thus, changes in TH expression probably occur at the transcriptional level, as has recently been shown for the induction of Hsc70 by ethanol [127]. The dose response, time course and magnitude of TH induction by ethanol are similar to that for Hsc70 and MHCI (section 1.4.4). Therefore, its possible that similar cis-acting element(s) and cognate binding protein(s) might mediate the ethanol-responsiveness of these genes. A more in depth discussion of common ethanol-induced regulatory elements of TH, Hsc 70 and MHCI is presented in chapter 5.

Chapter IV

ETHANOL-INDUCED CHANGES IN cAMP-STIMULATED TH EXPRESSION

4.1 INTRODUCTION

Tyrosine hydroxylase is transynaptically induced and activated in both the peripheral and central nervous system. The primary messengers that mediate this transynaptic regulation are acetylcholine, peptidergic agonists of the secretin-glucagon family (i.e. secretin, vasoactive intestinal peptide, peptide histidine isoleucine amide, rat growth hormone-releasing factor), adenosine, and the growth factors, EGF and NGF (section 1.2). These hormones and neurotransmitters stimulate cAMP, Ca^{++}, and/or DAG formation, which in turn, increases TH activity and protein levels (section 1.2).

One of the most prominent regulators of increased TH expression is increased intracellular cAMP levels. Cyclic-AMP has been shown to regulate TH activity in adrenal glands [44], chromaffin cells [64], nictating membranes [170], salivary gland [171], iris [171], vas deferens [172], PC12 cells [156], striatal slices and synaptosomes [173]. Agents which elevate cAMP levels and also increase TH activity and protein levels, include peptidergic, purinergic, and nicotinic agonists, as well as growth factors. For example, VIP, secretin, and glucagon acutely increase adenylyl cyclase activity and intracellular cAMP levels as well as TH activity in superior cervical ganglia [171], chromaffin cells [174] or in PC12 cells [60]. Adenosine agonists stimulate cAMP production in PC12 cells and in pheochromocytoma cells [52]. Adenosine released from PC12 cells will also act as an autocrine regulator of basal TH levels. For example, Roskoski et. al. demonstrated that the adenosine receptor

antagonist, theophylline, and enzymes that metabolize extracellular adenosine, such as, adenosine deaminase or AMP deaminase, will decrease both basal cyclic AMP levels and TH activity in PC12 cells [52]. Cyclic AMP levels can also be elevated by less classical mechanisms. NGF treatment increases cAMP levels and PKA activation in PC12 cells [175] and in superior cervical ganglia [176]. Stimulation of nicotinic receptors by selective nicotinic agonists also increases cAMP levels and TH activity in bovine chromaffin cells [177]. Acetylcholine when bound to the nicotinic receptor results in depolarization of the cell by increasing the permeability to Na^+ and Ca^{++}. Depolarization of the cell leads to the release of chemical neurotransmitters from synaptic vesicles. It is thought that the released neurotransmitters then feedback onto the cell in an autocrine manner to increase cAMP levels.

Thus, cAMP is an important regulator of TH expression. Acute and chronic ethanol exposure alter cAMP generation (section 1.3). Therefore it is possible that changes in cAMP generation produced by ethanol could be responsible for altered TH expression. To test this hypothesis, I measured changes in TH promoter activity in N1E-C3 cells exposed to various agents which elevate intracellular cAMP levels, i.e. PGE_1, adenosine agonists, and 8-BrcAMP, in the presence and absence of ethanol. Receptor agonists were used to determine the effects of ethanol-induced changes in receptor-effector coupling on TH expression. The non-hydrolyzable cAMP analog, 8-BrcAMP, was used to distinguish receptor-coupled events from those following cAMP production. I also measured ethanol-induced changes in cAMP-stimulated CAT activity directed by the minimal cAMP responsive portion of the human VIP promoter [178]. This latter construct was used to determine whether CRE-mediated transcription could be induced by ethanol in the absence of other regulatory elements.

4.2   METHODS

4.2.1   *Materials* - Same source as described previously

4.2.2   *Stable Transfection* - N1E-115 cells were co-transfected with p25VIPCAT and pSV3neo by the calcium phosphate transfection technique described previously [127]. After 14 days, viable colonies were picked, expanded and screened for CAT activity. Four individually isolated clones were obtained and analyzed. The p25VIPCAT plasmid was a generous gift of H. Goodman (Oregon Health Science Univ.). It contains the minimum (25 base pair) promoter portion of the human vasoactive intestinal peptide, demonstrated to mediate cAMP-induced gene transcription, fused upstream to the CAT coding sequence [178]. Furthermore, Hyam et. al. have also demonstrated that the CRE element in the TH promoter and the CRE element in p25VIPCAT bind the same trans-acting factor, presumably CREB [179].

4.2.3   *CAT Assay* - CAT assays were performed as described previously (section 3.2.5).

4.3   RESULTS

4.3.1   Ethanol Increases cAMP-Regulated TH Expression - To determine whether ethanol alters cAMP-regulated TH expression, N1E-C3 cells were treated for 24 hours with PGE_1 (1 nM-1 μM) in the presence or absence of 100 mM ethanol. Figure 11 shows that TH-promoter directed CAT activity increased in a dose-dependent manner with PGE_1 treatment. When cells were exposed to ethanol in combination with PGE_1, an increase in TH promoter activity was seen at each concentration of PGE_1.
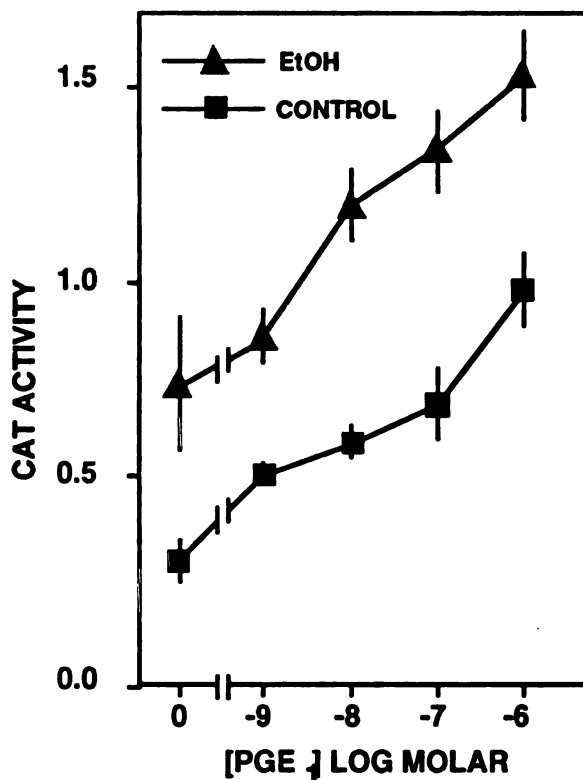
FIG 11. **Stable transfection analysis of ethanol-induced changes in PGE_1-responsive TH promoter activity.** N1E-C3 cells were either co-incubated for 24 hours with 100 mM ethanol plus indicated concentrations of PGE_1 or with various concentrations of PGE_1 alone. CAT activity is expressed as a percent of total chloramphenicol incorporated into product and represents the mean ± S.D. from quadruplicate cultures for each data point. Results are representative of experiments repeated at least four times.

I next assessed whether the ethanol-mediated increase in PGE_1 stimulated TH promoter activity was specific for the PGE_1 receptor or extended to other receptors coupled positively to adenylyl cyclase. Therefore the effects of ethanol on adenosine receptor agonist $((-)-N^6-(R-$ phenylisopropyl)-adenosine (PIA)) stimulation of TH promoter activity were determined. Binding of PIA to adenosine receptors stimulates cAMP generation. Increased TH promoter activity was seen when either PIA or PGE_1 was used in combination with ethanol (Fig. 12), suggesting that the ethanol response was not confined to PGE_1 receptor stimulation. The relatively modest effect of PIA- compared to PGE_1-stimulated CAT activity correlates with the magnitude of PIA- compared to PGE_1-stimulated cAMP levels (data not shown).

FIGURE 12

DRUG	CONT		PGE$_1$		PIA		8-BrcAMP	
EtOH	-	+	-	+	-	+	-	+
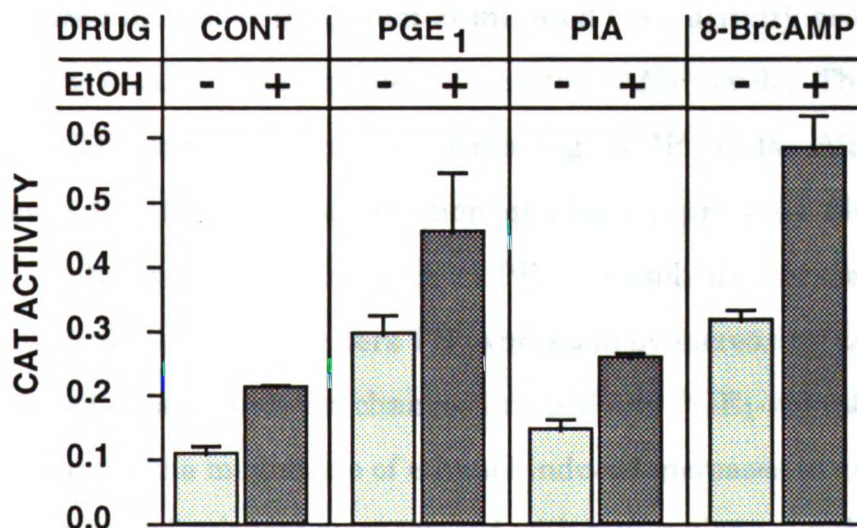
TABLE 3

Drug condition	EtOH	PGE$_1$	PGE$_1$+ EtOH vs PGE$_1$	8-BRcAMP	8-BRcAMP + EtOH vs 8-BRcAMP
% control	197 ± 13	215± 19	206 ± 14	425 ± 93	193 ± 24
N	14	14	12	3	3
p value	.0001	.0001	.0001	.07	.06

FIG.12 and TABLE 3 **Ethanol effects on cAMP-responsive TH promoter activity.** FIG. 12, N1E-C3 cells were incubated for 24 hours with either 1 µM PGE$_1$, 10 µM PIA, or 0.5 mM 8-BrcAMP in the presence or absence of 100 mM ethanol. CAT activity is expressed as a percent of total chloramphenicol incorporated into product and represents the mean ± S.D. from triplicate cultures for each data point. Table 3, cumulative data of 3 to 14 experiments similar to the experiment in Fig. 12. Values represent the mean percent differences between treatments and controls ± SEM. N represents the number of experiments performed. P represents the significance from a single group, two-tailed t-test.

Ethanol-induced increases in agonist-stimulated CAT activity are consistent with the effects of ethanol on agonist-stimulated cAMP levels. Thus, ethanol may increase TH expression by first increasing cAMP levels. Alternatively, ethanol could be affecting the function of effector proteins distal to the receptor-adenylyl cyclase system, such as PKA, phosphodiesterase, or CREB. To determine whether ethanol alters TH expression by increasing cAMP levels, I first measured ethanol-induced changes in basal and PGE$_1$-stimulated cAMP levels to determine the magnitude of ethanol-induced increases in cAMP levels. In Figure 13, parallel cultures were treated with buffer alone, 1 µM PGE$_1$, 100 mM ethanol or PGE1 plus 100 mM ethanol. The levels of cAMP produced after a 30 minute drug treatment were measured and CAT activity in lysates was measured 24 hours later in parallel cultures. Cells treated with 1 µM PGE$_1$

elicited 2-3 fold increases in intracellular cAMP by 15 minutes (Fig. 13A), which corresponds to the magnitude of changes seen in TH promoter directed CAT activity. However, 100 mM ethanol only increases basal and PGE_1-stimulated cAMP levels by approximately 30% while TH-directed promoter CAT activity was increased by approximately 200% with either 100 mM ethanol or ethanol and PGE_1 together as compared to mock-treatment and PGE_1 alone, respectively (Fig. 13). It is possible that relatively small ethanol-induced changes in cAMP levels lead to large changes in CRE-mediated gene expression. It is also possible, however, that receptor proximal events are regulating ethanol-induced increases in cAMP-stimulated TH expression.

In order to distinguish between receptor-proximal and distal mechanisms of ethanol action, I determined the effects of ethanol on 8-bromoadenosine 3':5'-cyclic monophosphate (8-BrcAMP)-stimulated CAT activity. Increased TH promoter activity was seen following concurrent treatment of cells with ethanol and 8-BrcAMP as compared to either agent alone (Fig. 12 and Table 3), similar to results seen with PGE_1 and PIA. In all cases studied, ethanol treatment resulted in a 2-fold increase in both basal and stimulated TH promoter activity (Table 3).
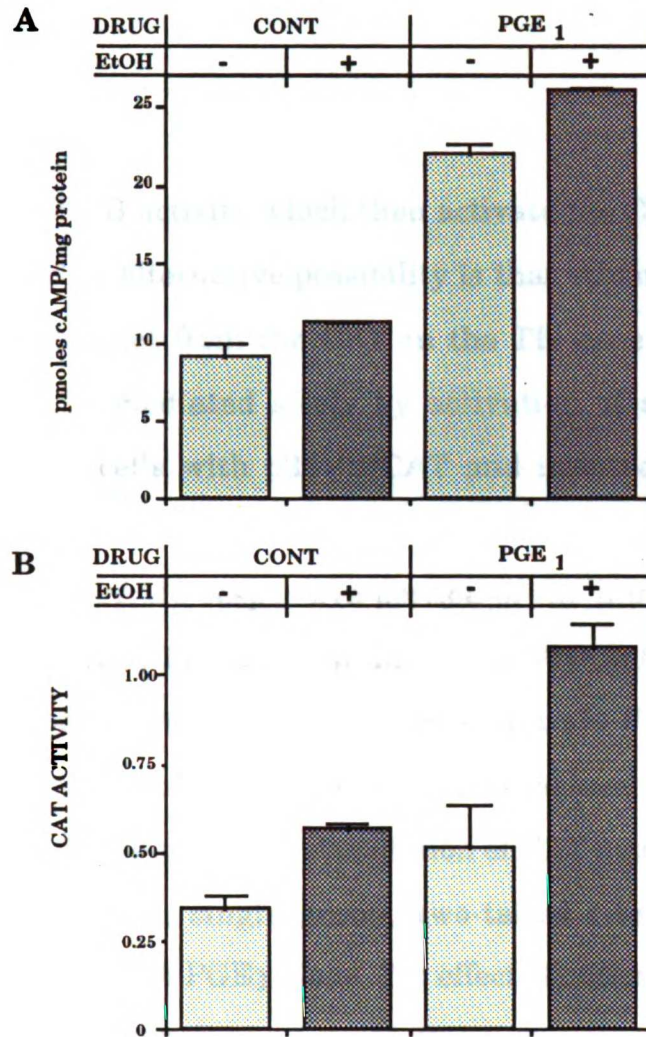
Fig. 13. Comparison of ethanol-induced changes in basal and PGE$_1$-stimulated cAMP levels and TH-promoter directed CAT activity. Parallel cultures of N1E-C3 cells were treated as follows. Panel A, cells were incubated with indicated treatment for 15 min. Cells were then lysed and cAMP levels were measured as described in experimental procedure (chapter 2). Panel B, cells were incubated with the indicated treatments for 24 hours and cell lysates (15 μg) were analyzed for CAT activity (methods) Values are the mean of triplicate data points. Error bars represent standard deviation.

4.3.2 *Ethanol-induced changes in basal and cAMP-stimulated CRE (VIP)*

activity- The above data suggests that ethanol treatment of N1E-115 cells

increases cAMP-mediated induction of TH expression through events distal to

receptor coupling. Ethanol-induced increases may be mediated by

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increased PKA or CREB activity which then activate the CRE element in the TH promoter. Another alternative possibility is that ethanol is influencing a response element distinct from the CRE in the TH gene. To determine if potentiation could be mediated solely by activation of a CRE element, I transfected N1E-115 cells with p25VIPCAT and isolated clones with this plasmid stably incorporated into their genome. The plasmid p25VIPCAT contains the minimal cAMP responsive portion of the human VIP promoter [178]. A subclone of N1E-115 cells containing p25VIPCAT was treated with 100 mM ethanol for 24 hours in the presence of 1 μM PGE$_1$ or 0.5 mM 8-BrcAMP. Interestingly, ethanol had no effect on basal levels of CAT activity in these cells (Fig 14). However, PGE$_1$ stimulation of CAT activity was increased 22% $\pm$ 4% (n=8, p<.0005, single group, two-tailed t-test) by ethanol as compared to treatment with PGE$_1$ alone. No effect of ethanol was observed on 8-BrcAMP stimulation of TH promoter activity.

FIGURE 14

DRUG	CONT		PGE$_1$		8-BrcAMP	
EtOH	-	+	-	+	-	+
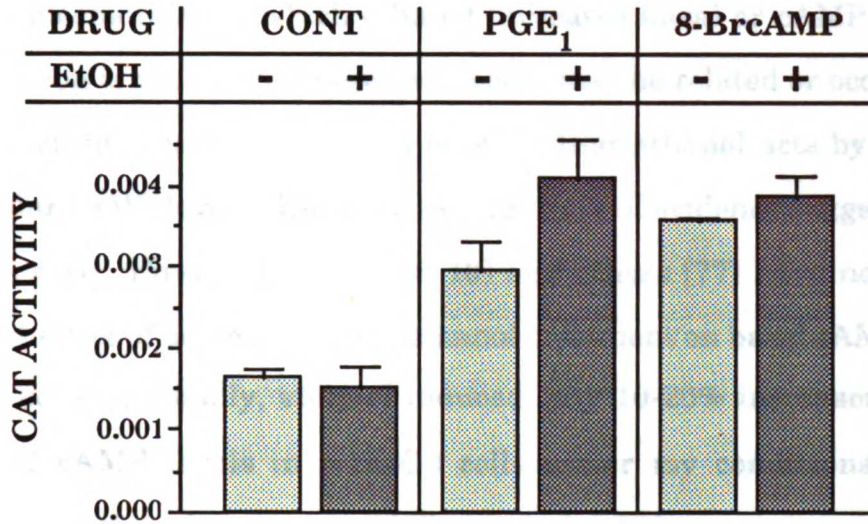
TABLE 4

Drug condition	EtOH	PGE$_1$	PGE$_1$+ EtOH vs PGE$_1$	8-BRcAMP	8-BRcAMP + EtOH vs 8-BRcAMP
% control	107 ± 8	256 ± 16	122 ± 4	306 ± 39	103 ± 11
N	8	10	9	4	4
p	N.S.	.0001	.003	.013	N.S.

FIG. 14. and TABLE 4 **Ethanol effects on basal and cAMP-responsive CRE-directed CAT activity.** An N1E-115 clone containing p25VIPCAT was incubated for 24 hours with either 1 μM PGE$_1$ or 0.5 mM 8-BR-cAMP in the presence or absence of 100 mM ethanol. CAT activity is expressed as a percent of total chloramphenicol incorporated into product and represents the mean ± S.D. from triplicate cultures for each data point. Table 4, cumulative data of 4 to 10 experiments similar to the experiment in Fig. 12. Values represent the mean percent differences between treatments and controls ± SEM. N represents the number of experiments performed. p represents the significance from a single group, two-tailed t-test.

4.4   DISCUSSION

The mechanism by which ethanol increases basal or cAMP-dependent TH expression is not clear. These two events may be related or occur through totally different mechanisms. It is possible that ethanol acts by increasing intracellular cAMP levels. However, several lines of evidence suggest that this is not the case. First, I (Fig. 13 and 10) and others [77] have not seen any significant effect of acute or chronic ethanol treatment on basal cAMP levels in N1E-115 cells. Secondly, ethanol induced only 10-20% increases in PGE_1-stimulated cAMP levels in N1E-C3 cells under my conditions (Fig. 13). Furthermore, ethanol increases cAMP-responsive TH expression even when 8-BrcAMP is used as the inducing agent (Fig. 12 and Table 3). It seems unlikely that small changes in cAMP levels produced by ethanol would have measurable effects on TH expression in the presence of relatively large amounts of a diffusible cAMP analog. Finally, no induction of CRE-directed promoter activity by ethanol was seen in cells containing the p25VIPCAT construct. These data suggest that basal levels of cAMP in ethanol-treated cells are not raised sufficiently to significantly activate CRE-directed promoter activity. In addition to no effects of ethanol on basal CRE-directed promoter activity, 8-BrcAMP-mediated activation of p25VIPCAT was not affected by ethanol coincubation. These result suggest that ethanol-induced changes in TH expression are not at the level of PKA or CREB since activation of these proteins should lead to increases in CAT activity directed by CRE alone. These results further suggest that events separate from the cAMP second messenger cascade are important for ethanol-induced changes in TH gene expression.

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PGE$_1$-mediated stimulation of CRE (p25VIPCAT) promoter activity in the presence of 100 mM ethanol was, however, increased by 22% over PGE$_1$ treatment alone (Table 4 and Fig. 14). This result could be explained by ethanol-induced changes in receptor-effector coupling. The 22% increase is very similar to the relative amounts of ethanol-induced stimulation of PGE$_1$-mediated increases in intracellular cAMP levels. This result taken together with the observation that ethanol had no effect on 8-BrcAMP-stimulation of CRE-directed activity, suggest that an event following the activation of PKA is mediating the effect of ethanol on PGE$_1$-stimulated CRE activity. Thus, ethanol-induced changes in receptor-effector coupling might have a role in the expression of genes that are regulated by cAMP. This effect may have particular importance in the nervous system since many genes (e.g., somatostatin, proopiomelanocortin, proenkephalin, and α-chorionic gonadotropin) important for neuronal signaling are regulated transcriptionally by cAMP [97, 98]. In the case of TH, this effect is probably masked by the relatively large ethanol-induced increase in TH expression produced through a separate mechanism.

In addition to the effects described above, ethanol and cAMP may regulate TH expression through distinct cis-acting elements that are capable of independent or dependent interactions. The TH promoter region used in our transfection experiments contains regulatory sequences for several other known signaling pathways in addition to the cAMP system. For example, glucocorticoid, HSE, AP-1, AP-2, POU/OCT-1, and SP1 response elements are contained in the 773 bp of the TH promoter [157]. Ethanol-induced changes in PKC may be important since AP-1 and AP-2 elements mediate PKC-stimulated gene expression. Another potential ethanol-induced response

element is the heat shock element (HSE) in the rat TH promoter. The role of these enhancer elements will be discussed in chapter 5.

It is possible that one or several of these elements play a role in the basal and cAMP-stimulated regulation of TH by ethanol. One could envision, for example, that chronic ethanol treatment causes activation of a trans-acting factor that is capable of positive synergistic interactions with CREB. An analogous situation exists where the glucocorticoid response element has been shown to interact with the phorbol ester-activated AP-1 (fos/jun) transcription factor [180]. One could also envision that ethanol induces TH gene expression through a response element and its respective DNA binding protein, independent of CRE and CREB. In this later case, ethanol-induced increased cAMP-stimulation of TH promoter activity would be additive and TH expression the effect of two independent elements working separately. Deletion analysis of the TH promoter should separate the role of the CRE element itself from any ethanol-responsive element mediating basal induction and enhancement of cAMP-responsiveness.

Chapter V

DELETION ANALYSIS OF THE TH PROMOTER

5.1 INTRODUCTION

In the previous chapters, I demonstrated that exposure of N1E-115 cells to ethanol resulted in increased TH mRNA and protein levels. Ethanol also induced TH promoter directed CAT activity in cells stably transfected with the TH promoter. In addition, ethanol-induced increases in CAT activity were specific to the TH promoter since neither the mouse sarcoma virus promoter nor the 25 base pair cAMP-responsive portion of the human VIP promoter were stimulated by ethanol exposure. These data suggest that cis-acting elements specific to the TH promoter other than or in addition to the CRE element mediate ethanol-induced activation of TH promoter activity.

In the TH promoter, most of the classic regulatory response elements characterized thus far are located in the first 220 nucleotides proximal to the transcription initiation site. For example, this region contains the enhancer elements important for basal and tissue specific expression of TH [167] as well as induction by growth factors [73], and cAMP [167]. The importance of the enhancer elements in the first 220 nucleotides in TH regulation is demonstrated by its conservation between rat and human TH promoters [167] (Fig. 15). This region also contains CRE, Sp-1 and AP-1 elements which are identical between the two species both in sequence and location from the TATA box (Fig. 15). Also located in this region is a POU/OCT-like binding region which may function as a negative regulator of TH expression since deletions in this region result in increased promoter activity [181]. Finally, two consensus AP-2

binding sites are located at position -218 to -210 and -140 to -132 of the rat TH promoter (Fig. 15).

Glucocorticoids have been shown to induce endogenous TH transcription in PC12 cells [68] and mice [54]. However, dexamethasone exposure of PC12 cells stably transfected with the rat 773 base pair TH promoter showed no induction of CAT activity while endogenous TH mRNA levels were increased, suggesting that other regions outside the -773 to +25 region mediate glucocorticoid inducibility [68]. Interestingly, there is a region homologous to the consensus glucocorticoid regulatory element described for the human metallothionein IIa gene [182] located at -452 to -443, adjacent to a third consensus AP-2 sequence (Fig. 15). However, this GRE consensus sequence is not conserved between species and therefore may not functionally bind the glucocorticoid receptor (Fig.15).

An interesting candidate DNA regulatory sequence that may mediate ethanol-responsiveness is the three heat shock consensus elements, nGAAn, located between bases -546 to -525 of the rat TH promoter (Fig. 15 and 16). This sequence may be a functional heat shock element since heat shock binding factors will bind with high affinity to a promoter if nGAAn is arranged either head to head (nGAAnnGAAn) or tail to tail (nGAAnnTTCn) [183]. The heat shock factor binding sequence is of interest because it is also found in the Hsc70 and pig MHCI genes (Fig. 16). The promoters of these genes are activated with similar time course and magnitude as TH (section 1.4).

Common regulatory elements are not restricted to the HSE. For example, Sp-1, AP-1 and AP-2-like enhancer elements are common to TH, Hsc70 and pig MHCI genes (Fig. 16). The mechanism of ethanol-induced activation of these three promoters is unknown, but is likely to involve common regulatory DNA-binding factors and their respective enhancer

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elements. Therefore, the HSE, AP-1, A-2 and Sp-1 elements are all likely candidates.
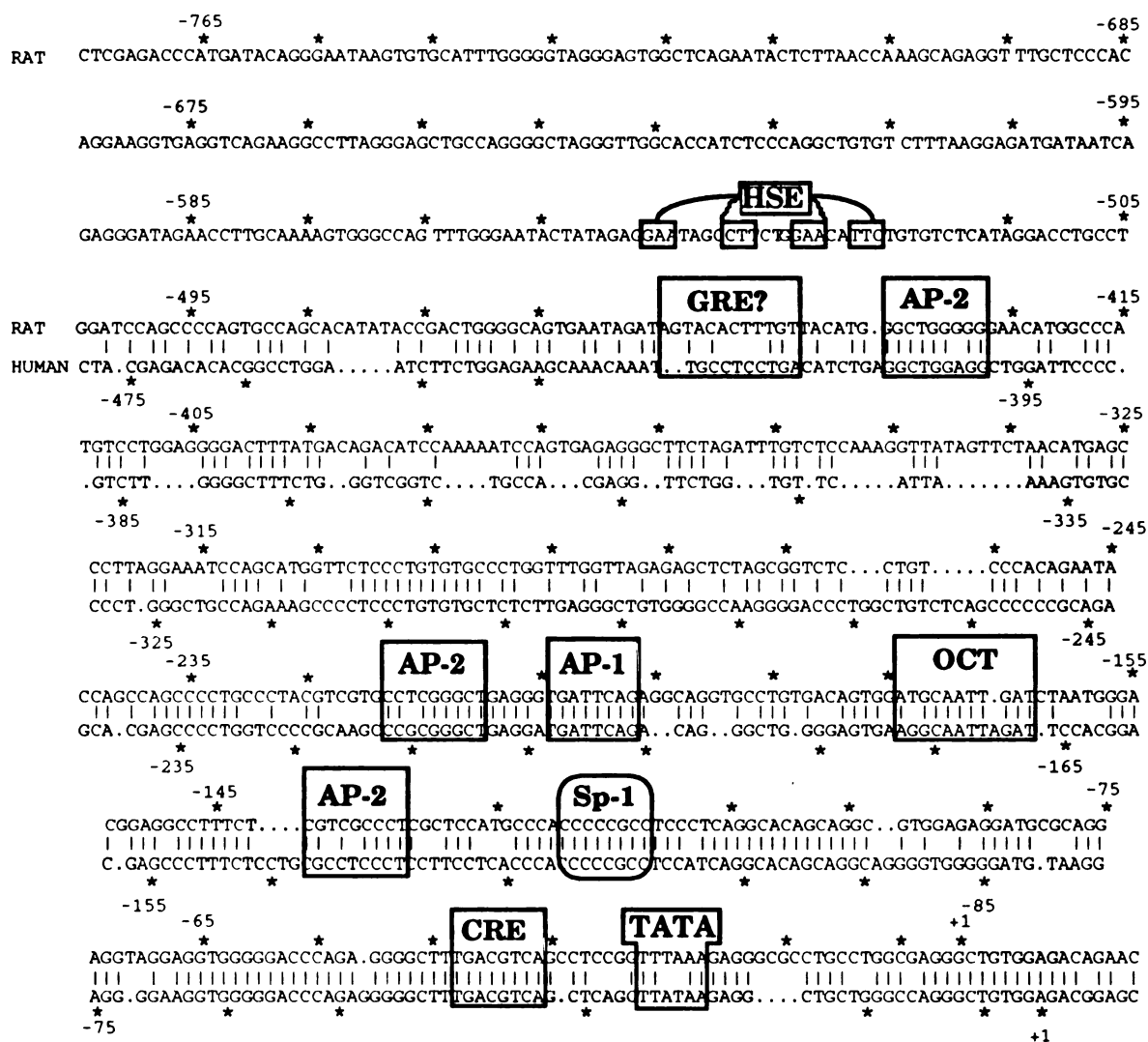
```
        -765                                                                                    -685
         *       *        *         *         *        *          *          *        *
RAT  CTCGAGACCCATGATACAGGGAATAAGTGTGCATTTGGGGGTAGGGAGTGGCTCAGAATACTCTTAACCAAAGCAGAGGT TTGCTCCCAC

        -675                                                                                    -595
         *       *        *         *         *        *          *          *        *
     AGGAAGGTGAGGTCAGAAGGCCTTAGGGAGCTGCCAGGGGCTAGGGTTGGCACCATCTCCCAGGCTGTGT CTTTAAGGAGATGATAATCA

        -585                                                        ┌──HSE──┐                    -505
         *       *        *         *                             ┌─┤       ├─┐        *         *
     GAGGGATAGAACCTTGCAAAAGTGGGCCAG TTTGGGAATACTATAGAC GAA TAGC CTT CT GAA CA TTC TGTGTCTCATAGGACCTGCCT

        -495                                        ┌────────┐         ┌──────┐                  -415
         *       *        *         *               │ GRE?   │         │ AP-2 │        *         *
RAT  GGATCCAGCCCCAGTGCCAGCACATATACCGACTGGGGCAGTGAATAGAT AGTACACTTTGT TACATG. GGCTGGGGG AACATGGCCCA
     I I II I II  III I I    I I  IIII I II  II I II II I I   II I  II IIIIII II  II III
HUMAN CTA.CGAGACACACGGCCTGGA.....ATCTTCTGGAGAAGCAAACAAAT..TGCCTCCTGA CATCTGA GGCTGGAGC CTGGATTCCCC.
        -475                                                                                    -395
        -405 *          *         *         *          *          *          *        *       -325
         *                                                                                       *
     TGTCCTGGAGGGGACTTTATGACAGACATCCAAAAATCCAGTGAGAGGGCTTCTAGATTTGTCTCCAAAGGTTATAGTTCTAACATGAGC
     III I   III IIII II  I I     IIII  IIII I III II   III   II II
     .GTCTT....GGGGCTTTCTG..GGTCGGTC....TGCCA...CGAGG..TTCTGG...TGT.TC.....ATTA.......AAAGTGTGC
        -385 *          *         *         *          *          *          *        *       -335
        -315                                                                                    -245
     CCTTAGGAAATCCAGCATGGTTCTCCCTGTGTGCCCTGGTTTGGTTAGAGAGCTCTAGCGGTCTC...CTGT.....CCCACAGAATA
     II I II    IIII I I   IIIIIIIIIIIIII II  I  II  I I I I II II I I    IIII    III I I I I
     CCCT.GGGCTGCCAGAAAGCCCCTCCCTGTGTGCTCTCTTGAGGGCTGTGGGGCCAAGGGGACCCTGGCTGTCTCAGCCCCCCGCAGA
        -325                                                                                    -245
        -235  *          *        ┌────────┐  ┌───────┐   *          *     ┌────────┐           -155
         *                        │ AP-2   │  │ AP-1  │                     │  OCT   │            *
     CCAGCCAGCCCCTGCCCTACGTCGTG CCTCGGGC GAGGG GATTCA AGGCAGGTGCCTGTGACAGTG ATGCAATT.GA TAATGGGA
     II I IIIIIIIII I I II      IIIII I    IIIIIII II   IIIIII III II IIIII  IIIIII III I    III
     GCA.CGAGCCCCTGGTCCCCGCAAGC CCGCGGGC GAGGA GATTCA A...CAG..GGCTG.GGGAGTGA AGGCAATTAGAT.TCCACGGA
        -235                                                                                    -165
        -145  *                   ┌────────┐       *      ╭─────╮     *          *        *      -75
         *                        │ AP-2   │              │ Sp-1│                                 *
     CGGAGGCCTTTCT.... CGTCGCCCT CGCTCCATGCCCA CCCCGCG TCCCTCAGGCACAGCAGGC..GTGGAGAGGATGCGCAGG
     I III IIIIIII     IIIIIIII  III         II IIIIIIIIIIIIII II  I II  III    I III
     C.GAGCCCTTTCTCCTG GCCTCCCT CTTCCTCACCCA CCCCGCG TCCATCAGGCACAGCAGGCAGGGGTGGGGATG.TAAGG
        -155                                                                                    -85
        -65   *          *        ┌─────┐    ┌──────┐      *          *        *                  +1
         *                        │ CRE │    │ TATA │
     AGGTAGGAGGTGGGGGACCCAGA.GGGGCTT GACGTCA CCTCCG TTTAAA GAGGGCGCCTGCCTGGCGAGGGCTGTGGAGACAGAAC
     III I IIIIIIIIIIIIIIIIIII IIIIIIII IIIIIIII II III IIIIII IIIII III IIIIIIIIIIIIIII II I
     AGG.GGAAGGTGGGGGACCCAGAGGGGGCTT GACGTCA G.CTCAG TTATAA GAGG....CTGCTGGGCCAGGGCTGTGGAGACGGAGC
        -75                                                                                      +1
```

FIG. 15 **Comparison of the rat and human TH promoter 5' promoter regions.** Sequences were aligned to show maximum homology as was reported by Cambi et. al. [167]. Only 504 bases of the human TH promoter region are shown and aligned to the 773 bases of the rat TH promoter. The putative DNA transcription factor binding sites are shown.

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	HSE	SP-1	AP-2	AP-1
TH	GAATAGCCTTCTGGAACATTCTGT → ← →	CCCCGCC	GCCTCGGGC	TGATTCA
Hsc 70	GAACCCTTCTGGAAGGTTCTAA → ← →	CCCCGCC	GCCCCCGCG	TGAGTCA
MHC	GAATATTCTCACTAAAAGGTTTGGAA → ← →	CCCCGCC	GCCCCCGGG	TGATTCA
CONSENSUS	nGAAnnTTC or nTTCnnGAA → ← ← →	CCCCGCC	GCCNNNGGC	TGANTCA

**FIG. 16 Common enhancer sequences of the TH, Hsc70 and MHC 5'
promoter regions.**

In order to initially characterize which region of the TH promoter is
responsive to ethanol exposure, deletion analysis of the TH promoter was
performed. Using transient transfection, the activity of various promoter
regions in directing ethanol-induced CAT synthesis was determined.
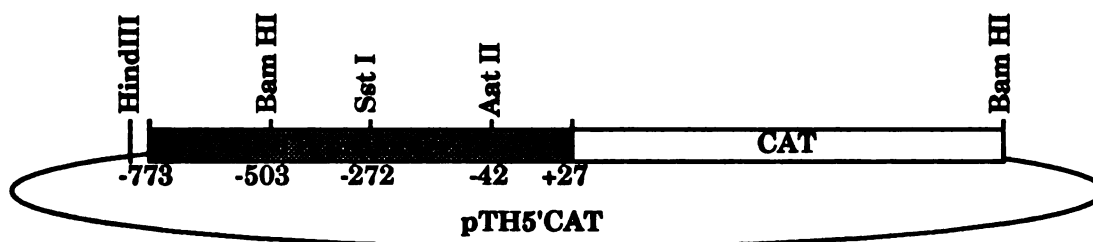
5.2   METHODS

FIG. 17 pTH5'CAT. Shown are the restriction enzyme sites in the TH
promoter used to construct promoter deletions. Shaded region is the TH
promoter and the open box is the CAT gene from pSV2CAT. The solid line
represents pUC13 sequences.

5.2.1   *Materials-* Enzymes were molecular biology grade and purchased from
either Promega or Boehringer Mannheim (B.M.). Electroporation chambers
were purchased from GIBCO-BRL. All other reagents were purchased from
either Sigma Chem., or B.M.

5.2.2 *Cell Cultures-* N1E-115 cells (passage 17-25) were cultured in 10% fetal calf serum in DMEM (section 2.2.2). Three days before transfection one million cells (a cell density resulting in 1/2 confluence on the day of transfection) were seeded into a T-175 flask (Falcon).

5.2.3 *Promoter deletions-* Ligation reactions, alkaline phosphatase treatments, restriction enzyme, and T4 DNA polymerase digestions, as well as procedures for agarose gel electrophoresis, were done exactly as described in Maniatis [152]. The sequence from -503 to -773 was deleted by first digesting p5'THCAT with Bam HI (see Fig. 17). The 2235 base pair DNA fragment containing the truncated TH promoter and CAT gene was then separated from the vector DNA by 0.8% agarose gel electrophoresis and purified using the Geneclean kit procedure (BIO 101). Next, the 2235 base pair fragment was treated with alkaline phosphatase to remove 5' phosphates. The reaction was stopped by phenol/chloroform extraction. Equal molar amounts of the truncated TH promoter and pGEM-2 (Promega) (which was previously digested with Bam HI followed by phenol/chloroform extraction) were ligated together using bacteriophage T4 DNA ligase. Finally, the ligated DNA was subcloned into subcloning efficiency DH5α competent bacteria (BRL) using the protocol provided with the bacteria.

Deletion of -773 to -272 base pair of the TH promoter was performed by first incubating p5'THCAT with restriction enzymes HindIII and SstI. The large 1857 base pair fragment was then separated from the 501 base pair distal promoter end (see Fig. 17) by 0.8% agarose gel electrophoresis. The large fragment was separated from the agarose gel by the Geneclean kit procedure. Overhanging ends were removed by digestion with bacteriophage T4 DNA

polymerase in the presence of dATP, dTTP, dGTP and dCTP. Blunt ends were ligated together with T4 DNA ligase. Finally, ligated DNA was subcloned into DH5α bacteria as described above.

5.2.4   *Electroporation-* Monolayers of N1E-115 cells in T-175 flasks were washed once with DMEM. Cells were removed from the flask and pelleted by centrifugation (1000 rpm for 5 min.). N1E-115 cells were then resuspended in DMEM at 6 million cells/ml. 0.8 ml. of the cell suspension was then placed into an electroporation chamber that contained 100 μg of DNA in 100 μl of TE buffer, pH 7.4. Cells and DNA were mixed by several inversions of the chamber. Electroporation was performed in a GIBCO-BRL cell porater system 1, at the setting of 1180 μf and 500 v. These settings provide for maximum transfection efficiency (data not shown). Following electroporation, the contents of one chamber was added to 20 ml of 10% FCS and mixed. Three ml/well was then aliquoted into each well of a 6 well plate (Falcon). Cells were allowed to recover for 24 hours before drug treatment. The drug treatment duration was for an additional 24 hours. Cells were lysed and CAT assays performed as described in section 3.2.5.

5.3   RESULTS

5.3.1   To test whether there are ethanol-regulated sequences in the rat TH promoter, I constructed a series of CAT-fusion genes containing various lengths of the 5'-flanking region of the rat TH gene. Ethanol-induced changes in both basal and cAMP-stimulated CAT activity were determined in N1E-115 cells transiently transfected with the TH promoter-CAT constructs. Ethanol-induced increases in both basal and cAMP-stimulated

CAT activity were demonstrated in N1E-C3 cells that contain p-773TH5'CAT stably integrated into their genome (Chapter 3 and 4). To confirm that similar ethanol-induced increases occur under transient transfection conditions, N1E-115 cells were transfected with p-773TH5'CAT and then incubated for 24 hours in the presence of 200 mM ethanol, with and without 0.75 mM 8-BrcAMP treatment. Ethanol exposure increased basal CAT activity by 40% in transiently transfected cells (Table 5). Large increases (12 to 14 fold) in CAT activity were seen with 24 hour treatment of 0.75 mM 8-BrcAMP. Ethanol coincubation with 8-BrcAMP led to a 48% increase in CAT activity when compared to cells incubated with 8-BrcAMP alone (Table 5). Ethanol coincubation with 1 μM PGE_1 produced similar increases in CAT activity when compared to cells treated with PGE_1 alone (data not shown). These results are similar to the observations made in the stable cell lines that contain p-773TH5'CAT. To define the promoter region responsible for mediating ethanol-induced increases in CAT activity, deletion constructs -503, and -272TH5'CAT were transiently transfected into N1E-115 cells (Fig. 18). Transfected cells were then treated using the same conditions as described above for p-773TH5'CAT. Ethanol exposure of cells transfected with p-503TH5'CAT produced variable results (Table 5). However, ethanol plus 8-BrcAMP stimulated CAT activity by an average of 64% when compared to 8-BrcAMP-treatment alone (Table 5). Ethanol exposure of cells transfected with p-272TH5'CAT resulted in a 57±20% increase in basal and a 93±61% increase in cAMP-stimulated CAT activity compared to their respective controls (Table 5). Ethanol-induced increases in cAMP-stimulated -503TH5' or -272TH5' directed CAT activity was seen whether 8BrcAMP or PGE_1 was used to

increase intracellular cAMP levels (data not shown). These results are similar
to the ethanol-induced changes in p-773TH5' directed CAT activity (Table 5).

The control plasmid, pMSVCAT, when transiently transfected in N1E-
115 cells, showed similar results to stable cell lines containing pMSVCAT. No
induction of pMSVCAT activity was seen in all 11 transient transfection
experiments (Table 5). As expected, since the MSV promoter has no CRE
elements, treatment with 8-BrcAMP had no effect on MSV-directed CAT
activity and no further increases were seen with ethanol and 8BrcAMP
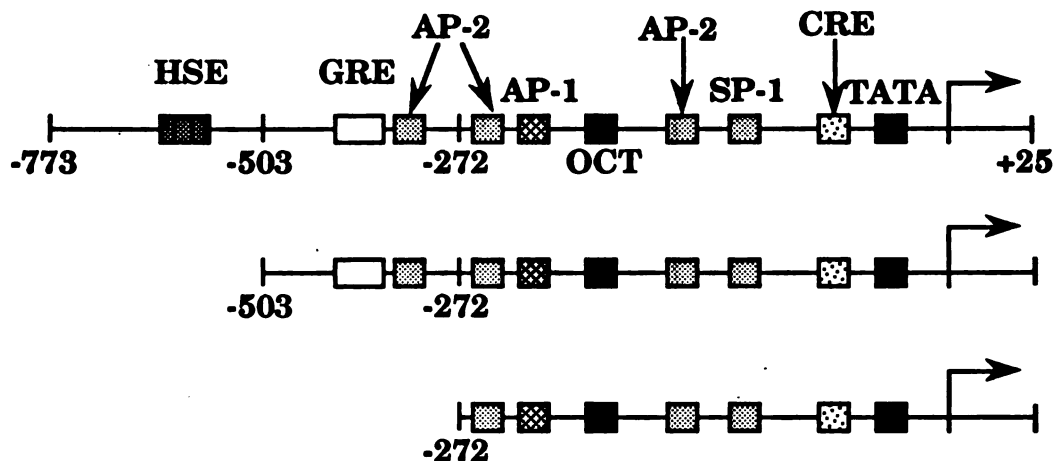coincubation (data not shown).

FIG. 18 **Putative enhancer sequences in the rat TH promoter.** TH
promoter CAT constructs used for experiments in Table 5 are shown above.
The putative enhancer sequences are shown (boxes).

TABLE 5

p(-773)TH5'CAT	EtOH	8-BrcAMP	8-BrcAMP + EtOH	8-BrcAMP + EtOH vs 8-BrcAMP
% control	140% ± 9%	1300% ± 370%	1900% ± 480%	148% ± 5%
n	12	5	5	13
P	.001	.03	.02	.02
p(-503)TH5'CAT				
% control	118% ± 14%	900% ± 300%	1500% ± 500%	160% ± 35%
n	7	4	4	4
P	N.S.	.04	.04	.08
p(-272)TH5'CAT				
% control	160% ± 20%	1500% ± 370%	2600% ± 670%	200% ± 61%
n	7	3	3	3
P	.03	.03	.03	N.S.

pMSVCAT	EtOH
% control	97% ± 4
n	11
P	N.S.

Table 5 **Ethanol effects on basal and 8-BrcAMP-stimulated TH promoter deletions.** N1E-115 were transiently transfected with either p-773TH5'CAT, p-503TH5'CAT, p-272TH5'CAT, or pMSVCAT as described in methods. Following transfection, cells were incubated for 24 hours with either buffer alone or 0.7 mM 8-BrcAMP, with and without 200 mM ethanol cotreatment. Cells were then lysed and CAT assays were performed (Methods). Percent of control represents the mean percent difference between control and treated cells ± SEM from independent experiments. N represents the number of experiments performed. P represents the significance from a single group, t- test.

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5.4 DISCUSSION

In chapters 3 and 4 it was demonstrated that -773 base pairs of the 5' proximal TH promoter encodes a specific nucleotide sequence(s) that mediates ethanol-induced increases in promoter activity. In this chapter, TH promoter CAT fusion constructs, -773, -503, and -272TH5'CAT, were transiently transfected into N1E-115 cells in order to further delimit the ethanol-responsive cis-acting sequence. Using these constructs the region responsible for ethanol-induced promoter activation was found to be located between -272 and +25 base pair of the TH promoter.

To determine that transient transfection analysis could produce similar results to those obtained using stable transfection analysis, I transfected N1E-115 cells with the -773TH5'CAT construct and then incubated the cells with 200 mM ethanol in the presence and absence of 0.75 mM 8-BrcAMP for 24 hours. I found that ethanol treatment increased CAT activity in cell lysates by $40 \pm 14\%$ (mean±S.E.M, n=7) when compared to lysates from mock-treated cells. In addition, 8-BrcAMP stimulated CAT activity was also enhanced after ethanol exposure by $65 \pm 13\%$ (mean±S.E.M, n=5) when compared to CAT activity in lysates treated with 8-BrcAMP alone. These results indicate that ethanol has similar effects on TH promoter activity whether measured in stable cell lines or by using transient transfection assays. The magnitude of the ethanol-induced increases in the transient transfection analysis is lower than those seen using the stable transfection analysis (compare Table 3 to Table 5) and may be due the differences in cell density which may alter the levels of basal TH expression. Also, serum was used in the transient transfection analysis. Serum growth factors may lead to

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higher background levels of TH and a decrease in induction by ethanol exposure.

I next determined whether the -503, and -272TH5' promoter regions could mediate ethanol-induced increases in CAT activity. These promoter lengths were used to determine the role of various elements in mediating ethanol induction and were constructed by taking advantage of unique restriction endonuclease sites in the rat TH promoter. Construct -503TH5'CAT is interesting because of the three heat shock consensus sequences (HSE), located between -546 and -525, removed (Fig 18). The consensus sequence similarity of the TH HSE to other HSE elements (Fig 18), the observation that mild heat shock (45°C for 1 hour) will specifically increase -773TH5' directed CAT activity in N1E-C3 cells approximately 40% over control cultures (Fig 19, appendix), and gel-retardation studies which demonstrate that the -773TH5' promoter can prevent HSE DNA binding proteins from binding to the minimal HSE element in the HSC70 promoter (Fig. 20, appendix), suggest that the TH-HSE is functional. Since, HSE sequences are found in other ethanol-induced promoters (section 1.4), the -503TH5'CAT construct can be used to determine the role of the HSE in mediating ethanol induction. I found that ethanol incubation of cells transiently transfected with -503TH5'CAT increased both basal and cAMP-stimulated CAT activity in lysates compared to their respective controls (Table 5) However, the ethanol-induced increases in -503TH5' directed CAT activity are relatively low compared to ethanol-induced increases seen using -773TH5'CAT, 118% vs 140%, respectively (Table 5). The relatively low induction may be real or may be a consequence of variable results between experiments. The -272TH5'CAT construct has the HSE element as well as the putative GRE and AP-2 sequences deleted. This later construct can therefore also be used to determine the role of HSE in mediating

ethanol-induced increases. I found ethanol exposure of cells transfected with -272TH5'CAT led to increases in both basal and 8-BrcAMP-stimulated promoter activity as compared to their respective controls. Furthermore, the magnitude of ethanol-induced increases were similar to those seen when the -773TH5'CAT construct was used (Table 5). The results obtained using the -503TH5'CAT and the -272TH5'CAT construct suggest that the HSE element is not responsible for ethanol-induced increases in TH expression. The HSE element may, however, be important in the regulation of TH expression by heat or other stressors. The involvement of the HSE element in the regulation of TH warrants further study since it represents a potential mechanism for regulation of TH by physiologic stress.

These results, taken together, suggest that ethanol-responsive nucleotide sequences are located between -272 and +25 base pair of the rat TH promoter. This region contains several known cis-acting sequences, such as, AP-1, AP-2, POU/OCT and Sp-1 enhancer elements. The AP-1, AP-2 and Sp-1 elements are also found in other genes regulated by ethanol and are therefore targets for future studies. The AP-1 element in the TH promoter is a functional element that has been shown to bind c-Fos and c-Jun and activate transcription in response to NGF treatment of PC12 cells [114]. In addition to NGF, c-Fos expression is increased by a variety of stimuli, including heat shock, phorbol esters, serum [113, 184]. It is possible that ethanol-induced activation of gene expression is mediated through increased expression of c-Fos or other AP-1 binding proteins. This possibility can be tested by transfection analyses similar to those described in this chapter using constructs that have base substitutions in the AP-1 element, thus rendering it nonfunctional. Alternatively, direct measurements of c-Fos mRNA and protein levels can be measured to determine if ethanol-induced changes in c-Fos expression occur.

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Similarly, point mutations can be made in the AP-2 enhancer element. AP-2 elements, like AP-1, are regulated by activation of PKC [121]. It is possible that ethanol-induced increases in PKC are involved in the regulation of TH. It has been demonstrated, in PC12 cells, that an ethanol-induced increase in voltage sensitive Ca^{++} channels is mediated by PKC [11, 93, 121]. Furthermore, the expression of some PKC isotypes in both PC12 and NG108-15 neuroblastoma cells are increased by chronic ethanol exposure (Messing, personal communication). Finally, the role of the Sp-1 element in ethanol-induced changes in gene expression can not be ruled out, since this element is also found in genes which are induced by ethanol.

It is also possible that a yet unidentified enhancer sequence is responsible for mediating ethanol-induced changes in TH expression. To identify the ethanol-responsive sequence, successive 5' deletions of linearized -272TH5'CAT can be made by digestion with the processing exonuclease Bal31 or ExoIII/Mung Bean nuclease. Various promoter deletion mutants can then be assayed for ethanol-induced changes in CAT expression. Once the ethanol-responsive region is isolated the DNA binding protein(s) can be purified and identified by heparin-agarose chromatography, Sephacryl S-300, and sequence-specific affinity columns, similar to the methods used to purify AP-1 and AP-2 [121]. The isolation of a putative ethanol-responsive DNA-binding protein is important because, once isolated, the specific mechanism of ethanol-induced changes in gene expression could be determined. For example, it is possible that ethanol-induced changes in a second messenger cascade result in the phosphorylation or dephosphorylation of the putative ethanol-responsive DNA-binding protein, which in turn, alters its affinity for its specific nucleotide sequence or perhaps its ability to stimulate/stabilize the RNA-polymerase complex. Alternatively, the DNA-binding protein may be a direct target for the

ethanol molecule itself. It is possible that the ethanol molecule may cause conformational changes in the DNA-binding protein resulting in altered DNA-binding or interactions with other transcriptional regulatory proteins. This mode is however less likely in light of the known effects of ethanol on second messenger regulation. It is more probable that ethanol alters DNA-binding proteins through ethanol-induced changes in regulatory cascades.

Finally, it is now clear that gene promoter regulatory regions with multiple enhancer elements are regulated by complex DNA-binding protein interactions. Miner and Yamamoto, in a recent review, describe several models for complex regulatory interactions that have been reported to regulate transcription [185]. The first model describes DNA-binding proteins from different families interacting specifically with each other at specific DNA sequences resulting in co-occupancy. The second describes protein-protein contact between members of different DNA-binding protein superfamilies in which only one factor binds to DNA. Finally, two factors from different gene families can interact with each other without binding to DNA resulting in mutual inhibition. Since the -272 to +25 portion of the TH promoter contains many enhancer elements complex regulatory interactions may be involved in ethanol's action. Defining the regulatory regions in the TH promoter that are responsive to ethanol is the first step in determining the mechanisms of ethanol-induced changes in transcriptional regulation.

Ethanol-induced increases in cAMP stimulation may result from an additive effect of two separate response elements (i.e., CRE and a ethanol responsive element) or may result from a more complex interaction of DNA-binding proteins. The simplest scenario would be that there is two separate enhancer sequences in the TH promoter. Since, the CRE element in the TH promoter is located close to the TATA box, it is possible to delete more distal

sequences without affecting the CRE element. If two separate enhancer sequences are involved, deletion analysis should separate out the effects of ethanol and cAMP on TH expression. Alternatively, a more complex interaction of CREB with other DNA binding proteins may be involved such as those described in section 1.4. The nucleotides sequence surrounding the CRE element are highly conserved between rat and human TH promoters (Fig. 15), suggesting that other DNA-binding proteins may interact with this region and thus in the regulation of TH transcription. It would be interesting to determine, either by gel-retardation analysis or DNA-nuclease protection assays, if ethanol alters DNA-binding protein patterns at the sequence surrounding the CRE element in the TH promoter. It is not known if ethanol exposure has effects on cAMP-stimulated TH promoter activity in other cell types besides N1E-115 cells. Diamond et. al. has demonstrated that positive interactions between glucocorticoid receptors and c-Jun are cell-type specific [180]. Future studies are needed to address these issues.
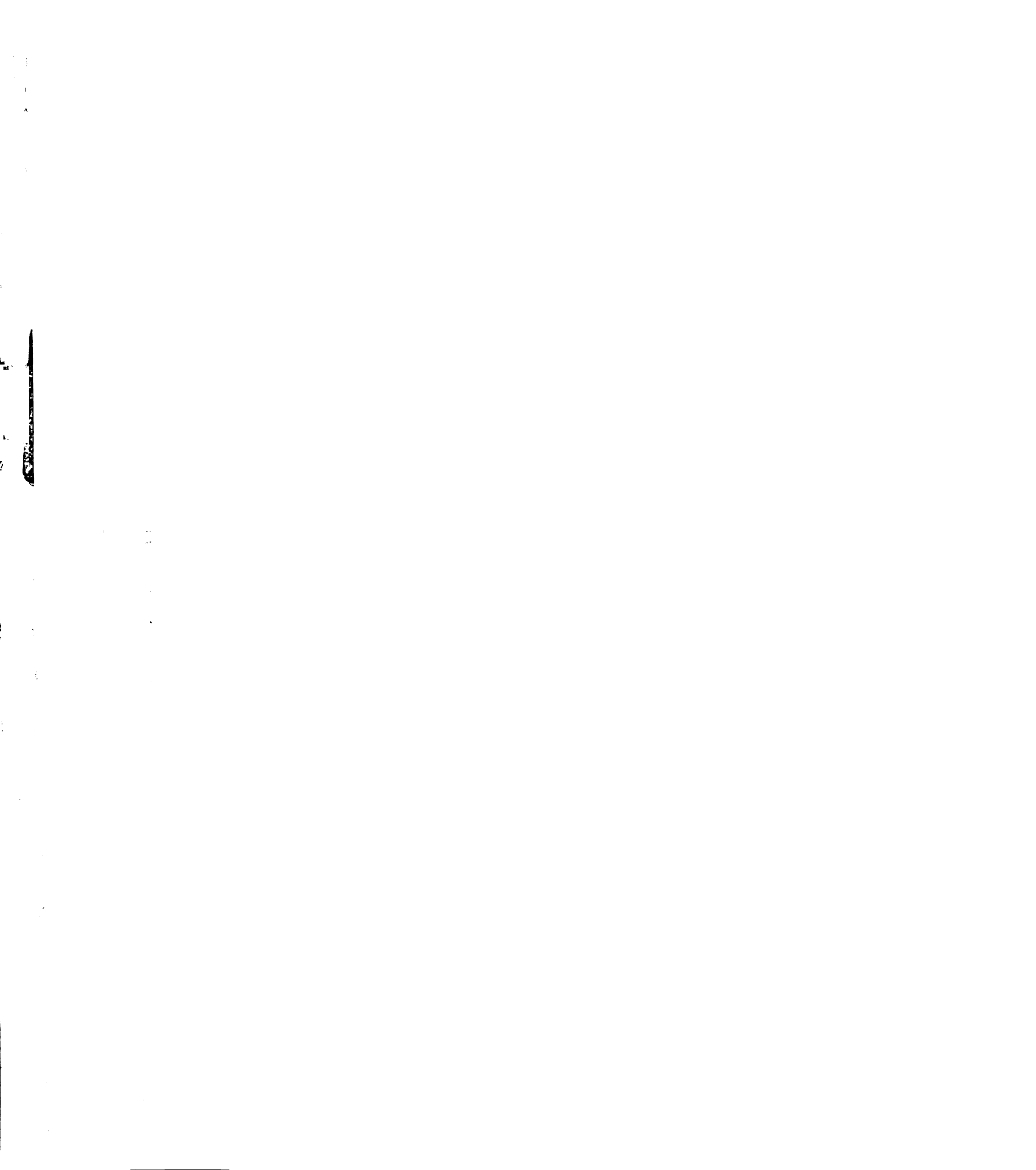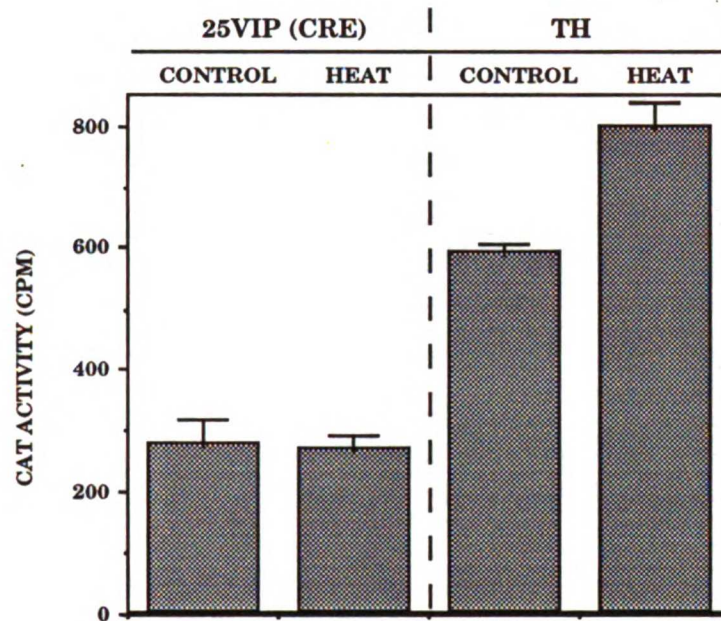
5.5 APPENDIX

FIG. 19 **Heat shock induction of TH promoter activity.** N1E-C3 cells which contain the -773THCAT promoter expression vector and the clone containing p25VIPCAT used in Fig. 14 were incubated at 45 °C for one hour and compared to parallel cultures incubated at 37 °C. Cells were lysed and CAT activity was measured. CAT activity is expressed as the mean ± S.D. from triplicate cultures for each data point. Results are representative of experiments repeated 3 times.
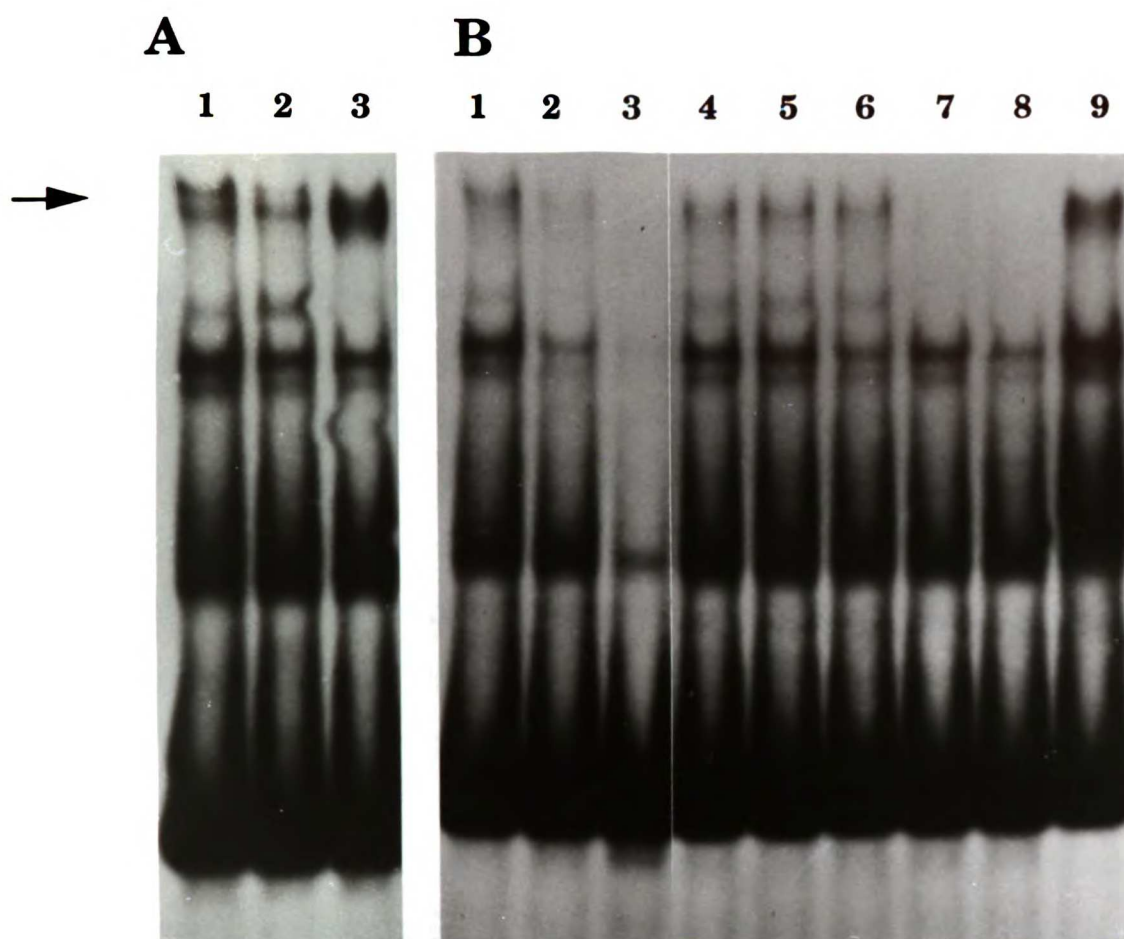
A

1 2 3

B

1 2 3 4 5 6 7 8 9

FIG. 20 **TH promoter competes for heat shock binding factor--gel retardation analysis.** Methods used in this figure were identical to those of Sorger and Pelham [186]. The DNA sequence between -86 and -30 of the rat Hsc 70 promoter was used as the radiolabeled probe. This DNA sequence contains three heat shock tandem elements and a Sp1 element [187]. In panel A, lysates were obtained from control cells (lane 1), cells treated with 200 mM ethanol for 24 hours (lane 2), and cells incubated at 45 °C for one hour (lane 3). Panel B, various unlabeled DNA constructs were used to compete for heat shock factor binding to the radiolabeled Hsc 70 promoter. These competitors include; no competitor (lane 1), 10 (lane 2) and a 25 (lane 3) time molar excess of unlabeled Hsc 70 promoter, Hse 12 (lane 4) which is a yeast heat shock promoter with the heat shock elements mutated [187], commercial Sp1 (lane 5) and AP1 (lane 6) oligonucleotides, -773 bp of the TH promoter (lane 7), Hsc 1/3 (lane 8) which contains the bases between -63 to +15 of the Hsc 70 promoter (this promoter portion contains the three heat shock elements minus the Sp1 element), and finally a mutant form of Hsc 1/3 which has base substitutions in each heat shock element. Arrow indicates the location of putative heat shock factor.

Chapter VI

CONCLUSION

I have found that exposure of N1E-115 cells to clinically relevant concentrations of ethanol produced increases in TH promoter activity, mRNA, and protein levels. Many studies using a variety of animal models indicate that catecholaminergic activity is increased upon chronic ethanol consumption. My studies suggest that ethanol-induced increases in TH expression may underlie the increased catecholaminergic tone seen in animals after long term ethanol consumption. In addition, since the catecholaminergic system is important in the development of tolerance to ethanol, increased TH expression may be an important molecular event in the development of tolerance to ethanol.

I also found that ethanol exposure of N1E-115 cells leads to significant increases in cAMP-stimulated TH expression. This observation is important for several reasons. First, it is well known that transynaptic-induction of TH expression, via increases in intracellular cAMP, is an important form of TH regulation, *in vivo*. Therefore ethanol-induced increases in cAMP-mediated TH expression may further increase the expression of TH after transynaptic induction. Second, ethanol-induced increases in cAMP-mediated gene expression are not restricted to the TH gene. I found ethanol-induced increases in PGE_1-stimulated p25VIPCAT activity. This latter plasmid contains the minimal cAMP responsive portion of the human VIP promoter. This result indicates that expression of many genes regulated by cAMP may be affected by ethanol-induced changes in cAMP generation. Finally, these data demonstrate for the first time that ethanol-induced changes in cAMP generation can have important effects on gene expression.

Finally an attempt was made to define the specific nucleotide sequence responsible for ethanol-induced increases in TH expression. I have narrowed down the ethanol-responsive nucleotide sequence to the first 272 bases of the TH promoter. Studies now can be performed to determine the exact regions that mediate ethanol induction. It is the hope of our laboratory that by understanding how ethanol regulates TH expression, insights may be gained as to how ethanol regulates gene expression in general. Understanding ethanol's effects on gene expression are vital because alterations in the levels of specific neural proteins may lead to long term behavioral changes in the brain, such as, tolerance and physical dependence.

BIBLIOGRAPHY

1.   Gordis, E., et. al. Seventh special report to the U.S. congress on alcohol and health. 1990.

2.   Charness, M. E., R. P. Simon and D. G. Greenberg. Ethanol and the nervous system. N. Engl. J. Med. 321(7): 442-454, 1989.

3.   Jaffe, J. "Drug addiction and drug abuse." The pharmacological basis of therapeutics. Gilman, Goodman, Rall and Murad ed. 1985 Macmillan Publishing Company. New York.

4.   Chin, J. H., L. M. Parsons and D. B. Goldstein. Increased cholesterol content of erythrocyte and brain membranes in ethanol-tolerant mice. Biochem. Biophys. Acta. 513: 358-363, 1978.

5.   Goldstein, D. B. and A. Goldstein. Possible role of enzyme inhibition and repression in drug tolerance and addiction. Biochem. Pharmacol. 8: 48-49, 1961.

6.   Charness, M. E., L. A. Querimit and I. Diamond. Ethanol increases the expression of functional delta-opioid receptors in neuroblastoma x glioma NG108-15 hybrid cells. J. Biol. Chem. 261(7): 3164-3169, 1986.

7.   Valverius, P., P. L. Hoffman and B. Tabakoff. Effect of ethanol on mouse cerebral cortical β-adrenergic receptors. Mol. Pharmacol. 32: 217-222, 1987.

8.   Charness, M. E., L. A. Quermit and M. Henteleff. Ethanol differentially regulates G proteins in neural cells. Biochem. Biophys. Res. Commun. 155(1): 138-143, 1988.

9.   Gordon, A. S., K. Collier and I. Diamond. Ethanol regulation of adensoine receptor-stimulated cAMP levels in a clonal neural cell line: an *in vitro* model of cellular tolerance to ethanol. Proc. Natl. Acad. Sci. USA. 83: 2105-2108, 1986.

10.   Mochly-Rosen, D., F.-H. Chang, L. Cheever, M. Kim, I. Diamond and A. S. Gordon. Chronic ethanol causes heterologous desensitization of receptors by reducing α_s messenger RNA. Nature. 333: 848-850, 1988.

11.   Messing, R. O., C. L. Carpenter, I. Diamond and D. A. Greenberg. Ethanol regulates calcium channels in clonal neural cells. Proc. Natl. Acad. Sci. USA. 83: 6213-6215, 1986.

12.   Deitrich, R. A., T. V. Dunwiddie, R. A. Harris and V. G. Erwin. Mechanism of action of ethanol: initial central nervous system actions. Pharmacol. Rev. 41(4): 489-536, 1989.

13.   Lieber, C. S. and L. M. DeCarli. The role of the hepatic microsomal ethanol oxidizing system (MEOS) for ethanol metabolism in vivo. J. Pharmacol. Exp. Ther. 81: 279-287, 1972.

14.   Lindblad, B. and R. Olsson. Unusually high levels of blood alcohol? J. Amer. Med. Assn. 236: 1600-1602, 1976.

15.   Melchior, C. L. and B. Tabakoff. Modification of environmentally cued tolerance to ethanol in mice. J. Pharmacol. Exp. Ther. 219(1): 175-180, 1981.

16.   Edwards, F., V. V. Schabinsky, D. M. Jackson, G. A. Starmer and D. Jenkins. Involvement of catecholamines in acute tolerance to ethanol in mice. Psychopharm. 79: 246-250, 1983.

17.   Ritzmann, R. F. and B. Tabakoff. Dissociation of alcohol tolerance and dependence. Nature. 263: 418-420, 1976.

18.   Tabakoff, B. and R. F. Ritzman. The effects of 6-hydroxydopamine on tolerance to and dependence on ethanol. J. Pharmacol. Exp. Ther. 203(2): 319-331, 1977.

19.   Friedler, G., H. N. Bhargava, R. Quock and E. L. Way. The effect of 6-hydroxydopamine on morphine tolerance and physical dependence. J. Pharmacol. Exp. Ther. 183: 49-55, 1972.

20. Le, D. A., J. M. Khanna, H. Kalant and A. E. LeBlanc. Effect of L-tryptophan on the acquisition of tolerance to ethanol-induced motor impairment and hypothermia. Psychopharm. 61: 125-129, 1979.

21. Le, D. A., J. M. Khanna, H. Kalant and A. E. LeBlance. Effect of modification of brain serotonin (5-HT), norepinephrine (NE), and dopamine (DA) on ethanol tolerance. Psychopharm. 75: 231-235, 1981.

22. Hoffman, P. L., R. F. Ritzman, R. Walter and B. Tabakoff. Vasopressin maintains ethanol tolerance. Nature. 276: 614-616, 1978.

23. Hoffman, P. L., C. L. Melchior and B. Tabakoff. Vasopressin maintenance of ethanol tolerance requires intact brain noradrenergic systems. Life Science. 32: 1065-1071, 1983.

24. Eriksson, K. and M. Rosi. "Finnish selection studies on alcohol-related behaviors." Development of animal models as pharmacologic tools. McCleran, Deitrich and Erwin ed. 1981 Government Printing Office. Rockville.

25. Kiianmaa, K. and K. Hellevuo. The alcohol tolerant and alcohol nontolerant rat lines selected for differential sensitivity to ethanol: a tool to study mechanism of the actions of ethanol. Annal. Med. 22: 283-287, 1990.

26. Hellevuo, K., K. Kilanmaa and E. R. Korpi. GABA turnover in the brain of rat lines developed for differential ethanol-induced motor impairment. Pharmacol. Biochem. Behav. 34: 905-909, 1989.

27. McClearn, G. E. and R. Kakihana. "Selective breeding for ethanol sensitivity: Short-sleep and long-sleep mice." Development of animal models as pharmacologic tools. McCleran, Deitrich and Erwin ed. 1981 Government Printing Office. Rockville.

28. French, T. A., J. M. Masserano and N. Weiner. Ethanol-induced changes in tyrosine hydroxylase activity in adrenal glands of mice selectively bred for

differences in sensitivity to ethanol. J. Pharmacol. Exp. Ther. 232: 315-321, 1985.

29.   Baizer, L., J. M. Masserano and N. Weiner. Ethanol-induced changes in tyrosine hydroxylase activity in brains of mice selectively bred for differences in sensitivity to ethanol. Pharmacol. Biochem. Behav. 15: 945-949, 1981.

30.   Imperato, A. and G. Di Chiara. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J. Pharmacol. Exp. Ther. 239: 219-239, 1986.

31.   Bacopoulus, N., R. Bhatnagar and C. VanOrden. The effects of subhypnotic doses of ethanol on regional catecholamine turnover. J. Pharm. Exp. Ther. 204: 1-10, 1978.

32.   Hunt, W. A. and E. Majchrowicz. Alterations in the turnover of brain norepinephrine and dopamine in alcohol-dependent rats. J. Neurochem. 23: 549-552, 1974.

33.   Griffiths, P. J., J. M. Littleton and A. Ortiz. Changes in monoamine concentrations in mouse brain associated with ethanol dependence and withdrawal. Br. J. Pharmac. 50: 489-498, 1974.

34.   Ahtee, L. and M. Svartström-Fraser. Effect of ethanol dependence and withdrawal on the catecholamines in rat brain and heart. Acta pharmacol. et toxicol. 36: 289-298, 1975.

35.   Pohorecky, L. A. Effects of ethanol on central and peripheral noradrenergic neurons. J. Pharmacol. Exp. Ther. 189: 380-391, 1974.

36.   Carlsson, A., T. Magnusson, T. Svennson and B. Waldeck. Effects of ethanol on the metabolism of brain catecholamines. Psychopharm. 30: 27-36, 1973.

37.	Levitt, M., S. Spector, A. Sjoerdsma and S. Undenfriend. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. J. Pharmacol. Exp. Ther. 148: 1-8, 1965.

38.	Brenneman, A. R. and S. Kaufman. The role of tetrahydropteridines in the enzymatic conversion of tyrosine to 3,4-dihydroxyphenylalanine. Biochem. Biophys. Res. Comm. 17(2): 177-183, 1964.

39.	Fuxe, K., T. Hokfelt, L. F. Agnati, D. Johannson, M. Goldstein, M. Perez de la Mora, R. Tapia, L. Teran and R. Palacios. "Mapping out central catecholamine neurons immumohistochemical studies on catecholamine-synthesizing enzymes." Psychopharmacology: A generation of progress. Lipton, DeMascio and Killman ed. 1978 Raven Press. New York.

40.	Yamanaka, Y. and T. Egashira. Effects of ethanol on catecholamine levels and related enzyme activities in different brain regions of rats. Japan. J. Pharmacol. 32: 599-606, 1982.

41.	Masserano, J. M. and N. Weiner. The rapid activation of adrenal tyrosine hydroxylase by decapitation and its relationship to a cyclic AMP-dependent phosphorylating mechanism. Mol. Pharmacol. 16: 513-528, 1979.

42.	Cohen, L. B., E. M. Sellers, E. A. Sellers and K. V. Flattery. Ethanol and sympathetic denervation effects on rat adrenal catechlamine turnover. J. Pharmacol. Exp. Ther. 212(2): 425-430, 1980.

43.	Masserano, J. M., G. S. Takimoto and N. Weiner. Tyrosine hydroxylase activity in the brain and adrenal gland of rats following chronic administration of ethanol. Alcohol. Clin. Exp. Res. 7(3): 294-298, 1983.

44.	Fluharty, S. J., G. L. Snyder, E. M. Stricker and M. J. Zigmond. Short- and long-term changes in adrenal tyrosine hydroxylase activity during insulin-induced hypoglycemia and cold stress. Brain Res. 267: 384-387, 1983.
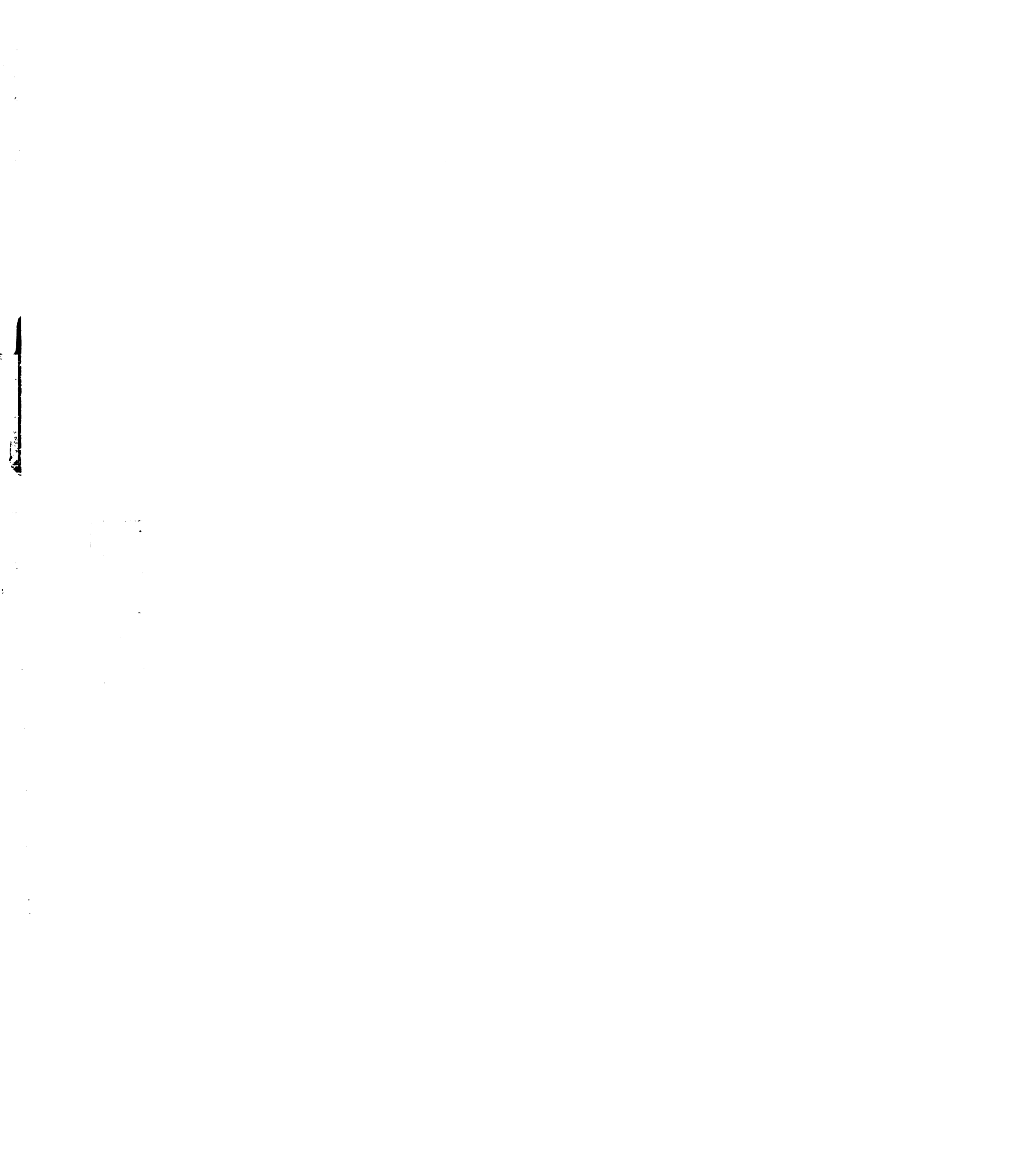
45.	Masserano, J. M., G. S. Takimoto and N. Weiner. Electoconvulsive shock increases tyrosine hydroxylase activity in the brain and adrenal gland of the rat. Science. 214: 662-665, 1981.

46.	Kvetnansky, R., V. K. Weise, G. P. Gewirtz and I. J. Kopin. Synthesis of adrenal catecholamines in rats during and after immobilization stress. Endocrinology. 89: 46-49, 1971.

47.	Zivkovic, B., A. Guidotti and E. Costa. Effects of neuroleptics on striatal tyrosine hydroxylase: changes in affinity for the pteridine cofactor. Mol. Pharmacol. 10: 727-735, 1974.

48.	McTigue, M., J. Cremins and S. Halegoua. Nerve growth factor and other agents mediate phosphorylation and activation of tyrosine hydroxylase. A convergence of multiple kinase activities. J. Biol. Chem. : 9047-9056, 1985.

49.	Nose, P. S., L. C. Griffith and H. Schulman. Ca^{2+} -dependent phoshporylation of tyrosine hydroxylase in PC12 cells. J. Cell Biol. 101: 1182-1190, 1985.

50.	Roskoski, R., L. White, R. Knowlton and L. M. Roskoski. Regulation of tyrosine hydroxylase activity in rat PC12 cells by neuropeptides of the secretin family. Molec. Pharmacol. 36: 925-931, 1989.

51.	Cahill, A. L. and R. L. Perlman. Phosphorylation of tyrosine hydroxylase in the superior cervical ganglion. Biophys. Acta. 805: 217-226, 1984.

52.	Roskoski, R. and L. M. Roskoski. Adenosine receptor activation and the regulation of tyrosine hydroxylase activity in PC12 and PC18 cells. J. Neurochem. 53: 1934-1940, 1989.

53.	Bucciarelli de Simone, A. and M. C. Rubio. Differential effects of prostaglandins E_1 and E_2 on tyrosine hydroxylase activity of the superior cervical ganglion of the cat. Gen. Pharmacol. 11: 539-541, 1980.
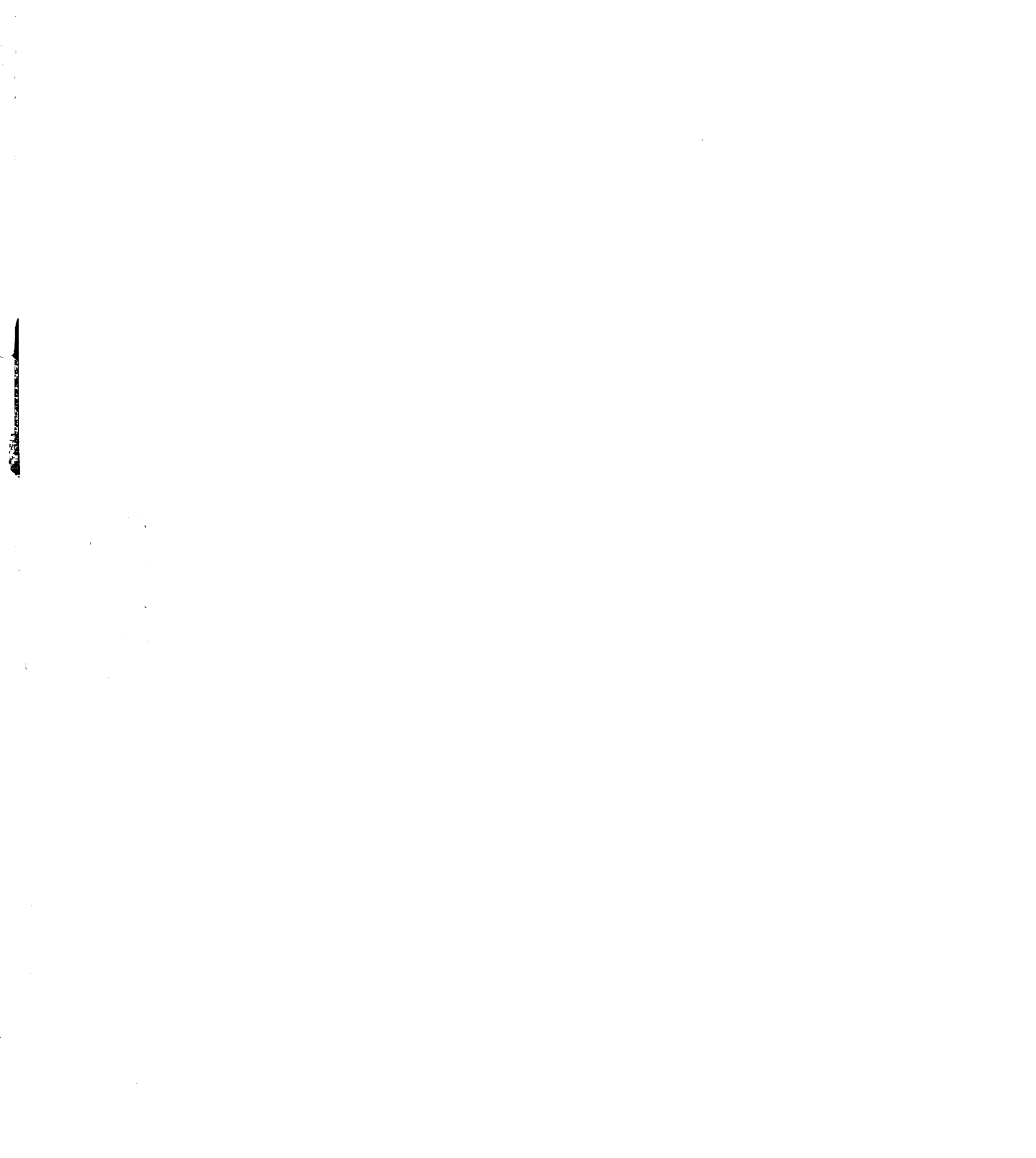
54.   Tank, A. W., P. Curella and L. Ham. Induction of mRNA for tyrosine hydroxylase by cyclic AMP and glucocorticoids in a rat pheochromocytoma cell line: evidence for the regulation of tyrosine hydroxylase synthesis by multiple mechanisms in cells exposed to elevated levels of both inducing agents. Mol. Pharmacol. 30: 497-503, 1986.

55.   Tank, A. W., E. J. Lewis, D. M. Chikaraishi and N. Weiner. Elevation of RNA coding for tyrosine hydroxylase in rat adrenal gland by reserpine treatment and exposure to cold. J. Neurochem. 45: 1030-11033, 1985.

56.   Kurosawa, A., A. Guidotti and E. Costa. Induction of tyrosine 3-monooxygenase in adrenal medulla: role of protein kinase activation and translocation. Science. 193: 691-693, 1976.

57.   Black, I. B., M. Chikaraishi and E. J. Lewis. Trans-synaptic increase in RNA coding for tyrosine hydroxylase in a rat sympathetic ganglion. Brain Res. 339: 151-153, 1985.

58.   Leonard, D. G. B., E. B. Ziff and L. A. Greene. Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. Mol. Cell. Biol. 7: 3156-3167, 1987.

59.   Lewis, E. J. and D. M. Chikaraishi. Regulated expression of the tyrosine hydroxylase gene by epidermal growth factor. Mol. Cell. Biol. 7(9): 3332-3336, 1987.

60.   Wessels-Reiker, M., J. W. Haycock, A. C. Howlett and R. Strong. Vasoactive intestinal polypeptide induces tyrosine hydroxylase in PC12 cells. J. Biol. Chem. 266(15): 9347-9350, 1991.

61.   Tachikawa, E., A. W. Tank, D. H. Weiner, W. F. Mosimann, N. Yanagihara and N. Weiner. Tyrosine hydroxylase is activated and phosphorylated on different sites in rat pheochromocytoma PC12 cells treated with phorbol ester and forskolin. J. Neurochem. 46: 1366-1376, 1987.

62.   Tachikawa, E., A. W. Tank, N. Yanagihara, W. Mosimann and N. Weiner. Phosphorylation of tyrosine hydroxylase on at least three sites in rat pheochromocytoma PC12 cells treated with 56 mM K$^+$: Determination of the sites on tyrosine hydroxylase phosphorylated by Cyclic AMP-dependent and calcium/calmodulin-dependent kinases. Mol. Pharmacol. 30: 476-485, 1986.

63.   Roskoski, R. and L. M. Roskoski. Activation of tyrosine hydroxylase in PC12 cells by the Cyclic GMP and Cyclic AMP second messenger systems. J. Neurochem. 48: 236-242, 1987.

64.   Waymire, J. C., J. P. Johnston, K. Hummer-Lickteig, A. Lloyd, A. Vigny and G. L. Craviso. Phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase. J. Biol. Chem. 263(25): 12439-12447, 1988.

65.   Campbell, D. G., D. G. Hardie and P. R. Vulliet. Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. J. Biol, Chem. 261(23): 10489-10492, 1986.

66.   Roskoski, R., R. P. Vulliet and D. B. Glass. Phosphorylation of tyrosine hydroxylase by Cyclic GMP-dependent kinase. J. Neurochem. 48: 840-845, 1987.

67.   Yamauchi, T., H. Nakata and H. Fujisawa. A new activator protein that activates tryptophan %-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca^{2+}-calmodulin-dependent protein kinase. Purification and Charaterization. J. Biol. Chem. 256: 5404-5409, 1991.

68.   Lewis, E. J., C. A. Harrington and D. M. Chikaraishi. Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. Proc. Natl. Acad. Sci. USA. 84: 3550-3554, 1987.

69.   Vyas, S., N. F. Biguet and J. Mallet. Transcriptional and post-transcriptional regulation of tyrosine hydroxylase gene by protein kinase C. EMBO J. 9(11): 3707-3712, 1990.
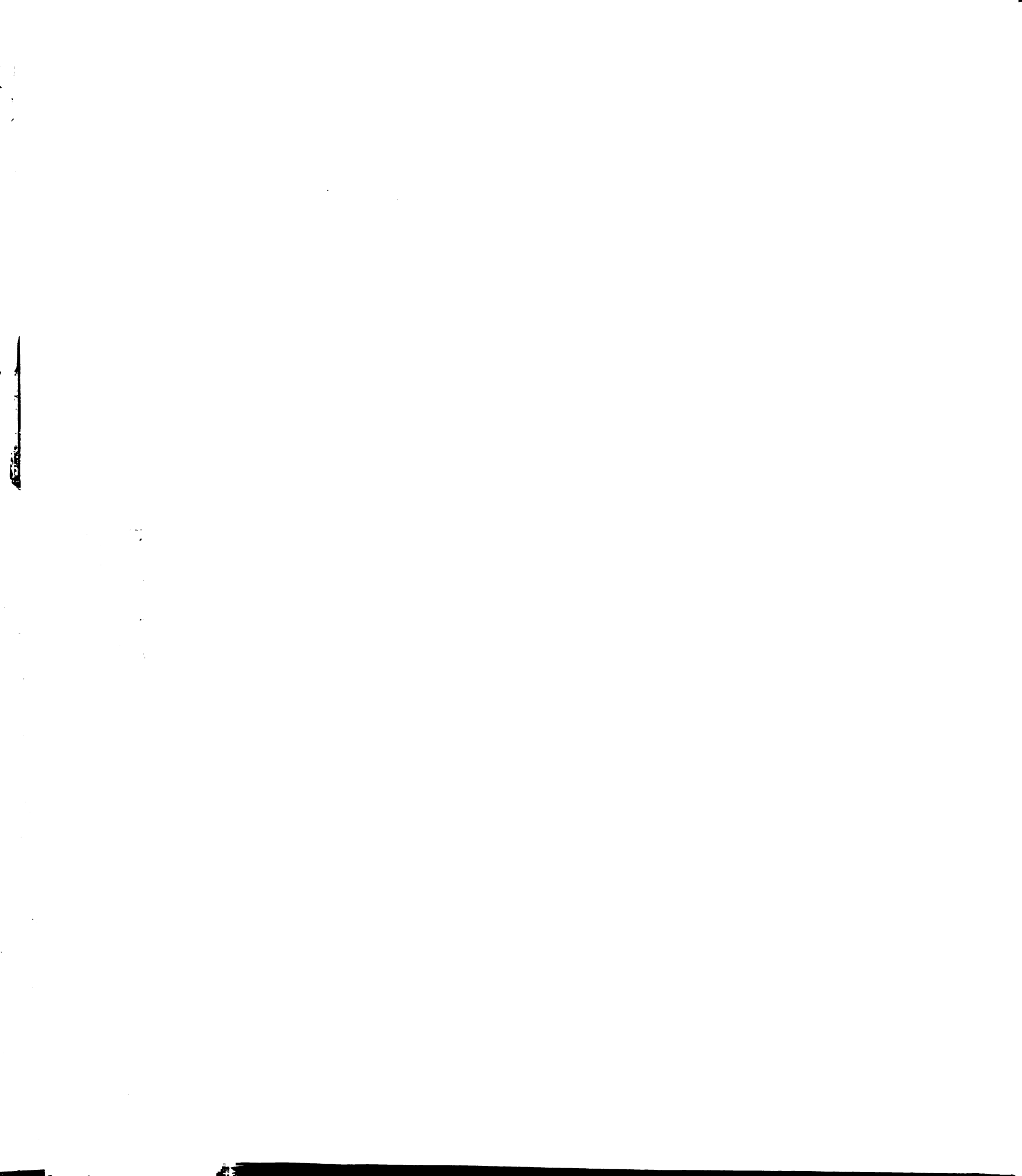
70.   Stachowiak, M. K., H. K. Jiang, A. M. Poisner, R. K. Tuominen and J. Hong. Short and long term regulation of catecholamine biosynthetic enzymes by angiotensin in cultured adrenal medullary cells. J. Biol. Chem. 265(8): 4694-4702, 1990.

71.   Acheson, A., D. Edgar, R. Timpl and H. Thoenen. Laminin increases both levels and activity of tyrosine hydroxylase in calf adrenal chromaffin cells. J. Cell Biol. 102: 151-159, 1986.

72.   Summerhill, E. M., K. Wood and M. C. Fishman. Regulation of tyrosine hydroxylase gene expression during differentiation of neuroblastoma cells. Mol. Brain. Res. 2: 99-103, 1987.

73.   Gizang-Ginsberg, E. and E. B. Ziff. Nerve growth factor regulates tyrosine hydroxylase gene transcription though a nucleoprotein complex that contains c-Fos. Genes & Development. 4: 477-491, 1990.

74.   Huidobro-Toro, J. P., V. Blec, A. M. Allan and R. A. Harris. Neurochemical actions of anesthetic drugs on the gamma-aminobutyric acid receptor-chloride channel complex. J. Pharmacol. Exp. Ther. 242: 963-969, 1987.

75.   Lyon, R. C., J. A. McComb, J. Schreurs and D. B. Goldstein. A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. J. Pharmacol. Exp. Ther. 218: 669-675, 1981.

76.   Rabin, R. A. and P. B. Molinoff. Activation of adenylate cyclase by ethanol in mouse striatal tissue. J. Pharmacol. Exp. Ther. 216: 129-134, 1981.

77.   Stenstrom, S. and E. Richelson. Acute effects of ethanol prostaglandin E-mediated cyclic AMP formation by a murine neuroblastoma clone. J. Pharmacol. Exp. Ther. 221: 334-341, 1982.

78.   Diamond, I., B. Wrubel, W. Estrin and A. Gordon. Basal and adenosine receptor-stimulated levels of cAMP are reduced in lymphocytes from alcoholic patients. Proc. Natl. Acad. Sci. USA. 84: 1413-1416, 1987.

79.   Saito, T., J. M. Lee and B. Tabakoff. Effects of chronic ethanol treatment on the β-adrenergic receptor-coupled adenylate cyclase system of mouse cerebral cortex. J. Neurochem. 48(6): 1817-1822, 1987.

80.   Rabin, R. A. and P. B. Molinoff. Multiple sites of action of ethanol on adenylate cyclase. J. Pharmacol. Exp. Ther. 227: 551-556, 1983.

81.   Luthin, G. R. and B. Tabakoff. Activation of adenyate cyclase by alcohols requires the nucleotide-binding protein. J. Pharmacol. Exp. Ther. 228: 579-587, 1984.

82.   Gonzales, R. A., C. Theiss and F. T. Crews. Effects of ethanol on stimulated inositol phospholipid hydrolysis in rat brain. J. Pharmacol. Exp. Ther. 237(1): 92-98, 1986.

83.   Hoek, J. B., A. P. Thomas, R. Rubin and E. Rubin. Ethanol-induced mobilization of calcium by activation of phosphoinositide-specific phospholipase C in intact hepatocytes. J. Biol. Chem. 262: 682-691, 1987.

84.   Simonsson, P., E. Hansson and A. C. Ethanol potentiates seotonin stimulated inositol lipid metabolism in primary astroglial cell cultures. Biochem. Pharmacol. 38(17): 2801-2805, 1989.

85.   Nishizuka, Y. Turnover of inositol phospholipids and signal transduction. Science. 225: 1365-1370, 1984.

86.   Rabe, S. C. and F. W. Weight. Effects of ethanol on neurotransmitter release and intracellular free calcium in PC12 cells. J. Pharmacol. Exp. Ther. 244(2): 417-422, 1988.
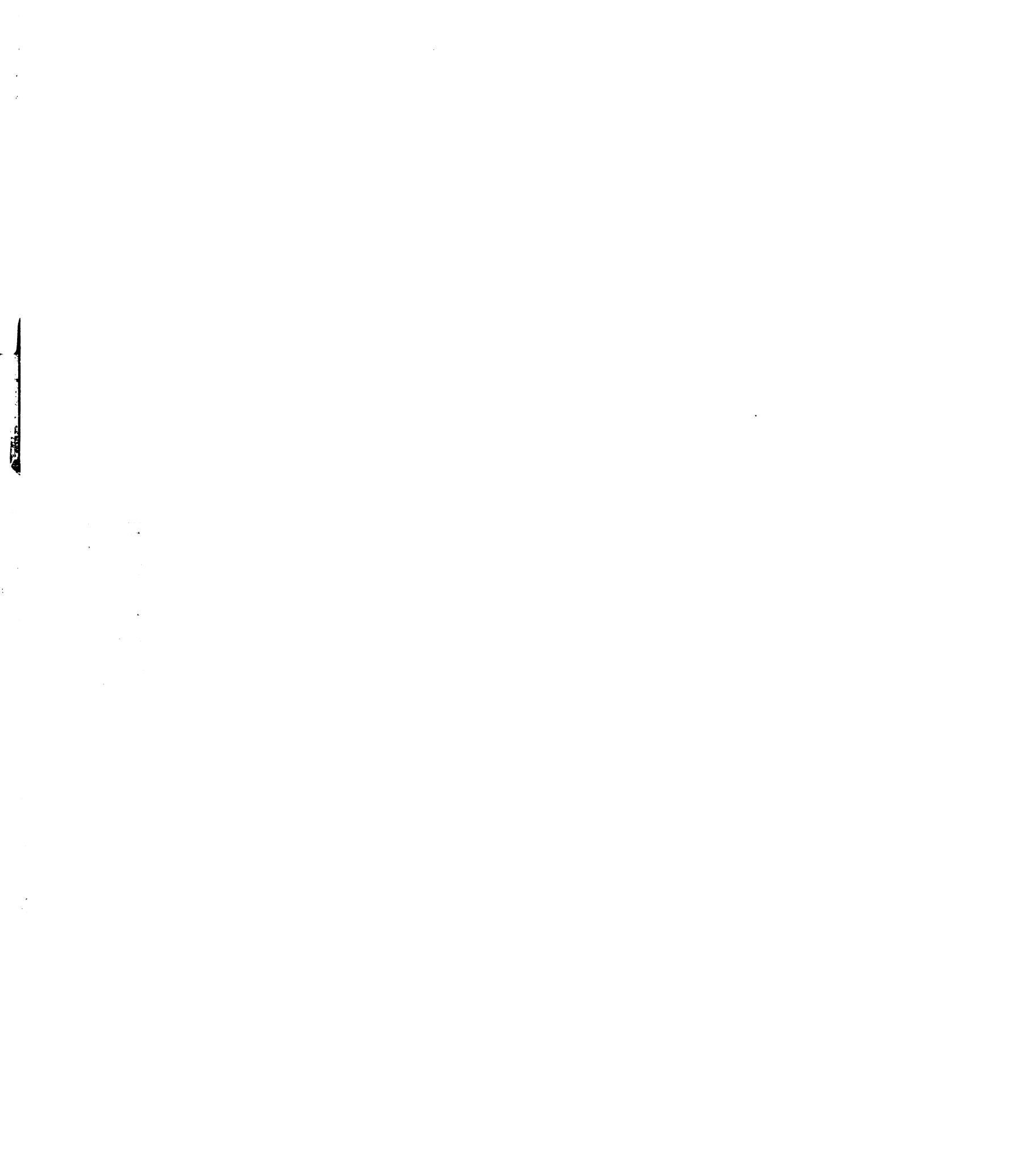
87.   Daniell, L. C., E. P. Brass and R. A. Harris. Effect of ethanol on intracellular ionized calcium concentrations in synaptosomes and hepatocytes. Mol. Pharmacol. 32: 831-837, 1987.

88.   Shah, J. and H. C. Pant. spontaneous calcium release induced by ethanol in the isolated rat brain microsomes. Brain. Res. 474: 94-99, 1988.

89.   Michaelis, M. L. and E. K. Michaelis. Alcohol and local anesthetic effects on Na+- dependent Ca2+ fluxes in brain synaptic membrane vesicles. Biochem. Pharm. 32(6): 963-969, 1983.

90.   Yamamoto, H. and R. A. Harris. Effects of ethanol and barbiturates on Ca^{2+}-ATPase activity of erythrocyte and brain membranes. Biochem. Pharmacol. 32(18): 2787-2791, 1983.

91.   Michaelis, M. L., E. K. Michaelis, E. W. Nunley and N. Galton. Effects of chronic alcohol administration on synaptic membrane Na^+-Ca^+ exchange. Brain Research. 414: 239-244, 1987.

92.   Leslie, S., E. Barr, J. Chandler and R. P. Farrar. Inhibition of fast- and slow-phase depolarization-dependent synaptosomal colcium uptake by ethanol. J. Pharmacol. Exp. Ther. 225(3): 571-575, 1983.

93.   Messing, R. O., A. B. Sneade and B. Savidge. Protein kinase C participates in up-regulation of dihydropyridine-sensitive calcium channels by ethanol. J. Neurochem. 55: 1383-1389, 1990.

94.   Hoffman, P. L., C. S. Rabe, F. Moses and B. Tabakoff. N-Methyl-D-apartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. J. Neurochem. 52: 1937-1940, 1989.

95.   Grant, K. A., P. Valverius, M. Hudspith and B. Tabakoff. Ethanol withdrawal seizures and the NMDA receptor complex. Eur. J. Pharmac. 176: 289-296, 1990.
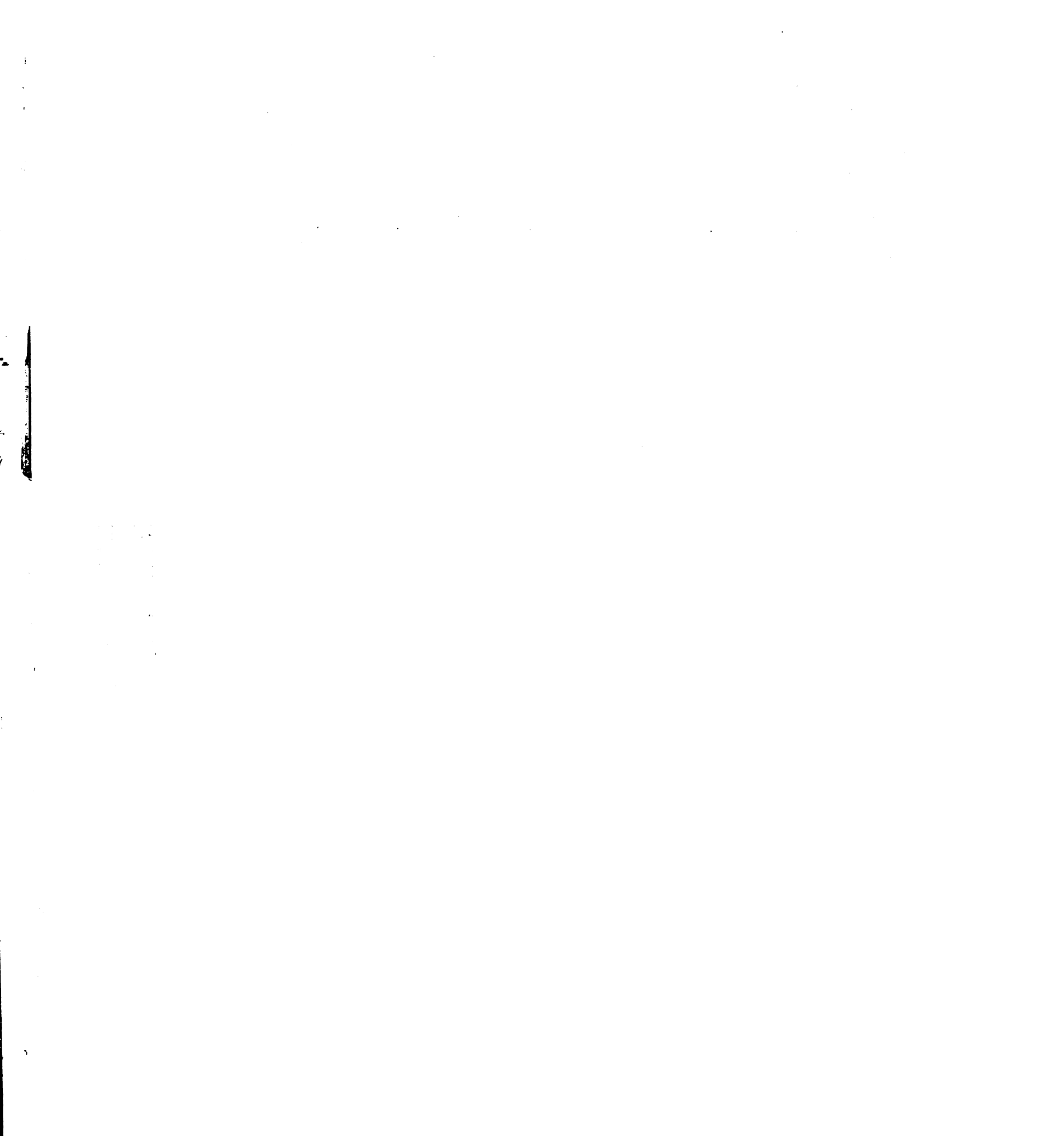
96.   Freed, W. J. and E. K. Michaelis. Glutamic acid and ethanol dependence. Pharmac. Biochem. Behav. 8: 509-514, 1978.

97.   Roesler, W. J., G. R. Vandenbark and R. W. Hanson. Cyclic AMP and the induction of eukaryotic gene transcription. J. Bio. Chem. 263(19): 9063-9066, 1988.

98.   Montminy, M. R., G. A. Gonzalez and K. K. Yamamoto. Regulation of cAMP-inducible genes by CREB. TINS. 13(5): 184-188, 1990.

99.   Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel and R. H. Goodman. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA. 83: 6682-6686, 1986.

100.   Yamamoto, K. K., G. A. Gonzalez, B. W.H. and M. R. Montminy. Phosphorylation-induced binding and trancriptional efficacy of nuclear factor CREB. Nature. 334: 494-498, 1988.

101.   Gonzalez, G. A. and M. R. Montminy. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell. 59: 675-680, 1989.

102.   Sheng, M., G. McFadden and M. E. Greenberg. Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. Neuron. 4: 571-582, 1990.

103.   Sheng, M., M. A. Thompson and M. E. Greenberg. CREB: A Ca2+-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science. 252: 1427-1430, 1991.

104.   Mitchell, P. J. and R. Tjian. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science. 245: 371-378, 1989.

105.   Hoeffler, J. P., T. E. Meyer, G. Waeber and J. F. Habener. Multiple adenosine 3',5'-monophosphate response element DNA-binding proteins

generated by gene diversification and alternative exon splicing. Molecular Endocrinology. 9: 920-930, 1990.

106. Kara, C. J., H. Liou, L. B. Ivashkiv and L. H. Glimcher. A cDNA for a human cyclic AMP response element-binding protein which is distinct from CREB and expressed preferentially in brain. Mole. Cell. Biol. 10(4): 1347-1357, 1990.

107. Hai, T., L. Fang, W. J. Coukos and M. R. Green. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. 3: 2083-2090, 1989.

108. Rehfuss, R. P., K. M. Walton, M. M. Loriaux and R. H. Goodman. The cAMP-regulated enhancer-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. J. Biol. Chem. 266(28): 18431-18434, 1991.

109. Foulkes, N. S., E. Borelli and P. Sassone-Corsi. CREM gene: Use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. Cell. 64: 739-749, 1991.

110. Greenberg, M. E., E. B. Ziff and L. A. Greene. Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science. 234: 80-84, 1986.

111. Bravo, R., M. Neuberg, J. Burckhardt, J. Almendral, R. Wallich and R. Muller. Involvement of common and cell type-specific pathways in c-fos gene control: stable induction by cAMP in macrophages. Cell. 48: 251-260, 1987.

112. Ran, W., M. Dean, R. A. Levine, C. Henkle and J. Campisi. Induction of c-fos and c-myc mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. Proc. Natl. Acad. Sci. USA. 83: 8216-8220, 1986.

113. Greenberg, M. E. and E. B. Ziff. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature. 311: 433-438, 1984.

114. Krijer, W., D. Schubert and I. M. Verma. Induction of the proto-oncogene fos by nerve growth factor. Proc. Natl. Acad. Sci. USA. 82: 7330-7334, 1985.

115. Cochran, B. H., J. Zullo, I. M. Verma and C. D. Stiles. Expression of the c-fos gene and an fos-related gene is stimulated by platelet-derived growth factor. Science. 226: 1080-1082, 1984.

116. Stumpo, D. J. and P. J. Blackshear. Insulin and growth factor effects on c-fos expression in normal and protein kinase C-deficient 3T3-L1 fibroblast and adipocytes. Proc. Natl. Acad. Sci. USA. 83: 9453-9457, 1986.

117. Ransome, L. J. and I. M. Verma. Nuclear proto-oncogenes fos and jun. Annu. Rev. Cell. Biol. 6: 539-557, 1990.

118. Auwerx, J. and P. Sassone-Corsi. IP-1: A dominant inhibitor of fos/jun whose activity is modulated by phosphorylation. Cell. 64: 983-993, 1991.

119. Hai, T. and T. Curran. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding spedificity. Proc. Natl. Acad. Sci. USA. 88: 3720-3724, 1991.

120. Sassone-Corsi, P., L. J. Ransone and I. M. Verma. Cross-talk in signal transduction: TPA-inducible factor jun/AP-1 activates cAMP-responsive enhancer elements. Oncogene. 5: 427-431, 1990.

121. Imagawa, M., R. Chiu and M. Karin. Transcription factor AP-2 mediates induction by two different signal-tranduction pathways: protein kinase C and cAMP. Cell. 51: 251-260, 1987.

122. Jackson, S. P., J. J. MacDonald, S. Lees-Miller and R. Tjian. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell. 63: 155-165, 1990.

123. Tanaka, M. and W. Herr. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell. 60: 375-386, 1990.

124. Sorger, P. K. and H. R. B. Pelham. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell. 54: 855-864, 1988.

125. Miles, M. F., J. E. Diaz and V. DeGuzman. Ethanol-responsive gene expression in neural cell cultures. Biochem. Biophys. ACTA. in press: 1992.

126. Schlesinger, M. J. Heat shock proteins. J. Biol. Chem. 265(21): 12111-12114, 1990.

127. Miles, M. F., J. E. Diaz and V. S. DeGuzman. Mechanisms of neuronal adaptation to ethanol. Ethanol induces Hsc 70 gene transcription in NG108-15 neuroblastoma x glioma cells. J. Biol. Chem. 266(4): 2409-2414, 1991.

128. Parent, L. J., R. Ehrlich, L. Matis and D. S. Singer. Ethanol: an enhancer of major histocompatibility complex antigen expression. FASEB J. 1: 469-473, 1987.

129. Kolber, M. A., R. M. Walls, M. L. Hinners and D. S. Singer. Evidence of increased class I MHC expression on human peripheral blood lymphocytes durein acute ethanol intoxication. Alcohol. Clin. Exp. Res. 12(6): 820-823, 1988.

130. Bjorkman, P., M. Saper, B. Samraoui, W. Bennet, J. Strominger and D. Wiley. Structure of the human class I histocompatibility antigen, HLA-A2. Nature. 329: 506-518, 1987.

131. Kapoun, A. M., B. W. Geer, P. W. Heinstra H., V. Corbin and S. W. McKechnie. Molecular control of the induction of alcohol dehydrogenase by ethanol in Drosophila melanogaster larvae. Genetics. 124: 881-888, 1990.

132. Song, B., H. V. Gelboin, S. Park, C. S. Yang and F. J. Gonzalez. Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P-450's. Transcriptional and post-transcriptional regulation of the rat enzyme. J. Biol. Chem. 261(35): 16689-16697, 1986.

133. Dave, J. R., L. E. Eiden, J. W. Karaman and R. L. Eskay. Ethanol exposure decreases pituitary corticotropin-releasing factor binding, adenylate cyclase activity, proopiomelanocortin biosynthesis and plasma β-endorphin levels in the rat. Endocrinology. 118: 280-286, 1986.

134. Morrow, L. A., P. Montpied, A. Lingford-Hughes and S. M. Paul. Chronic ethanol and pentobarbital administration in the rat: effects on GABAA receptor function and expression in brain. Alcohol. 7: 237-244, 1990.

135. Held, I. R., S. T. Sayers and J. A. McLane. Acetylcholine receptor gene expression in skeletal muscle of chronic ethanol-fed rats. Alcohol. 8(3): 173-177, 1991.

136. Hunt, W. A. and E. Majchrowicz. "Alterations in neurotransmitter function after acute and chronic treatment with ethanol." Biochemistry And Pharmacology Of Ethanol. Majchrowicz and Noble ed. 1979 Plenum Press. New york.

137. Richelson, E., S. Stenstrom, C. Forray, L. Enloe and M. Pfenning. Effects of chronic exposure to ethanol on the prostaglandin E_1 receptor-mediated response and binding in a murine neuroblastoma clone (N1E-115). J. Pharmacol. Exp. Ther. 239: 687-692, 1986.

138. Nagy, L. E., I. Diamond, D. J. Casso, C. Franklin and A. S. Gordon. Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. J. Biol. Chem. 265(4): 1946-1951, 1990.

139. Amano, T., E. Richelson and M. Nirenberg. Neurotransmitter synthesis by neuroblastoma clones. Proc. Nat. Acad. Sci. USA. 69(1): 258-263, 1972.

140. Lloyd, T. and F. H. Schneider. Stimulation of tyrosine hydroxylase activity in cultured mouse neuroblastoma cells by monocarboxylic acids. J. Neurochem. 30: 1641-1643, 1978.

141.   Wastek, G. J., J. R. Lopez and E. Richelson. Demonstration of a muscarinic receptor-mediated cyclic GMP-dependent hyperpolarization of the membrane potential of mouse neuroblastoma cells using [^{3}H] tetraphenylphosphonium. Molec. Pharmacol. 19: 15-20, 1981.

142.   Richelson, E. Histamine H1 receptor-mediated guanosine 3',5'-monophosphate formation by cultured mouse neuroblastoma cells. Science. 201: 69-71, 1978.

143.   Amar, S., J. Mazella, F. Checler, P. Kitabgi and J. Vincent. Regulation of cylcic GMP levels by neurotensin in neuroblastoma clone N1E115. Biochem. Biophysic. Res. Comm. 129(1): 117-125, 1985.

144.   Greene, L. A. and A. S. Tischler. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA. 73(7): 2424-2428, 1976.

145.   Rabe, C. S., P. R. Giri, P. L. Hoffman and B. Tabakoff. Effect of ethanol on cyclic AMP levels in intact PC12 cells. Biochem. Pharmacol. 40(3): 565-567, 1990.

146.   Van Buskirk, R., T. Corcoran and J. A. Wagner. Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ornithine decarboxylase in response to nerve growth factor but not to adenosine agonists. Molec. Cell. Biol. 5(8): 1984-1992, 1985.

147.   Wolfe, R. A., G. H. Sato and D. B. McClure. Continous culture of rat C6 glioma in serum-free media. J. Cell. Biol. 87: 434-441, 1980.

148.   Bottenstein, J. E. "Growth and differentiation of neural cells in defined media." Cell culture in the neurosience. Bottenstein and Sato ed. 1985 Plenum Press. New York.

149. Ham, R. G. and W. L. McKeehan. "Media and growth requirements." Methods in enzymology. Jakoby and Pastan ed. 1979 Academic Press. New York.

150. Patterson, M. K. "Measurement of growth and viability of cells in culture." Methods in enzymology. Jakoby and Pastan ed. 1979 Academic Press. New York.

151. Cathala, G., J.-F. Savouret, B. Mendez, B. L. West, M. Karin, J. A. Martial and J. D. Baxter. A method for isolation of intact, translationally active ribonucleic acid. DNA. 2: 329-335, 1983.

152. Sambrook, J., E. F. Frisch and T. Maniatis. Molecular cloning. A loboratory manual. Ford, Nolan and Ferguson ed. 1989 Cold Spring Harbor Laboratory Press.

153. Feinberg, A. P. and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-13, 1983.

154. Tank, A. W., L. Ham and P. Curella. Induction of tyrosine hydroxylase by cyclic AMP and glucocorticoids in a rat pheochromocytoma cell line: effect of the inducing agents alone or in combination on the enzyme levels and rate of synthesis of tyrosine hydroxylase. Mol. Pharmacol. 30: 486-496, 1986.

155. Cooper, D. M. F. and C. londos. "Adenosine receptors." Receptor biochemistry and methodology. Venter and Harrison ed. 1988 Alan R. Liss, Inc. New York.

156. Lewis, E. J., A. W. Tank, N. Weiner and D. M. Chikaraishi. Regulation of tyrosine hydroxylase mRNA by glucocorticoid and cyclic AMP in a rat pheochromocytoma cell line. J. Biol. Chem. 258: 14632-14637, 1983.

157. Coker, G. T., L. Vinnedge and K. L. O'Malley. Characterization of rat and human tyrosine hydroxylase genes: functional expression of both promoters in
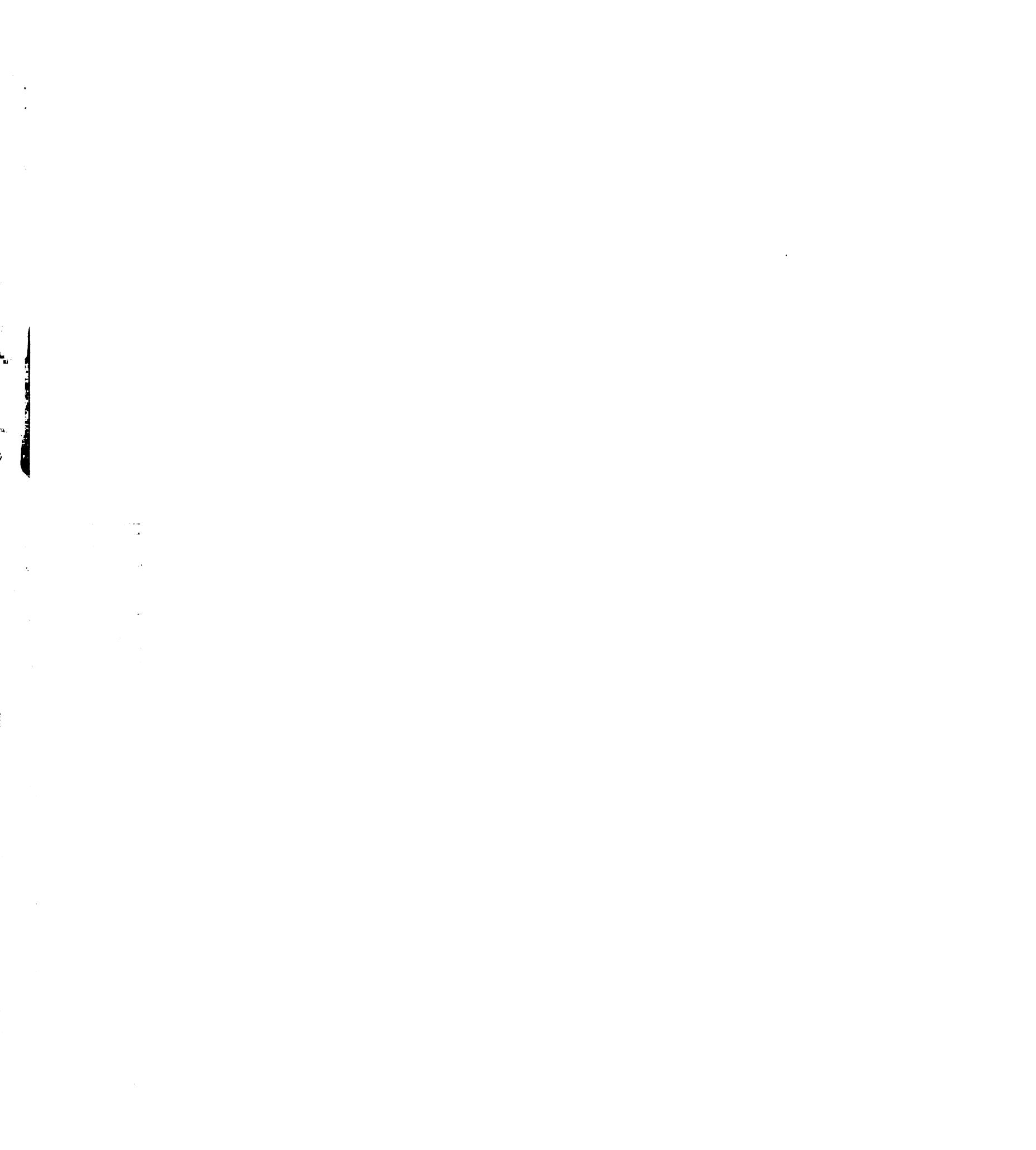
neuronal and non-neuronal cell types. Biochem. Biophysic. Res. Comm. 157(3): 1341-1347, 1988.

158. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

159. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(259): 680-685, 1970.

160. Towbin, H., T. Staehelin and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76(9): 4350-4354, 1979.

161. Hollander, M. C. and A. J. Fornace Jr. Estimation of relative mRNA content by filter hybridization to a polythymidylate probe. BioTechniques. 9: 174-179, 1990.

162. Southern, P. J. and P. Berg. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1: 327-341, 1982.

163. Laimins, L. A., G. Khoury, C. Gorman, B. Howard and P. Gruss. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA. 79: 6453-6457, 1982.

164. Seed, B. and J.-Y. Sheen. A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene. 67: 271-277, 1988.

165. Rohrer, H., A. L. Acheson, J. Thibault and H. Thoenen. Development potential of quail dorsal root ganglion cells analyzed in vitro and in vivo. J. Neurosci. 6(9): 2616-2624, 1986.
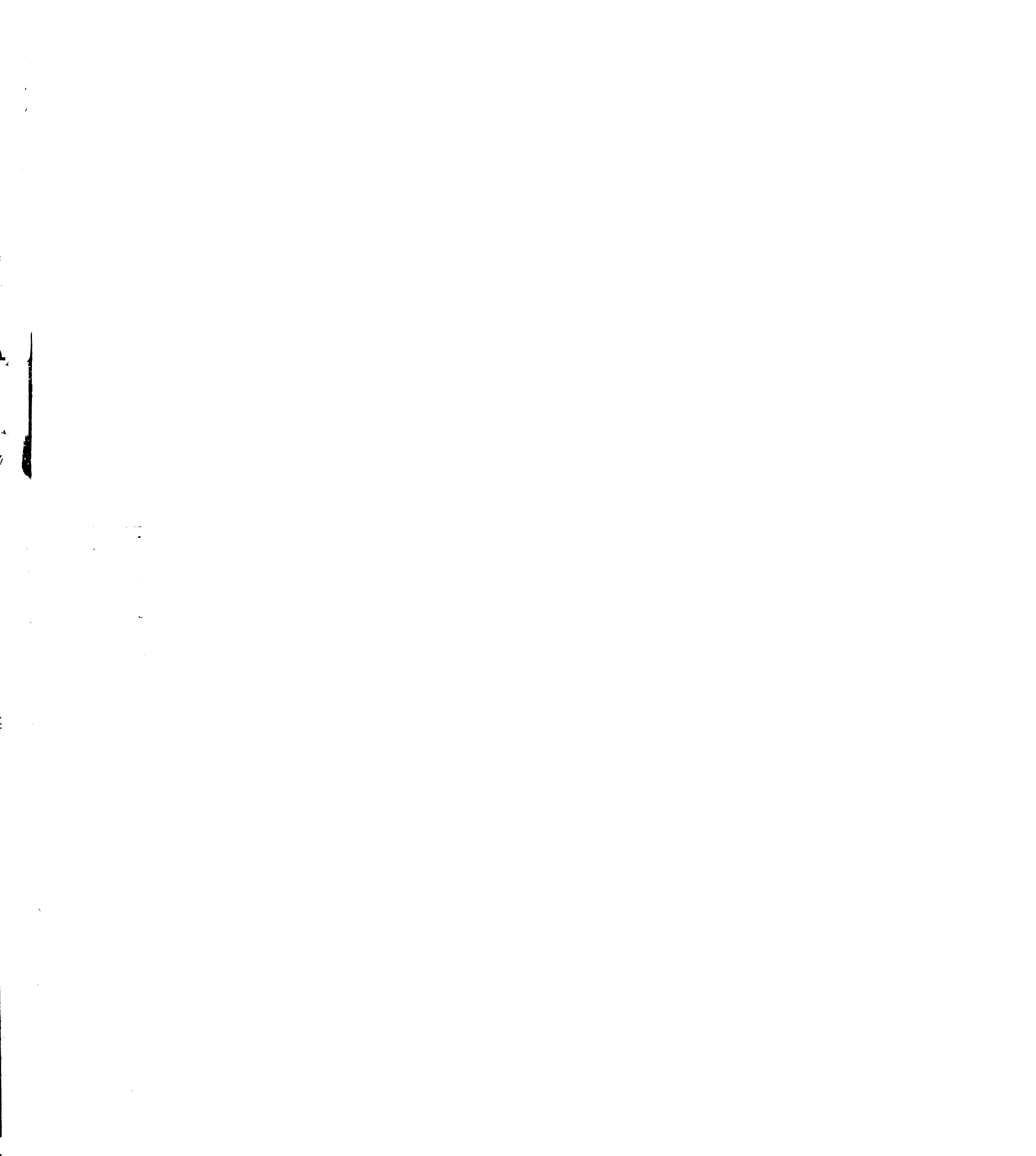
166. Hall, J. L., L. Dudley, P. R. Dobner, S. A. Lewis and N. J. Cowan. Identification of two human β-tubulin isotypes. Mol. Cell. Biol. 3: 854-862, 1983.

167. Cambi, F., B. Fung and D. Chikaraishi. 5' flanking DNA sequences direct cell-specific expression of rat tyrosine hydroxylase. J. Neurochem. 53: 1656-1659, 1989.

168. Goldstein, D. B. Alcohol withdrawal reactions in mice: effects of drugs that modify neurotransmission. J. Pharmacol. Exp. Ther. 186: 1-9, 1973.

169. Goldstein, D. B. and N. Pal. Alcohol dependence produced in mice by inhalation of ethanol: Grading the withdrawal reaction. Science. 172: 288-290, 1971.

170. Rubio, M. C. Effects of db cAMP on tyrosine hydroxylase activity of ganglia and nerve endings. Naunyn Schmiedeberg's Arch. Pharmacol. 299: 69-75, 1977.

171. Schwarzchild, M. A. and R. E. Zigmond. Secretin and vasoactive intestinal peptide activat tyrosine hydroxylase in sympathetic nerve endings. J. Neurosci. 9(1): 160-166, 1989.

172. Weiner, N., F. L. Dreyer and E. Barnes. The activation of tyrosine hydroxylase in noradrenergic neurons during acute nerve stimulation. Life Sci. 22: 1197-1215, 1978.

173. Chowdhury, M. and M. Fillenz. K+-dependent stimulation of dopamine synthesis in striatal synaptosomes is mediated by protein kinase C. J. Neurochem. 50: 624-629, 1988.

174. Wilson, S. P. Vasoactive intestinal peptide elevates cyclic AMP levels and potentiates secretion in bovine adrenal chromaffin cells. Neuropeptides. 11: 17-21, 1988.

175. Schubert, D., M. LaCorbiere, C. Whitlock and W. Stallcup. Alterations in the surface properties of cells responsive to nerve growth factor. nature. 273: 718-723, 1978.

176. Nikodijevic, B., O. Nikodijevic, M. Yu, H. Pollard and G. Guroff. The effect of nerve growth factor on cyclic AMP levels in superior cervical ganglia of the rat. Proc. Natl. Acad. Sci. USA. 72: 4769-4771, 1975.

177. Pocotte, S. L., R. W. Holz and T. Ueda. Cholinergic receptor-mediated phosphorylation and activation of tyrosine hydroxylase in cultured bovine adrenal chromaffin cells. J. Neurochem. 46: 610-622, 1986.

178. Tsukada, T., G. Fink, G. Mandel and R. H. Goodman. Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J. Biol. Chem. 262: 8743-8747, 1987.

179. Hyam, S. E., M. Comb, Y. Lin, J. Pearlberg, M. R. Green and H. M. Goodman. A common trans-acting factor is involved in transcriptional regulation of neurotransmitter genes by cyclic AMP. Molec. Cell. Biol. 8(10): 4225-4233, 1988.

180. Diamond, M. I., J. N. Miner, S. K. Yoshinaga and K. R. Yamamoto. Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. Science. 249: 1266-1271, 1990.

181. Yoon, S. O. and D. M. Chikaraishi. Tissue-specific transcription of the rat tyrosine hydroxylase gene requires a synergy between an E-box containing dyad and an overlapping AP1 motif which responds to elevated level of cAMP. Soc. Neurosci. Abstr. 17(2): 1514, 1991.

182. Karin, M. and R. I. Richards. Human metallothionein genes-primary structure of the metallothionein-II gene and a related processed gene. Nature. 299: 797-802, 1982.
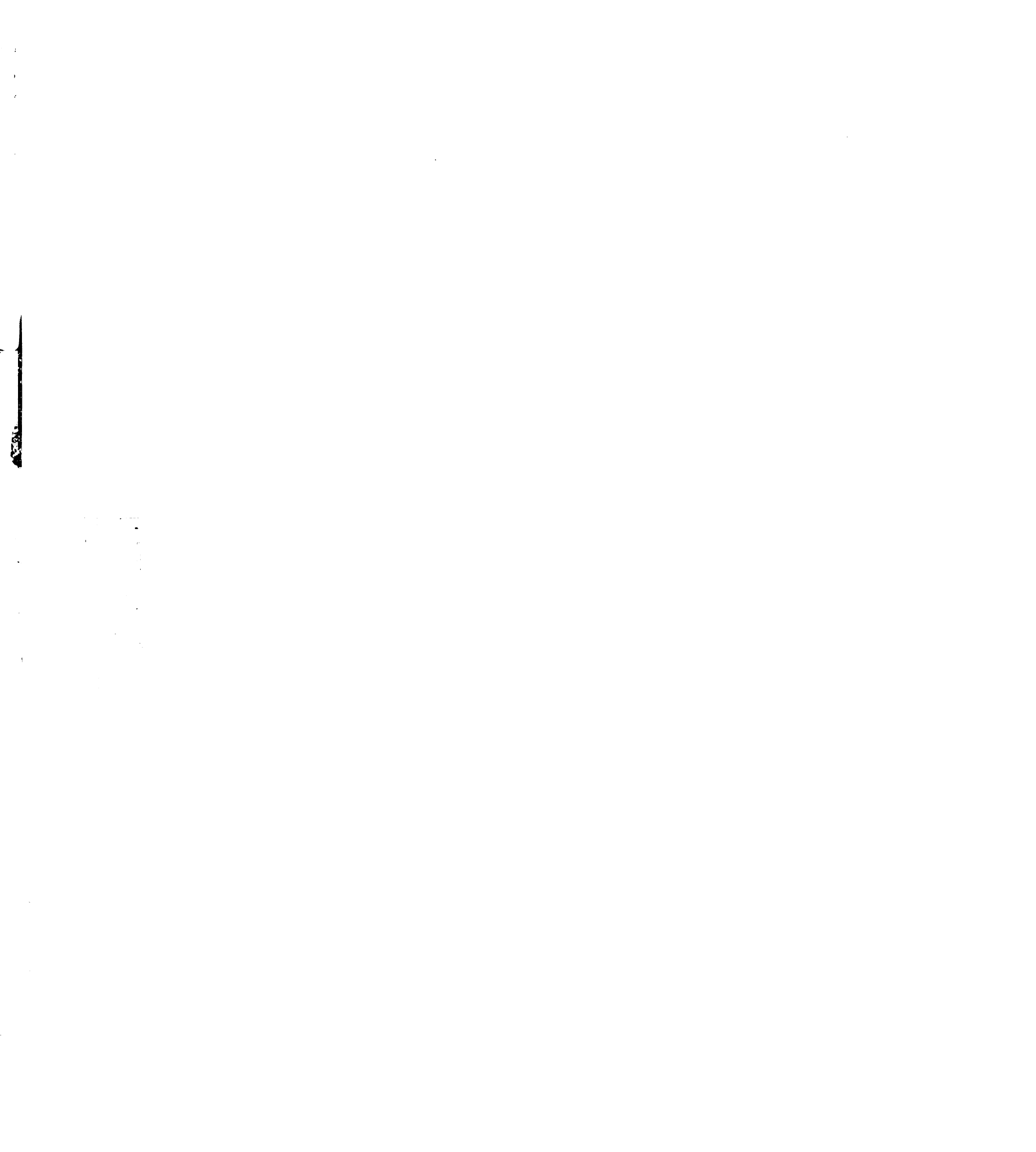
183. Sorger, P. K. Heat shock and the heat shock factor. Cell. 65: 363-368, 1991.

184. Dragunow, M., R. W. Currie, H. A. Robertson and R. Faull. Heat shock induces c-fos protein-like immunoreactivity in glial cells in adult rat brain. Exp. Neurol. 106: 105-109, 1989.

185. Miner, J. N. and K. R. Yamamoto. Regulatory crosstalk at composite response elements. Trends Biochem. Science. 16: 423-426, 1991.

186. Sorger, P. K. and H. R. B. Pelham. Purification and characterization of a heat-shock element binding protein from yeast. EMBO. 6(10): 3035-3041, 1987.

187. Sorger, P. K. and H. R. B. Pelham. Cloning and expression of a gene encoding hsc73, the major hsp70-like protein in unstressed rat cells. EMBO. 6(4): 993-998, 1987.

YEAH!!!

FOR REFERENCE