Endocrine (1995) 3, 39–47

Effects of acute ethanol administration and cold exposure on the hypothalamic-pituitary-thyroid axis

R. Thomas Zoeller[1], Agnes Simonyi, Olimpia Butnariu, Don L. Fletcher[1], P. Kevin Rudeen, Sue McCrone & Sandra L. Petersen[1]

Department of Anatomy and Neurobiology, University of Missouri School of Medicine, M304, Medical Science Building, Columbia, Missouri 65212; [1]Neuroscience and Behavior Program and Department of Biology, University of Massachusetts, Morrill Science Center, Amherst, Massachusetts 01003, U.S.A.

A single dose of ethanol increases cellular levels of the mRNA encoding thyrotropin-releasing hormone (TRH) in neurons of the paraventricular nucleus (PVN), but blocks the cold-induced increase in the levels of this mRNA. Because the thyrotropic response to cold is dependent upon TRH secretion, we hypothesized that ethanol uncouples the stimulus-induced regulation of TRH secretion from the stimulus-induced regulation of TRH expression. We employed two complementary strategies to test this hypothesis. The first was to determine whether ethanol alters pituitary sensitivity to TRH. Animals given a single intraperitoneal injection of ethanol (3 g/kg) that produced a blood alcohol concentration of nearly 300 mg/100 mL exhibited the same increase in circulating levels of TSH following an intravenous infusion of TRH. Thus, ethanol does not appear to alter pituitary sensitivity to TRH. Second, we tested whether ethanol blocks the cold induction of c-*fos* expression in TRH neurons of the PVN. Both cold exposure and ethanol induced the expression of c-*fos* in the PVN and in TRH neurons; the effects of cold and ethanol on c-*fos* expression were additive. Thus, ethanol clearly does not block the cold activation of TRH neurons.

Keywords: ethanol; HPT-axis; thyroid; paraventriculum nucleus; cold exposure

Introduction

Circulating levels of thyroid hormones (thyroxine, T4 and triiodothyronine, T3) are regulated by a classic neuroendocrine system in which neurons containing thyrotropin-releasing hormone (TRH) play a dominant role (Martin & Reichlin, 1987). TRH is stored in high concentrations in axon terminals of the median eminence (Liao *et al.*, 1985; Ishikawa *et al.*, 1988) and is released into the pituitary-portal blood for transport to the pituitary gland (Fink *et al.*, 1983; Rondeel *et al.*, 1989). Although TRH-containing neurons are widely distributed in the central nervous system, TRH in the median eminence is synthesized primarily by neurons of the hypothalamic paraventricular nucleus (PVN) (Aizawa & Greer, 1981; Brownstein *et al.*, 1982; Palkovits *et al.*, 1982).

Several kinds of physiological signals are known to alter circulating levels of thyroid hormones in part by changing the activity of TRH neurons in the PVN. For example, T3 itself exerts a negative feedback effect on TSH both by a direct action on pituitary thyrotropes (Shupnik *et al.*, 1986; Franklyn *et al.*, 1987; Mirell *et al.*, 1987; Shupnik & Ridgway, 1987), and by an action on hypothalamic TRH neurons. Elements of the negative feedback effect of T3 on TRH neurons include a reduction in TRH release from the median eminence (Rondeel *et al.*, 1988), a decrease in TRH content of the median eminence and PVN (Mori & Yamada, 1987; Yamada *et al.*, 1989), and a reduction in cellular levels of TRH mRNA specifically in neurons of the PVN (Koller *et al.*, 1987; Segersen *et al.*, 1987).

TRH neurons in the PVN are also regulated by neural signals. For example, acute exposure to cold stimulates TRH neurons by what is generally regarded as an adrenergic mechanism. Cold exposure increases catecholamine turnover in the PVN (Annunziato *et al.*, 1977; Schettini *et al.*, 1979), and a central inhibitor of epinephrine synthesis (SKF64139) blocks the thyrotropic response to cold (Terry, 1986), as do a variety of alpha adrenergic receptor antagonists (Krulich *et al.*, 1977; Arancibia *et al.*, 1989). TRH neurons of the PVN also receive adrenergic synapses (Liposits *et al.*, 1987). Elements of the stimulatory effect of cold on TRH neurons include an immediate increase in TRH release from the median eminence (Szabo & Frohman, 1977; Arancibia *et al.*, 1983; Ishikawa *et al.*, 1984; Arancibia *et al.*, 1989; Rondeel *et al.*, 1989; Rondeel *et al.*, 1991) and a subsequent increase in cellular levels of TRH mRNA in neurons of the PVN (Zoeller *et al.*, 1990; Uribe *et al.*, 1991; Uribe *et al.*, 1993; Rage *et al.*, 1994). The observed parallelism between changes in TRH release and changes in cellular levels of TRH mRNA indicate that signals regulating TRH release also regulate TRH synthesis, as has been proposed for other neuroendocrine peptides (Young & Zoeller, 1987; Armstrong & Montminy, 1993).

TRH neurons, and the hypothalamic-pituitary-thyroid (HPT) axis, are also affected by drugs of abuse. Specifically, acute ethanol exposure reduces circulating levels of thyroid hormones but serum TSH remains unchanged (Portolés *et al.*, 1985; Zoeller & Rudeen, 1992). This may indicate that ethanol reduces pituitary sensitivity to TRH and/or to T3. However, ethanol-treated animals exposed to cold exhibit a normal increase in TSH and thyroid hormones (Zoeller & Rudeen, 1992), indicating that ethanol does not alter the ability of cold exposure to stimulate TRH release, or affect pituitary sensitivity to TRH. Ethanol also increases resting levels of TRH mRNA in the PVN, indicating that TRH release is also elevated if these two processes are regulated in parallel (Zoeller & Rudeen, 1992). In contrast, ethanol blocks the cold-

Correspondence: R. Thomas Zoeller
Received 18 April 1994; accepted 2 August 1994

induced increase in TRH mRNA. Thus, a single administration of ethanol appears to decrease basal TRH release while increasing TRH mRNA levels, and it permits the cold-induced increase in TRH release while blocking an additional increase in TRH mRNA levels. One interpretation of these data is that ethanol uncouples the regulation of TRH mRNA levels from the regulation of TRH release.

The present studies were initiated to test two elements of this hypothesis. First, does ethanol reduce pituitary-sensitivity to TRH? If so, it would indicate that changes in circulating levels of TSH are not reflective of TRH release. We approached this question by testing whether a single administration of ethanol affected TRH-stimulated TSH secretion. Second, does ethanol block the cold-activation of TRH neurons? If so, it would suggest that ethanol blocks the cold-induced TRH release and TRH synthesis. We approached this question by testing whether ethanol blocks the cold-induction of c-*fos* expression in TRH neurons of the PVN. The expression of the proto-oncogene c-*fos* can be induced by neuronal activation (Armstrong & Montminy, 1993), and its expression has been used widely as a marker of neuronal activation (Sager *et al.*, 1988).

Results

Effect of Ethanol on TRH-Stimulated TSH In vivo

The effects of ethanol on circulating levels of TSH and its response 15 and 30 min after TRH injection (iv) are illustrated in Figure 1. The 3-way ANOVA revealed a significant effect of ethanol, TRH and time on circulating levels of TSH [$F(1,48) = 4.5$, $P < 0.05$; $F(1,48) = 152$, $P < 0.0001$; and $F(2,48) = 40.5$, $P < 0.0001$, respectively]. Circulating levels of TSH were significantly higher in animals injected with TRH compared with control animals at both 15 and 30 min after injection. However, the level of TSH achieved following TRH injection in saline-pretreated animals was not different from that achieved following TRH injection in ethanol-pretreated animals. TSH levels in ethanol-treated animals injected with TRH were significantly lower than that of saline-treated animals 30 min after TRH (Figure 1). Ethanol-pretreated animals exhibited blood alcohol levels of 297 ± 47.6 mg/100 mL at the time TRH was administered.

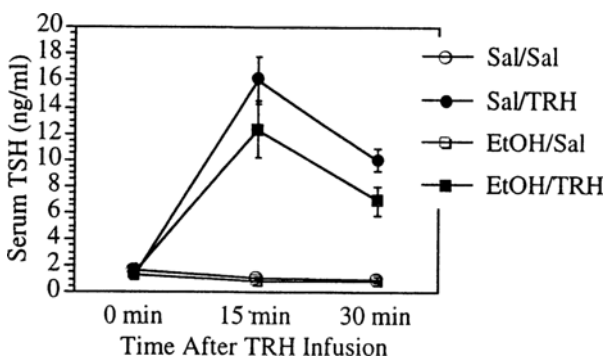

Figure 1 Effect of acute ethanol injection on the TSH response to TRH *in vivo*. Animals were prepared as described in the Methods. Blood was withdrawn before TRH (400 ng) or saline and 15 and 30 min after. Legend refers to animals pre-treated with saline (Sal/) or ethanol (EtOH/) and injected with saline (/Sal) or TRH (/TRH). TSH levels were significantly higher following TRH compared to saline at both 15 and 30 min. *Significantly different from TSH levels in saline pre-treated animals 30 min after TRH injection

Six-hour Time-Course of Effects of Ethanol on the HPT Axis

The MANOVA performed on all variables revealed a significant effect of ethanol and of temperature [$F_{(9,86)} = 18.42$, $P < 0.0001$].

Circulating levels of T3 were or tended to be significantly lower in ethanol-treated animals compared to the saline-treated controls maintained at the same temperature (Table 2). However, animals exposed to 5°C for 1 or 3 h exhibited elevated levels of circulating T3 whether or not they were injected with ethanol (Table 2).

Thyrotropin levels were significantly higher in animals exposed to cold compared to their respective 25°C controls (Figure 2A). Ethanol-treated animals did not exhibit TSH levels that were different from saline-treated controls maintained either at 25°C or placed at 5°C. The one exception was at 3 h, where TSH levels were slightly elevated in control animals at 25°C compared to those at 1 hr. In this case, TSH levels in cold-exposed animals did not differ from the controls.

The individual 3-way ANOVA for TRH mRNA indicated that there was a significant effect of treatment and time on TRH mRNA levels in neurons of the PVN (Figure 2B). TRH mRNA levels did not change significantly over time in control animals. However, TRH mRNA levels were significantly elevated in cold-exposed animals at 6 h. Ethanol-treated animals maintained at 25°C tended to exhibit elevated TRH mRNA levels at 3 h, and this did not change at 6 h. TRH mRNA levels were not further elevated by cold (Figure 2B).

There were no significant effects of treatment, temperature or time on levels of mRNAs encoding β-actin (data not shown).

c-fos *mRNA*

To determine whether cold induces c-*fos* expression in TRH neurons of the PVN, we first characterized the time-course of c-*fos* activation in the PVN using the oligonucleotide probe. All treated animals exhibited c-*fos* expression in the PVN at 1 h after treatments began (Figure 3A). This expression was transient in that fewer animals exhibited expression at 3 and 6 h following the initiation of cold exposure. We therefore measured the relative abundance of c-*fos* mRNA in animals at 1 h of cold exposure. Animals injected with ethanol exhibited c-*fos* mRNA levels that were similar to those exposed to 5°C. However, animals injected with ethanol and exposed to cold exhibited significantly higher levels of c-*fos* mRNA in the PVN (Figure 3B). Using dual-label ISH, we demonstrated that c-*fos* mRNA was induced by both treatments in TRH neurons of the PVN (Figure 4). We detected an average of 76 ± 6 digoxigenin-labeled cells (i.e., TRH mRNA-positive) in a single 12 micron section through the PVN; of these, $20 \pm 7\%$ were labeled with the c-*fos*

Table 1 Synthetic oligonucleotides

	Probe	*Sequence (5'-3')*[a]	*Base no.*	*Ref.*
1	TRH	GTCTTTTTCCTCCTCCTCCCTTTTGCCTGGATGCTGGCGTTTTGTGAT	319–366	(Lechan *et al.*, 1986)
2	c-*fos*	GATAAAGTTGGCACTAGAGACGGACAGATCTGCGCAAAAGTCCTGTGT	270–318	(Curran *et al.*, 1987)
3	b-Actin	GCCAGTGGTACGACCAGAGGCATACAGGGACAACACAGCCTGGATGGC	135–150[b]	(Nudel *et al.*, 1983)

[a]Sequences are listed from 5' to 3' and are *complementary* to the base number listed in the next column. They were synthesized using an Applied Biosystems model 380A DNA synthesizer according to the manufacturer's instructions and purified by RPC columns according to ABI'S instructions. [b]Refers to amino acid numbers. Note. The specificity of the ISH signal using these probes has been previously validated with the exception of c-*fos*. Methods of validation include Northern Analysis, comparison of anatomical distribution with immunocytochemistry, and comparison with a sense-strand probe. Specificity of the ISH using the c-*fos* probe was validated in the present experiments using northern analysis on total RNA extracted from the brain of a rat treated with kainic acid, and by comparison of the ISH labeling using the cRNA probe

Table 2 Serum triiodothyronine levels following cold-exposure in ethanol-treated rats

Time & Treatment	*T3* ng/dl
0 h	
Sal-25°C	55.6±3.87 (8)
1 h	
Sal-25°C	56.7±3.15 (8)
Sal-5°C	49.5±4.73 (8)
EtOH25°C	43.7±4.96 (7)
EtOH5°C	43.1±3.13 (8)
3 h	
Sal-25°C	41.8±5.83 (8)
Sal-5°C	64.7±3.41 (8)
EtOH25°C	30.4±5.35 (7)
EtOH5°C	47.9±3.79 (8)
6 h	
Sal-25°C	55.1±6.69 (8)
Sal-5°C	66.4±4.16 (8)
EtOH25°C	49.7±4.32 (8)
EtOH5°C	51.1±5.86 (8)

Values for T3 represent mean±SEM of (*n*) animals/group. The 3-way ANOVA for T3 indicated a significant effect of time, temperature and treatment [$F(2,84) = 4.3$, $P < 0.02$; $F(1,84) = 7.6$, $P < 0.01$; $F(1,84) = 17.29$, $P < 0.001$; respectively

probe in ethanol-treated animals exposed to cold. The proportion of TRH mRNA-positive neurons that were also c-*fos* positive was correlated with the abundance of c-*fos* labeling in the PVN. However, not all c-*fos*-positive cells were TRH mRNA-positive. In ethanol-treated animals exposed to cold, we detected an average of 23 ± 5 c-*fos*-positive cells in a single section through the PVN. The expression of c-*fos* was induced in all regions of the PVN, including the medial parvocellular division which contains both TRH and CRH neurons, and the dorsal magnocellular division containing vasopressin and oxytocin neurons.

Two-Hour Time-Course of Cold-Induced Changes in HPT Axis following Ethanol

The MANOVA performed on all variables revealed a significant effect of treatment and temperature [$F(3,24) = 23.61$, $P < 0.001$]. Serum T3 levels were significantly lower in ethanol-treated animals than in control animals at 60 and 120 min after cold exposure (3 h and 4 h after ethanol injection; Table 3). Ethanol-treated animals exposed to cold tended to have higher T3 levels compared to ethanol-treated animals maintained at 25°C, but this was not statistically significant.

In contrast, serum TSH in saline-treated animals exposed to cold were significantly elevated over that of saline-treated animals maintained at 25°C at all times (Figure 5A). Likewise, ethanol-treated animals exposed to cold exhibited serum TSH levels that were significantly higher than ethanol-treated animals maintained at 25°C at 60 and 120 min following cold. However, serum TSH levels declined in ethanol-treated animals maintained at 25°C, such that levels were significantly lower than that of saline-injected controls at 60 min and 120 min (3 h and 4 h after ethanol injection).

The 3-way ANOVA on TRH mRNA levels revealed a significant effect of temperature, and a significant interaction between temperature and time (Figure 5B). Control animals (saline-injected at 25°C) sacrificed 30 min after cold exposure (1330 h) exhibited TRH mRNA levels in the PVN that were significantly higher than control animals sacrificed 60 min and 120 min after cold exposure (1400 h and 1500 h, respectively). TRH mRNA levels in the PVN of animals exposed to cold were significantly higher than that of their 25°C counterparts at 60 min and 120 min of cold. Ethanol-treated animals maintained at 25°C had TRH mRNA levels in the PVN that were similar at all time points, while ethanol-treated animals transferred to 5°C had levels of TRH mRNA in the PVN that were similar to their respective controls.

c-fos mRNA. We evaluated c-*fos* mRNA in PVN using both the oligonucleotide probe for quantitation and the cRNA probe for dual-label ISH. The results were identical to those obtained earlier.

Discussion

The goal of these experiments was to test the hypothesis that ethanol uncouples stimulus-induced changes in TRH release from stimulus-induced changes in TRH gene expression. We employed two complementary strategies to test this hypothesis. The first was to determine whether ethanol alters pituitary sensitivity to TRH. If so, it would indicate that ethanol-induced changes in circulating levels of TSH reflect changes in pituitary function, not changes in TRH release. We found that ethanol-treated animals exhibited the same increase in circulating levels of TSH following TRH infusion as observed in control animals, indicating that a single administration of ethanol which produces blood alcohol levels of nearly 300 mg/100 mL does not alter pituitary-sensitivity to

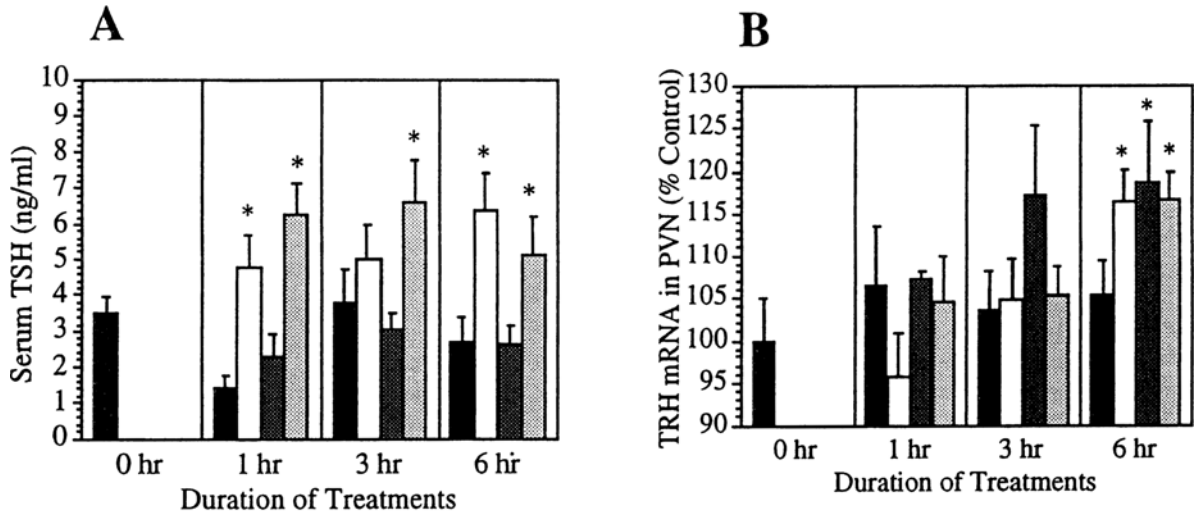

Figure 2 Effect of acute ethanol exposure on circulating levels of TSH (A) and cellular levels of TRH mRNA in PVN (B) in animals exposed to cold. Bars represent Mean ± SEM ($n = 8$/group). Group designations are: ■ Sal/25° □ Sal/5° ▨ EtOH/25° ▩ EtOH/5°. The 3-way ANOVA for TSH indicated a significant effect of temperature only [$F(1,84) = 25.53$, $P < 0.0001$]. For TRH mRNA measurements, there was a significant affect of treatment and time [$F(1,84) = 3.82$, $P < 0.05$; $F(2,84) = 4.776$, $P < 0.01$, respectively]. *Significant difference ($P < 0.05$ by Bonfferoni's t-test) from saline or ethanol control at the same time

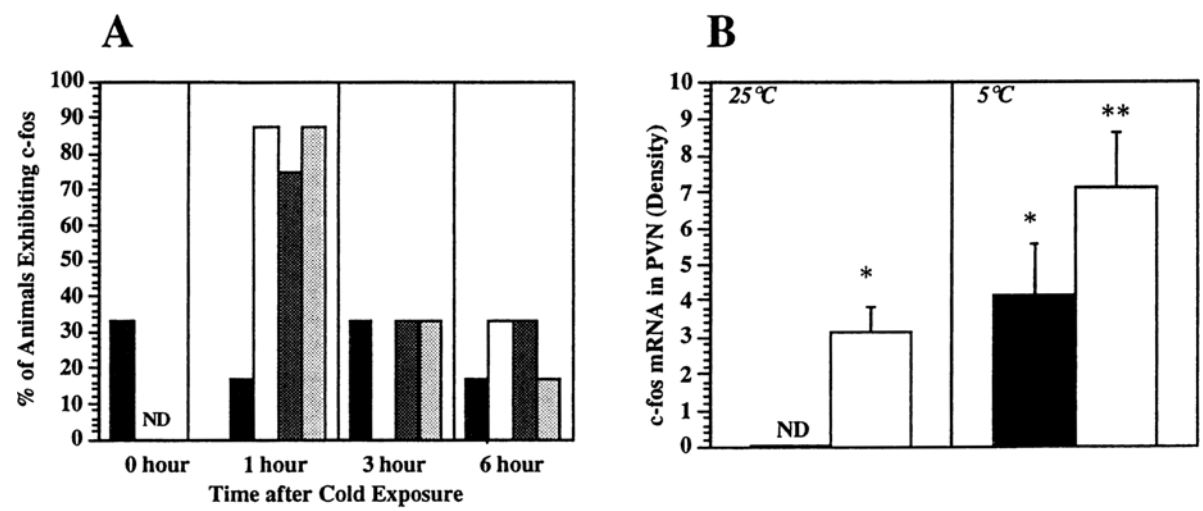

Figure 3 Time course (A) of effects of cold and ethanol exposure on c-*fos* mRNA expression in PVN, and effects of cold and ethanol exposure on c-*fos* mRNA levels in PVN after 1 h of treatment (B). Group designations in (A) are: ■ Sal/25° □ Sal/5° ▨ EtOH/25° ▩ EtOH/5° For (B), the solid bars represent saline-injected animals; the open bars represent ethanol-injected animals. Bars represent Mean ± SEM. At 1 h there was a significant treatment and temperature effect [$F(1,23) = 7.8$, $P < 0.01$; $F(1,23) = 13.5$, $P < 0.001$, respectively]. ND = Not Detected. *Significant difference ($P < 0.05$ by Bonfferoni's t-test) from saline control. **Significant difference ($P < 0.01$ by Bonfferoni's t-test) from saline control

TRH, and that ethanol-induced changes in circulating TSH reflect changes in TRH secretion. This is consistent with our previous observation that ethanol-treated animals exhibit the same percentage increase in serum TSH following cold-exposure as observed in control animals (Zoeller & Rudeen, 1992). TSH levels appeared to return toward baseline more rapidly in ethanol-treated animals following TRH infusion, suggesting that TSH clearance may be more rapid in ethanol-treated animals.

The second strategy we employed to test the hypothesis that ethanol uncouples stimulus-induced changes in TRH release from stimulus-induced changes in TRH gene expression was to determine whether ethanol blocks the cold-activation of TRH neurons. We reasoned that, as an immediate early gene whose expression is transiently increased following neuronal activation (Morgan & Curran, 1991), c-*fos* expression would provide a marker of TRH neuronal activation independent of measures such as changes in TRH mRNA levels and changes in circulating levels of TSH. We predicted that c-*fos* expression would be induced in TRH neurons by cold exposure because others had shown that TRH neurons express c-*fos* in response to stress (Koibuchi *et al.*, 1991).

We found that both cold exposure and ethanol injection induce the expression of c-*fos* mRNA in TRH neurons of the PVN, and the two treatments produced

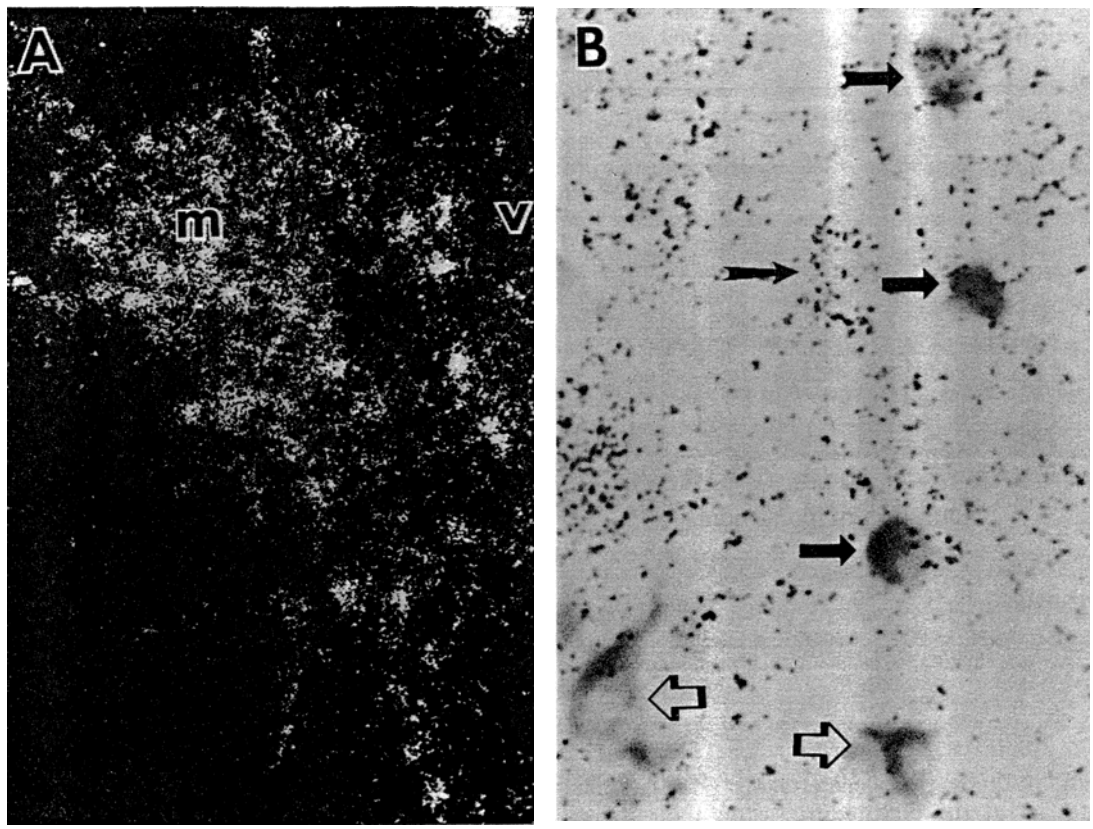

Figure 4 Darkfield (A) and bright-field (B) photomicrographs of the hypothalamic paraventricular nucleus following dual-label *in situ* hybridization with an ^{35}S-labeled cRNA probe complementary to c-*fos* mRNA and a digoxigenin-labeled cRNA probe complementary to TRH mRNA (see Methods). Only the ^{35}S signal is visible in (A) (c-*fos* mRNA); silver grains appear as white clusters over one side of the PVN shown. Note the widespread distribution of c-*fos* expression throughout both medial (parvocellular) and dorso-lateral (magnocellular) divisions of the PVN and the absence of signal outside the PVN. In (B), both silver grains (black clusters) identifying c-*fos*-positive cells and digoxigenin (dark cell bodies) identifying TRH-positive cells are visible in brightfield. **Solid arrow:** dual-labeled neurons; **Open arrow:** TRH-positive, c-*fos* -negative; **Arrowhead:** c-*fos* -positive, TRH-negative. **m,** magnocellular PVN; **v,** third ventricle

Table 3 Serum hormone levels following cold-exposure in ethanol-treated rats

Time & Treatment	*T3* ng/dl
30 min	
Sal-25°C	42.9±7.86 (4)
Sal-5°C	48.4±6.64 (4)
EtOH25°C	32.1±5.38 (4)
EtOH5°C	42.00±6.32 (4)
60 min	
Sal-25°C	67.4±2.07 (4)
Sal-5°C	63.5±17.48 (4)
EtOH25°C	24.0±3.31 (4)
EtOH5°C	23.7±4.69 (4)
120 min	
Sal-25°C	44.9±5.59 (4)
Sal-5°C	84.0±18.03 (4)
EtOH25°C	24.8±8.52 (4)
EtOH5°C	28.0±10.06 (4)

Values for T3 represent mean±SEM of (*n*) animals/group. The 3-way ANOVA revealed a significant effect of treatment [$F(1,36) = 26.52$, $P < 0.0001$] and temperature [$F(1,36) = 4.25$, $P < 0.05$], but not of time $F(1,36) = 0.030$, $P > 0.05$]

an additive effect (Figure 3B). Although these treatments induced c-*fos* expression in TRH neurons, we did not observe c-*fos* expression in all TRH neurons (Figure 4). The expression of c-*fos* mRNA was most robust in ethanol-treated animals exposed to cold, yet only 20% of TRH-positive neurons of the PVN were labeled with the c-*fos* probe. It is possible that all TRH neurons express c-*fos* but that our protocol failed to detect it. We have shown previously that cold-exposure increases cellular levels of TRH mRNA in all neurons of the PVN; analysis of single-cell levels of TRH mRNA throughout the PVN failed to identify subsets of TRH neurons that selectively exhibited an increase in TRH mRNA levels (Zoeller *et al.*, 1990). However, we cannot rule out the possibility that while TRH mRNA levels are elevated by cold in all TRH neurons, c-*fos* is induced in only a subpopulation of these neurons.

There are few reports of the effects of ethanol on c-*fos* induction in the central nervous system. Le *et al.* (1990 & 1992) have reported that acute ethanol administration does not induce the expression of c-*fos* as measured by northern analysis. However, considering the anatomical pattern and intensity of c-*fos* induction by ethanol we observe in the present experiments, it is unlikely that this would have been detected by northern analysis of total RNA extracted from whole rat brain.

The mechanism by which ethanol affects the expression of c-*fos* in rat brain in unclear. Le *et al.* (1990 & 1992) demonstrate that ethanol can block the induction

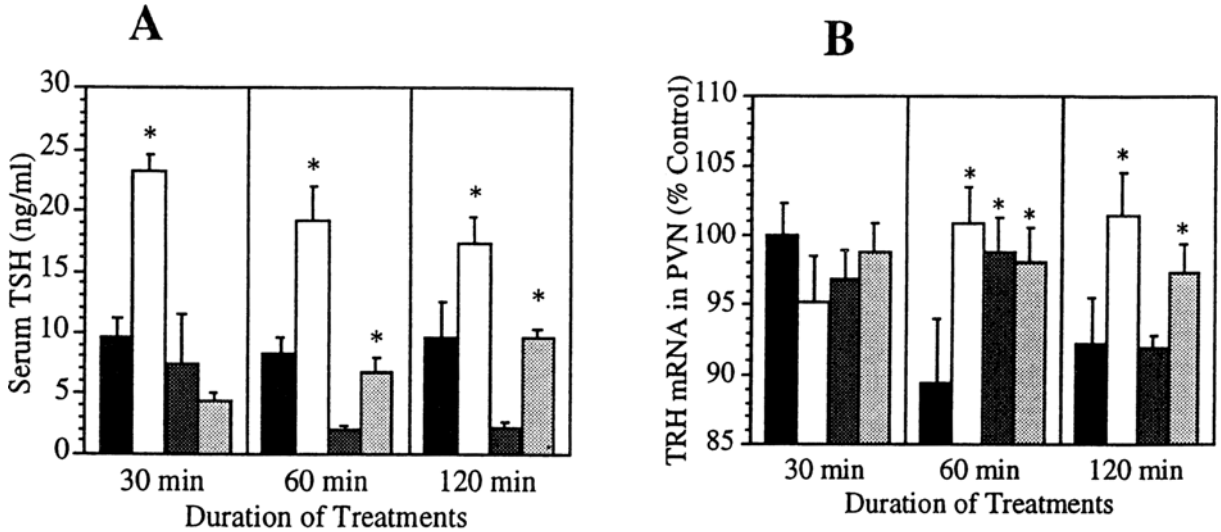

Figure 5 Effect of acute ethanol exposure on circulating levels of TSH (A) and cellular levels of TRH mRNA in PVN (B) in animals exposed to cold. Bars represent Mean ± SEM ($n = 4$/group). Group designations are: ■ Sal/25° □ Sal/5° ▨ EtOH/25° ▩ EtOH/5°. For TSH, the 3-way ANOVA revealed significant effects of temperature and treatment [$F(1,36) = 149$, $P < 0.0001$; $F(1,36) = 73$, $P < 0.0001$, respectively], but not of time. For TRH mRNA measurements, there were significant effects of temperature [$F(1,36) = 6.09$, $P < 0.01$]. *Significant difference ($P < 0.05$ by Bonfferoni's t-test) from saline control at the same time

of c-*fos* in brain by pentylenetetrazole (PTZ), which appears to act at the picrotoxin site on the gamma-aminobutyric acid-benzodiazepine receptor complex (GABA-BZ; Squires *et al.*, 1984). Thus, a single administration of ethanol appears capable of both inducing the expression of c-*fos* and blocking its induction by specific drugs, indicating that ethanol may affect c-*fos* expression by more than one mechanism. This would be most easily understood if these different effects are occurring in separate brain regions. Considering that ethanol induces the expression of c-*fos* in a neuroanatomical pattern that is identical to that of cold-exposure (Zoeller & Fletcher, 1994), it is possible that ethanol-induced c-*fos* expression is mediated by ethanol-induced hypothermia. Ethanol-induced hypothermia is similar in its duration and severity to that induced by cold-exposure, and ethanol exacerbates the hypothermia following exposure to cold (Zoeller & Rudeen, 1992). This may explain why cold and ethanol produce an additive effect on the expression of c-*fos* mRNA in the PVN.

We have previously demonstrated that ethanol simultaneously induces the expression of c-*fos* and reduces the expression of c-*jun* in the PVN (Zoeller & Fletcher, 1994). Since Fos protein must form a heterdimer with a member of the Jun family to function as a transcription regulator at the AP-1 site (Curran & Franza, 1988; Hai & Curran, 1991; Morgan & Curran, 1991; Armstrong & Montminy, 1993), the ethanol-induced Fos protein may lack or exhibit diminished function in the PVN if Jun protein is reduced. Considering that the TRH gene contains a functional AP-1 site (Lee *et al.*, 1988, 1993), it is tempting to speculate that ethanol blocks the cold-induced increase in TRH mRNA levels by altering the balance of Fos:Jun. However, it is unclear whether c-*fos* induced by ethanol in TRH neurons directly interacts with the TRH gene.

Although these findings support the contention that at least a subset of TRH neurons are activated by cold exposure in ethanol-treated animals at a time when circulating levels of TSH are reduced, the results of the time-course studies are difficult to reconcile with the concept that TRH release and TRH synthesis are tightly coupled. In the 6 h time course, animals were exposed to cold at 0900 h and sampled 1,3 and 6 h later. Serum TSH was elevated at the first sample time and c-*fos* expression was induced in the PVN, indicating that TRH neurons were activated, but TRH mRNA levels did not become elevated until after 6 h (Figure 2B). This temporal delay may suggest that the cold-induced increase in cellular levels of TRH mRNA are dependent upon the synthesis of new proteins (e.g., Fos) which then affect TRH transcription. Conversely, it may indicate that cold increases steady-state levels of TRH mRNA by affecting mRNA stability, which may require some time before levels are increased to a detectable level.

However, considering that others have reported a rapid effect of cold exposure on TRH mRNA levels (Uribe *et al.*, 1991; Uribe *et al.*, 1993; Rage *et al.*, 1994), and that there is a daily rhythm of changes in TRH mRNA (Covarrubias *et al.*, 1988; Zoeller *et al.*, 1990), another possibility is that the high morning levels of TRH mRNA mask the effect of cold exposure.

Therefore, we performed the second experiment in which cold exposure was initiated in the afternoon. We found that levels of TRH mRNA in the PVN were significantly higher in cold-exposed animals at 60 and 120 min than in control animals, suggesting that cold can increase TRH mRNA levels within 1 h if cold is initiated in the afternoon. However, the baseline level of TRH mRNA in the PVN declined in control animals over the course of the experiment. Thus, it remains unclear whether cold exposure prevents the daily decline in TRH mRNA or stimulates an increase in TRH mRNA levels which is masked by daily rhythm.

These experiments were initiated to determine the effects of acute ethanol exposure on interactions within the HPT axis. Our results do not support the hypothesis that ethanol alters pituitary sensitivity to TRH. Thus, ethanol-induced changes in circulating levels of TSH are likely to reflect changes in TRH release. It is paradoxical that ethanol simultaneously decreases TRH release and increases TRH mRNA levels in the PVN. Although this may be caused by the reduction in circulating levels of T3, this would have to occur rapidly. An alternate interpretation is that a single dose of ethanol alters specific intracellular signaling mechanisms within TRH neurons of the PVN, leading to an increase in TRH mRNA. This interpretation is based on studies demonstrating that cellular levels of neural mRNAs are regulated trans-synaptically by specific second messenger systems and nuclear regulatory mechanisms (Goodman, 1990; Habener, 1990; Morgan & Curran, 1991; Armstrong & Montminy, 1993). TRH mRNA levels in neurons of the PVN are regulated predominantly by T3 (Koller *et al.*, 1987; Segersen *et al.*, 1987), but also by cold exposure and by a 24 h rhythm (Zoeller *et al.*, 1990), all of which may be mediated by trans-synaptic effects on second messenger systems. Therefore, the observation that ethanol can selectively alter the regulation of TRH mRNA levels, while having no effect on the regulation of c-*fos* mRNA in the same neurons, suggests that ethanol can have very specific effects on intracellular signalling mechanisms within neurons.

Materials and methods

Animals

All animal treatments were performed in accordance with a protocol approved by the University of Missouri-Columbia Animal Care and Use Committee.

Effect of Ethanol on TRH-Stimulated TSH In Vivo

We performed this experiment to test the effect of ethanol on the ability of the pituitary gland to respond to TRH. Adult male Sprague-Dawley rats (220–260 g; Harlan) were maintained individually in plastic cages. After being acclimated to lab conditions, the animals were anesthetized with 80 mg/kg ketamine (i.p., KETASET, Aveco) and 10 mg/kg xylazine (i.p., Xylazine HCl, TechAmerica) and each was fitted with an in-dwelling jugular catheter exteriorized from the back of the neck. The following day, an initial blood sample (700 µl each) was withdrawn from the catheter between 1330 h and 1430 h. Five- to 10-min later, each animal was given ethanol (3 g/kg i.p. in 25%, v/v, in normal saline) or the equivalent volume of normal saline. Thirty-minutes after ethanol administration, the animals were infused through the catheter with TRH (400 ng/100g, BW, Calbiochem); controls received an equal volume of saline. Blood was withdrawn at 15 and 30 min after TRH administration. These serum samples were used to determine levels of TSH by RIA. Blood alcohol concentration (BAC) was determined in a separate experiment because the volume of blood required was too large to allow us to obtain repeated measurements of TSH and alcohol.

Six-hour Time-Course of Effects of Ethanol on HPT

The goal of this experiment was to determine whether ethanol blocks the cold-activation of TRH neurons in the PVN. We used c-*fos* expression as an index of neuronal activation, and because its induction is transient, we performed a time-course study beginning in the morning. Adult male Sprague-Dawley rats (175–200 g; Sasco; $n = 120$) were individually housed in wire-rack cages maintained in an environmental chamber with controlled lighting and temperature (12:12; L:D; light onset at 0600 h; 25°C). This 78 ft^2 environmental chamber was one of four identical units that opened into a small tissue preparation room where animals were sacrificed. Food and water were available at all times. At 0900 h on the morning of the 8th day of acclimation to the environmental chamber, eight animals were sacrificed as untreated controls. Remaining animals were injected with ethanol (3g/kg BW i.p.; 25% in sterile 0.9% NaCl) or saline alone. After the injections, one rack of animals was transferred to the adjacent chamber equilibrated at 5°C. To control for stress associated with this process, the remaining rack was moved out of the 25°C chamber into the preparation room and immediately returned. This process was completed in less than 1 min for each rack. Animals were decapitated (8/group) at 1 h, 3 h, or 6 h following the initiation of cold exposure. The brain was immediately removed from the cranium and frozen in pulverized dry ice. Trunk blood was collected and serum harvested and frozen for subsequent measurement of hormones by radioimmunoassay.

Two-hour Time-Course of Cold-Induced Changes in HPT Axis Following Ethanol

This experiment was performed to test the possibility that the effect of cold and/or ethanol on TRH mRNA levels is different in the afternoon. At 1100 h on the 8th day in the environmental chamber at 25°C, 48 animals were injected with ethanol or saline as described above. The animals remained at 25°C for 2 h following this injection, then half were transferred to a 5°C chamber; again, the remaining animals were moved out of the 25°C chamber and returned. Groups were sampled at 30 min, 60 min and 120 min (four animals per treatment group at each time); tissues and blood were collected and stored as described above.

In situ *hybridization (ISH)*

Twelve-micrometer coronal sections were made through the hypothalamus at the level of the PVN in a Reichert-Jung 2800N cryostat and thaw-mounted onto cold microscope slides (2 sections/slide) previously treated with two coats of a gelatin/chromalum solution. Thawed sections were then briefly dried on a slide warmer at 37°C before being stored at −70°C until hybridization. To prepare sections for hybridization, they were first warmed to room temperature and loaded into stainless steel slide racks that had been previously treated with diethylpyrocarbonate (DEPC) and autoclaved (Blumberg, 1987). Racks containing 100 slides each were then immersed in 4% formaldehyde/phosphate-buffered saline (PBS; 0.15 M NaCl/1.0 mM KH_2PO_4/6.0 mM Na_2HPO_4) for 5 min, rinsed in PBS for 2 min, and soaked for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% NaCl (pH 8.0). They were then rinsed in twofold concentrated (2X) standard saline citrate (SSC; 1XSSC = 150 mM NaCl/15 mM sodium citrate), dehydrated through a graded series of ethanol, delipidated in chloroform, rehydrated to 95% ethanol and air dried. All solutions used for prehybridization were previously treated with DEPC and autoclaved, or prepared with DEPC-treated/autoclaved stock buffers and water.

Fifty microliters of hybridization buffer were applied to each slide, covered with a parafilm coverslip and incubated at 37°C for 20 h in humid chambers. The hybridization buffer for oligonucleotide probes contained: 50% formamide; 4XSSC; transfer RNA (250 µg/ml); sheared, single-stranded salmon sperm DNA (100 µg/ml); Denhardt's solution (0.02% each of BSA, Ficoll and polyvinylpyrrolidone); 10% (w/v) dextran sulfate MW = 500 000; 100 mM dithiothreitol (DTT);

and 0.75 -1.0 × 10^6 c.p.m. probe (see Table 1). After hybridization, the coverslips were floated off in 1XSSC and washed two times in 1XSSC, then four times for 15 min each in 2XSSC/50% formamide at 40°C. Following two 30 min washes in 1XSSC at room temperature, the slides were dipped in distilled water, equilibrated in 70% ethanol and air-dried.

Dual-Label in situ Hybridization

The hybridization buffer for the dual-label ISH (Petersen *et al.*, 1993) contained 40% formamide and two separate probes, but all other components were the same. After 5 h at 52°C, the coverslips were removed in 1XSSC, the tissues soaked in 50% formamide/2XSSC for 5 min at room temperature, then 20 min at 52°C. Following two 1 min rinses in 2XSSC at room temperature, the sections were incubated in a solution containing 2XSSC and 100 μg/ml RNAse A at 37°C for 30 min. The slides were then rinsed twice briefly in 2XSSC and incubated for 10 min in 50% formamide/2XSSC at 52°C. The sections were then incubated for a minimum of 9 h in 2XSSC/0.05% Triton-X-100 and 2% normal rabbit serum (NRS). Following a 10 min rinse in 100 mM Tris-HCl/150 mM NaCl, pH 7.5, the sections were incubated in anti-digoxigenin peroxidase 1:100 in Tris/NaCl with 0.3% Triton X-100 and 1% NRS overnight at 4°C. The sections were then washed twice for 1 min in Tris/NaCl, then incubated in 0.1 M Tris pH 7.6 mixed with 10mg 3:3 diaminobenzydine (DAB) and 8 μl H_2O_2. The reaction was stopped in 0.1 M Tris, rinsed in water, dehydrated in 70% ethanol and air dried. These slides were then dipped in Kodak NTB3 nuclear track emulsion to visualize the radioactive probe.

Probe Preparation

Oligonucleotides were synthesized on an Applied BioSystems, Inc., Model 380B DNA synthesizer and purified by RPC columns according to the manufacturer's instructions. Purified oligomers (5 pmol, Table 1) were 3'end-labeled by incubating with 50 units of terminal deoxynucleotidyl transferase (Boehrenger-Mannheim, Indianapolis, IN), 50 pmol ^{35}S-dATP (New England Nuclear), 200 mM potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumin, 1.5 mM $CoCl_2$, (pH 6.6) for 15 min at 37°C.

c-RNA Probes For dual-label ISH, the c-*fos* cRNA was generated from a plasmid provided by Dr T. Curran (Roche Institute of Molecular Biology, Nuttely NJ). This plasmid containing a 2.1 kb insert in pSP65 was linearized with Bgl II and transcribed in the presence of 120 μM ATP, CTP and GTP, and 12 μM each of UTP and ^{35}S-UTP to generate an approximately 288 bp cRNA. The TRH cRNA was generated from a plasmid provided by Dr R.H. Goodman (Vollum Institute, Portland, Oregon). This plasmid contains a 1.2 kb insert in pSP64, and was linearized with HindIII and transcribed in the presence of 670 nM each of ATP, CTP, GTP and digoxigenin-labeled UTP (Boehringer-Mannheim), and 130 mM of UTP to generate a 1.2 kb cRNA.

The specificity of hybridization has been evaluated by a number of methods for each probe. All oligomers hybridize to a single-class of RNA appropriate for the target mRNA as determined by Northern analysis in our lab or others (Koller *et al.*, 1987; Young & Zoeller, 1987; c-*fos* data not shown). Specificity of the cRNA probes was verified both by Northern analysis and by using sense-strand probes (data not shown).

Autoradiography and Signal Quantitation

Specific labeling was detected with Kodak X-Omat AR film using techniques previously described (Zoeller *et al.*, 1990; Zoeller & Rudeen, 1992; Zoeller *et al.*, 1993). Slides were held against film with ^{14}C-standards (American Radiolabelled Chemicals, Inc.) in x-ray cassettes for 12 h (β-actin probe), 7 days (TRH probes), or 14 days (c-*fos* oligonucleotide probe). Microdensitometry was performed on the signal over the PVN using a Macintosh-based image analysis system (Image 1.42, Wayne Rasband, NIMH) interfaced with a Dage-MTI 72 series video camera equipped with a Nikon macro lens mounted onto a bellows system over a light box. Signal density was used as an index of mRNA levels; ^{14}C-standards were used to ensure that the film was not over-exposed. The hybridization signal was evaluated for each probe as follows. First, the average gray-level was measured for signal and background for each section. Background was subtracted from signal to provide a numerical value of average density over the specified region. This corrected density was averaged over the four brain sections measured for each animal before being evaluated for statistical significance.

Radioimmunoassay

TSH Serum levels of thyrotropin (TSH) were measured using the double-antibody NIDDK rat TSH radioimmunoassay kit including RP-2 standards. The detection limits for serum TSH were from 0.5–50 ng/ml. The intra-assay variation was 9.2%.

Thyroid hormones Serum triiodothyronine (T3) was measured using kits purchased from ICN Biomedicals, Inc. Detection limits for T3 were from 12.5 to 400 ng/dL; the average intra-assay variation (coefficient of variation) was 3.2%. All samples were measured in duplicate in the same assay.

Blood Alcohol Concentration was assayed using the enzyme-based kit purchased from Sigma (St. Louis) according to the manufacturer's instructions.

Data Analysis TSH levels measured in the first experiment were analysed using a three-way Analysis of Variance with ethanol, TRH, and time as main effects. For each time-course experiment, all measurements for each animal were analyzed simultaneously using a Multivariate Analysis of Variance (MANOVA). After obtaining a significant F in the MANOVA, individual 3-way ANOVAs (main effects of temperature *vs* ethanol for each time) were performed. Bonferroni's *t*-test, which uses the MSE from the individual 3-way ANOVA table as a point estimate of the pooled variance, was used to test for differences between means.

Acknowledgements
This work was supported by NIH Grant AA08887 to RTZ.

References

Aizawa, T. & Greer, M.A. (1981). *Endocrinol*, **109**, 1731–1738.

Annunziato, L., Renzo, G.F.D., Lombardi, G., Scopacasa, F., Schetini, G., Preziosi, P. & Scapagnini, U. (1977). *Endocrinol*, **100**, 738–744.

Arancibia, S., Tapia-Arancibia, L., Assenmacher, I. & Astier, H. (1983). *Neuroendocrinol*, **37**, 225–228.

Arancibia, S., Tapia-Arancibia, L., Assenmacher, I. & Astier, H. (1989). *Neurosci Lett.*, **100**, 169–174.

Armstrong, R.C. & Montminy, M.R. (1993). *Annu. Rev. Neurosci.*, **16,** 17–29.

Blumberg, D.D. (1987). *Guide to Molecular Cloning Techniques*, Berger, S.L., Kimmel, A.R. (eds). Academic Press: San Diego. 20–24.

Brownstein, M.J., Eskay, R.L. & Palkovits, M. (1982). *Neuropeptides*, **2,** 197–201.

Covarrubias, L., Uribe, R.M., Mendez, M., Charli, J.L. & Joseph-Bravo, P. (1988). *Biochem Biophys Res Commun.*, **151,** 615–622.

Curran, T., Gordon, M.B., Rubino, K.L. & Sambucetti, L.C. (1987). *Oncogene*, **2,** 79–84.

Curran, T. & Franza, B.R. (1988). *Cell*, **55,** 395–397.

Fink, G., Koch, Y. & Ben-Aroya, N. (1983). *Thyrotropin-Releasing Hormone*, Griffiths, E.C., Bennette, G.W., (eds). Raven Press: New York. 127–144.

Franklyn, J.A., Wood, D.F., Balfour, N.J., Ramsden, D.B., Docherty, K., Chin, W.W. & Sheppard, M.C. (1987). *Endocrinol*, **120,** 2279–2288.

Goodman, R.H. (1990). *Annu. Rev. Neurosci.*, **13,** 111–127.

Habener, J.F. (1990). *Mol. Endocrinol*, **4,** 1087–1094.

Hai, T., Curran, T. (1991). *Proc. Natl. Acad. Sci. USA.*, **88,** 3720–3724.

Ishikawa, K., Kakegawa, T. & Suzuki, M. (1984). *Endocrinol*, **114,** 352–358.

Ishikawa, K., Taniguchi, Y., Inoue, K., Kurosumi, K. & Suzuki, M. (1988). *Neuroendocrinol*, **47,** 384–388.

Koibuchi, N., Gibbs, R.B., Suzuki, M. & Pfaff, D.W. (1991). *Endocrinology*, **129,** 3208–3216.

Koller, K.J., Wolff, R.S., Warden, M.K. & Zoeller, R.T. (1987). *Proc. Natl. Acad. Sci. USA*, **84,** 7329–7333.

Krulich, L., Giachetti, A., Marchlewska-Koj, A., Hefco, E. & Jameson, H.E. (1977). *Endocrinol*, **100,** 496–505.

Le, F., Wilce, P., Cassady, I., Hume, D. & Shanley, B. (1990). *Neurosci Lett.*, **120,** 271–274.

Le, F.I., Wilce, P.A., Hume, D.A. & Shanley, B.C. (1992). *J. Neurochem*, **59,** 1309–1315.

Lechan, R.M., Wu, P., Jackson, I.M.D., Wolfe, H., Cooperman, S., Mandel, G. & Goodman, R.H. (1986). *Sci.*, **231,** 159–161.

Lee, S.L., Stewart, K. & Goodman, R.H. (1988). *J. Biol. Chem.*, **263,** 16604–16609.

Lee, S.L., Yang, L.M. & Lin, A. (1993). *Ann Meeting Endocrine Soc.*, Abs 1925.

Liao, N., Bulant, M., Nicolas, P., Vaudry, H. & Pelletier, G. (1985). *Neurosci. Lett.*, **85,** 24–28.

Liposits, Z., Paull, W.K., Wu, P., Jackson, I.M.D. & Lechan, R.M. (1987). *Histochem*, **88,** 1–10.

Martin, J.B. & Reichlin, S. (1987). *Clinical Neuroendocrinology*, Contemporary Neurology, F.A. Davis Company. 759.

Mirell, C.J., Yanagisawa, M., Lau, R., Pekary, A.E., Chin, W.W. & Hershman, J.M. (1987). *Mol. Endocrinol*, **1,** 408–412.

Morgan, J.I. & Curran, T. (1991). *Annu. Rev. Neurosci.*, **14,** 421–451.

Mori, M. & Yamada, M. (1987). *J. Endocrinol*, **114,** 443–448.

Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z. & Yaffe, D. (1983). *Nuc Acid Res.*, **11,** 1759–1771.

Palkovits, M., Eskay, R.L. & Brownstein, M.J. (1982). *Endocrinol*, **110,** 1526–1528.

Petersen, S.L., McCrone, S., Coy, D., Adelman, J.P. & Mahan, L.C. (1993). *Endocrine J.* (In Press):

Portolés, M., Sanchis, R. & Guerri, C. (1985). *Med Sci.*, **13,** 615–616.

Rage, F., Lazaro, J.-B., Benyassi, A., Arancibia, S. & Tapia-Arancibia, L. (1994). *J. Neuroendocrinology*, **6,** 19–23.

Rondeel, J.M.M., deGreef, W.J., Schoot, P.v.d., Karels, B., Klootwijk, W. & Visser, T.J. (1988). *Endocrinol*, **123,** 523–527.

Rondeel, J.M.M., deGreef, W.J., Vaart, P.D.M.v.d., Schhot, P.v.d. & Visser, T.J. (1989). *Endocrinology*, **125,** 971–975.

Rondeel, J.M.M., deGreef, W.J., Hop, W.C.J., Rowland, D.L. & Visser, T.J. (1991). *Neuroendocrinology*, **54,** 477–481.

Sagar, S.M., Sharp, F.R. & Curran, T. (1988). *Science*, **240,** 1328–1331.

Schettini, G., Quattrone, A., Renzo, G.D., Lombardi, G. & Preziosi, P. (1979). *Eur. J. Pharmacol.*, **56,** 153–157.

Segersen, T.P., Kauer, J., Wolfe, H.C., Mobtaker, H., Wu, P., Jackson, I.M.D. & Lechan, R.M. (1987). *Sci.*, **238,** 78–80.

Shupnik, M.A., Greenspan, S.L. & Ridgway, E.C. (1986). *J. Biol. Chem.*, **261,** 12675–12679.

Shupnik, M.A. & Ridgway, E.C. (1987). *Endocrinol*, **121,** 619–624.

Squires, R.F., Saederup, E., Crawley, J.N., Skolnick, P. & Paul, S.M. (1984). *Life Sci.*, **35,** 1439–1444.

Szabo, M. & Frohman, L.A. (1977). *Endocrinol*, **101,** 1023–1033.

Terry, L.C. (1986). *Neuroendocrinol*, **42,** 102–108.

Uribe, R.M., Charli, J.L., Rendondo, J.L., Martinez-Esclera, G., Aceves, C., Covarrubias, L. & Joseph-Bravo, T. (1991). *Annu Meeting Endocrine Soc.*, 1991:p. 134, Abstract 413.

Uribe, R.M., Redondo, J.L., Charli, J.L. & Joseph-Bravo, P. (1993). *Neuroendocrinol*, **58,** 140–145.

Yamada, M., Rogers, D. & Wilber, J.F. (1989). *Neuroendocrinol*, **50,** 560–563.

Young, W.S.I. & Zoeller, R.T. (1987). *Cell Mol Neurobiol.*, **7,** 353–366.

Zoeller, R.T., Kabeer, N. & Albers, H.E. (1990). *Endocrinol*, **127,** 2955–2962.

Zoeller, R.T. & Rudeen, P.K. (1992). *Molec Brain Res.*, **13,** 321–330.

Zoeller, R.T., Kabeer, N. & Albers, H.E. (1993). *Amer. Zool.*, **33,** 244–254.

Zoeller, R.T. & Fletcher, D.L. (1994). *Molec Brain Res.*, **24,** 185–191.