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Metrazole Induces the Sequential Activation of c-*fos*, Proenkephalin, and Tyrosine Hydroxylase Gene Expression in the Rat Adrenal Gland: Modulation by Glucocorticoid and Adrenocorticotropic Hormone

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SUMMARY

The immediate-early gene c-*fos* (a nuclear transcription factor) has been viewed as a nuclear "third messenger" or cellular "master switch." Both *in vitro* and *in vivo* studies have suggested that the proenkephalin (Penk) and tyrosine hydroxylase (TH) genes are potential targets of this immediate-early gene. We investigated the relationships between the activation of the c-*fos* gene and the activation of the Penk and TH genes in both rat hippocampus and adrenal using a commonly used model, metrazole (MTZ)-induced convulsions. The administration of MTZ produced a sequential elevation in c-*fos*, preproenkephalin (PPenk), and TH mRNAs. One hour after MTZ administration, c-*fos* mRNA was increased about 10-fold in rat hippocampus and about 5-fold in rat adrenal, without a significant change in spinal cord levels. Immunocytochemistry revealed that Fos-like immunoreactivity was greatly increased in both hippocampus and adrenal medulla at 3 hr after MTZ administration. The levels of PPenk and TH mRNAs were significantly increased (5-fold and 3-fold, respectively) in the adrenal 6 hr after MTZ treatment. The effects of MTZ on c-*fos*, PPenk, and TH mRNAs were dose dependent in both adrenal and hippocampus. In the adrenal, both the basal levels and the MTZ induction of PPenk mRNA were significantly attenuated by hypophysectomy (hypox) and were partially reinstated by adrenocorticotropic hormone (ACTH) replacement. In contrast, the basal levels of c-*fos* and TH mRNAs were not altered in hypox rat adrenal. ACTH treatment completely blocked the MTZ induction of adrenal c-*fos* mRNA and the subsequent induction of Fos-like immunoreactivity, whereas MTZ increased PPenk and TH mRNAs nearly 3-fold. Thus, in hypox rats MTZ can increase adrenal c-*fos* and TH mRNA levels without a corresponding increase in PPenk mRNA, whereas in ACTH-treated rats PPenk and TH mRNA levels in adrenal can be increased by MTZ without a preceding increase in c-*fos* mRNA. The MTZ induction of c-*fos* appears neither sufficient nor always necessary for the subsequent MTZ induction of Penk and TH gene expression. We conclude that c-*fos*, Penk, and TH genes can be differentially regulated in the adrenal of hypox rats or animals treated with ACTH, although they are co-localized in the same medullary cells.

It is well documented that transsynaptic activity can modulate Penk and TH gene expression (1–11). The Penk gene encodes the opioid peptides Met- and Leu-enkephalin, which are widely distributed in the CNS and several peripheral tissues and have been postulated to function as neurotransmitters, neuromodulators, and/or neurohormones (12). The adrenal gland is the major source of peripheral endogenous opioid peptides, which are co-stored and co-released with catecholamines in the chromaffin cells (13). These adrenal opioid peptides may be involved in some forms of stress-induced analgesia (14) and in the modulation of adrenal catecholamine secretion and actions (15). In addition, the adrenal medullary enkephalin system allows manipulation by surgery, explantation, or hormonal factors, in a manner not easily accomplished in CNS neurons expressing the Penk gene (4, 6, 8, 9).

In the rat adrenal gland, a number of studies have demonstrated that alterations in glucocorticoids (16, 17) or in transsynaptic activity (3, 7–9, 11) can either coordinately or differentially regulate Penk and TH gene expression. Increases in transsynaptic activity by different stressors have been shown to elevate both Penk and TH gene expression (3, 6). However, the mechanistic links between neurogenic stimulation and the alterations in Penk and TH gene expression are not clear, although some effort has been made to explain these links (18). Recently, it has been suggested that the IEG may be involved in the stimulus-transcription coupling cascade of late effector genes (19–22).

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ABBREVIATIONS: Penk, proenkephalin; ACTH, adrenocorticotropic hormone; AP-1, activating protein 1; Fos-LI, Fos-like immunoreactivity; hypox, hypophysectomy or hypophysectomized; IEG, immediate-early gene(s); MTZ, metrazole; PPenk, preproenkephalin; TH, tyrosine hydroxylase; FRA, *fos*-related antigen; CNS, central nervous system; bp, base pair(s).

IEG are those whose transcription is activated rapidly (usually within minutes of stimulation) and transiently at a transcriptional level by a mechanism that is independent of new protein synthesis (21). c-*fos*, a prominent member of the IEG family, encodes a nuclear transcription factor that can bind at the AP-1 site of target genes, predominately as heterodimers with other members of the AP-1 family such as Jun, to regulate late effector gene expression (19). Recently, it was reported that the c-*fos* gene was rapidly and transiently expressed in many tissues in response to various *in vitro* and *in vivo* stimuli (19, 21). For these reasons, it has been suggested that the *fos* gene might serve as a primary target of signal transduction and be capable of transforming the incoming signal into a change in gene expression. In this context the *fos* gene is viewed as a "third messenger system" or the "master switch" (19, 23, 24) in coupling extracellular signals to intracellular events. Recently, much effort has been directed at identifying the physiological target genes of the IEG (20, 22, 25–32). Both *in vitro* and *in vivo* studies have suggested that the Penk (20) and TH (30–32) genes may be potential targets of *fos*. However, the actual association between the induction of c-*fos* and Penk and TH gene expression is unclear, especially *in vivo*. In the present study, we have investigated the sequential changes in the levels of c-*fos*, PPenk, and TH mRNAs in rat adrenal and hippocampus after the administration of MTZ, a neuronal excitatory agent. By use of selected manipulations in intact rats, we have evaluated the role of glucocorticoid and ACTH in the regulation of adrenal c-*fos*, Penk, and TH gene expression.

Materials and Methods

Animals. Male Sprague-Dawley rats (175–275 g; Taconic) or male Lewis rats (225–275 g; Harlan) were used. Hypox was performed by the suppliers, and 5% dextran in water was supplied to the hypox animals. The animals were maintained on a 12/12-hr light/dark cycle.

Drug experiments. MTZ (pentylenetetrazol; Sigma Chemical Co., St. Louis, MO) was dissolved in saline and administrated subcutaneously. ACTH (H.P. Acthar gel; Armour Pharmaceutical Co., Kankakee, IL) (4 units/rat/day, subcutaneously) was started at day 5 with hypox or sham-operated rats and continued for 8 days; the last dose was given 2–3 hr before MTZ administration.

Preparation of total cellular RNA. After sacrifice by decapitation, the adrenal and hippocampus were dissected freehand. The spinal cord samples were 1.7 ± 0.09-cm (mean ± standard deviation) segments that included the L5 (lumbar) and L6, S1 (sacral) to S4, and C1 (coccygeal) portions of spinal cord. The tissues were immediately homogenized in RNA extraction buffer (11). Fifty micrograms of *Escherichia coli* tRNA carrier were added to the homogenizing buffer for tissues that weighed <50 mg. Total cellular RNA was extracted from tissues by a guanidine HCl-phenol extraction-ethanol precipitation method as described (11).

Preparation of RNA transcripts synthesized *in vitro*. ^{32}P-labeled riboprobes[1] (specific activity, 6.5×10^8 dpm/μg) for rat c-*fos*, PPenk, and TH mRNA were prepared by *in vitro* transcription as described previously (11). The plasmid for c-*fos* riboprobe was obtained from (Roche Institute of Molecular Biology) (33) and then a 970-bp *Bgl*II-*Sac*I fragment was subcloned into pSP73 (Promega); the PPenk plasmid was a 935-bp *Sac*I-*Sma*I fragment of pYSEA1 (a gift of Dr. S. Sabol, National Institutes of Health) (34), and the TH plasmid was a 384-bp *Eco*RI-*Kpn*I fragment (35) in pGEM-3 (a gift of Drs. Eveinger and Joh, Cornell University Medical College). Nonradiolabeled sense standard transcripts were also obtained from *in vitro* transcription, as described previously (11).

An 18 S riboprobe (specific activity, 1×10^7 dpm/μg) was obtained from a pSP65 derivative containing a portion of the human 18 S rRNA gene and was used for the determination of total cellular RNA levels (11).

Northern blot analysis. Northern blot analysis was carried out as described previously (16). Total cellular RNAs from rat tissues were denatured in 1 M glyoxal/50% (v/v) dimethylsulfoxide at 50° for 60 min, fractionated in a 1.6% agarose gel at room temperature with recirculation of 0.01 M sodium phosphate buffer, pH 7.1, and then transferred overnight to nitrocellulose (Schleicher & Schuell Inc.) by capillary blot procedure in the presence of 20× standard saline citrate (1× standard saline citrate is 0.15 M NaCl/0.015 M sodium citrate). The filter was hybridized in 1× TESS buffer [5 mM *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid, 5 mM EDTA, 0.15 M NaCl, 0.25% sodium dodecyl sulfate, pH 7.4] containing 1×10^6 cpm/ml c-*fos* riboprobe at 75° for 4 hr under mineral oil, washed, and exposured at −70° with an intensifying screen.

RNA quantitation. Total cellular RNA, PPenk, and TH mRNAs were determined by solution hybridization as described previously (11, 36). The levels of c-*fos* mRNA were also determined by a similar solution hybridization assay. The standard calibration curve for c-*fos* mRNA was linear from 1.95 to 250 pg of the full length c-*fos* sense transcript (i.e., c-*fos* mRNA), with a correlation coefficient of 0.997. In 10 consecutive experiments the interassay coefficient of variation averaged 7.4% and the intraassay coefficient of variation averaged 3.8% for duplicate aliquots of 30 different extracts. The specificity of the assay was confirmed by gel electrophoresis of ribonuclease-resistant products, which showed that the rat hippocampal samples and the sense transcript protected a single major labeled fragment of the expected size (970 bases in length). In contrast, an array of bands were present in samples in which the rat-derived riboprobe was hybridized to RNAs from hamster hippocampus (data not shown).

Immunocytochemistry. The immunocytochemistry was performed as described previously (37). Briefly, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 6 min. The brain and adrenal gland were then removed and further fixed by being placed in 30% sucrose in phosphate buffer at room temperature. The tissues were cut into 30-μm sections on a Vibratome. The sections were rinsed in 0.1 M Tris-buffered saline, pH 7.6, and incubated for 18–24 hr with a predetermined optimal dilution of 1/12,500 of the Fos Alu antibody (a gift from Dr. T. Curran, Roche Institute of Molecular Biology), which was obtained by using Fos peptides the sequence of amino acids 1–131. This antiserum recognizes both Fos and a number of proteins, termed FRAs, that share both structural and functional properties with Fos (38). Sites of antibody binding were visualized with the avidin-biotin-peroxidase procedure.

Statistics. The data are presented as mean ± standard error. For statistical analysis of the data on total RNA and c-*fos* and PPenk mRNA levels in Table 1, a logarithmic transformation of the raw data was performed because the raw data were skewed and the standard deviations were proportional to the means across eight treatment groups. Analysis of variance after *post hoc* Student-Newman-Keuls test was used to determine the difference among groups. A p value of <0.05 was accepted as the level of significance.

Results

MTZ sequentially increases the levels of c-*fos*, PPenk, and TH mRNAs. The administration of MTZ at a dose of 70 mg/kg, subcutaneously, produced twitches and a brief clonic convulsion in 95% of the rats, which is in agreement with a previous report (39). In the rat adrenal gland, a sequential increase in the levels of c-*fos*, PPenk, and TH mRNAs, as measured by solution hybridization, was observed (Fig. 1A). The increase in c-*fos* mRNA levels preceded the increases in PPenk and TH mRNAs, reached a peak (approximately 550% greater than saline control) at 1 hr, and returned to control within 6 hr after MTZ administration. The levels of PPenk and TH mRNAs rose 1 and 2 hr, respectively, after MTZ

[1] The terms "PPenk riboprobe," "c-*fos* riboprobe," "TH riboprobe," and "18 S rRNA riboprobe" refer to ^{32}P-labeled RNA transcripts complementary to portions of PPenk mRNA, c-*fos* mRNA, TH mRNA, and 18 S rRNA, respectively.

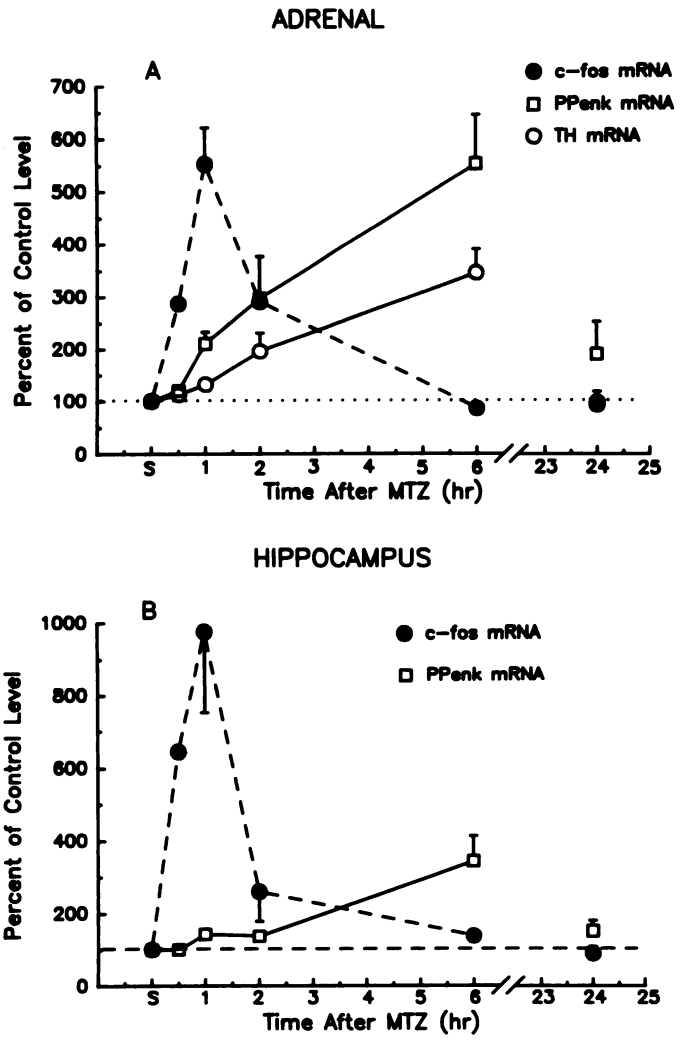

Fig. 1. A, Time course of induction of rat adrenal c-*fos*, PPenk, and TH mRNAs after MTZ treatment (70 mg/kg, subcutaneously). Total cellular RNA was obtained and the levels of each mRNA were determined by solution hybridization. The percentage of change was calculated by comparison with a parallel saline-treated group, which was given a value of 100. Each *point* represents the mean value from a group that averaged seven animals. B, Time course of induction of rat hippocampus c-*fos* and PPenk mRNAs after MTZ treatment (70 mg/kg, subcutaneously). Each *point* is the mean value from a group that averaged five animals.

administration, were induced 500% and 300%, respectively, above saline control at 6 hr, and then declined toward control at 24 hr. Northern blot analysis indicated that the sizes of c-*fos*, PPenk, and TH mRNAs were not changed with this treatment. The data for c-*fos* mRNA are shown in Fig. 2. Furthermore, Fos-LI in the adrenal was induced 3 hr after MTZ administration, as demonstrated by the use of immunocytochemistry (Fig. 3). The MTZ induction of Fos-LI occurred predominately in the adrenal medulla. Together with previous demonstrations that the Penk and TH genes are expressed only in the adrenal medulla (2, 13), these results suggest that the sequential increases in c-*fos*, PPenk, and TH mRNA levels may be co-localized in the adrenal medulla. A similar time course of increased c-*fos* mRNA, Fos-LI, and PPenk mRNA levels was observed in the rat hippocampus (Figs. 1B and 3) after MTZ treatment. However, this treatment did not alter the levels of c-*fos* (Fig. 2) and PPenk mRNAs (data not shown) in the spinal cord, as assessed by both Northern blot analysis and solution hybridization assay.

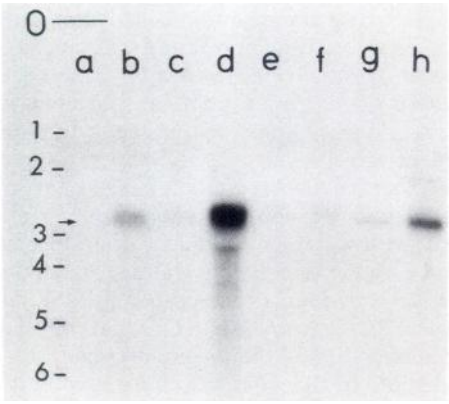

Fig. 2. Effect of MTZ on c-*fos* mRNA abundance. Northern blot analysis of total RNA extracts from rat adrenal (*lane a*, saline; *lane b*, MTZ), hippocampus (*lane c*, saline; *lane d*, MTZ), and spinal cord (*lane e*, saline; *lane f*, MTZ). Each lane contained an aliquot of 10 μg of total cellular RNA, which was obtained from tissues as indicated, denatured with glyoxal, and electrophoresed in a 1.6% agarose gel as described in Materials and Methods. *Lanes g* and *h*, 50 pg and 200 pg, respectively, of sense standards. *0*, origin of the electrophoresis. DNA size markers are as follows: *1*, 5148; *2*, 3530; *3*, 2027; *4*, 1584; *5*, 983; *6*, 564 bases. *Arrow*, position of the band (approximately 2.2 kilobases) that hybridized to the c-*fos* riboprobe.

Effects of MTZ on c-*fos*, PPenk, and TH mRNA levels are dose dependent. Fig. 4A shows the dose-response curve for MTZ induction of c-*fos* mRNA in both rat adrenal and hippocampus. The levels of c-*fos* mRNA were determined by solution hybridization 1 hr after MTZ administration. As indicated in Fig. 4A, MTZ was more potent in the induction of c-*fos* mRNA in rat adrenal than in hippocampus, with estimated EC_{50} values of 20 and 60 mg/kg, respectively. This differential dose-response relationship was also reflected in the changes in PPenk and TH mRNAs in the adrenal and PPenk mRNA in the hippocampus (Fig. 4B). In the hippocampus, neither c-*fos* (Fig. 4A) nor PPenk (Fig. 4B) mRNA levels were changed after administration of 30 mg/kg MTZ, whereas with a dose of 70 mg/kg MTZ both c-*fos* (Fig. 4A) and PPenk (Fig. 4B) mRNA levels were significantly increased. However, at a dose of either 30 mg/kg or 70 mg/kg MTZ significantly increased the c-*fos*, PPenk, and TH mRNAs in the adrenal (Fig. 4).

Penk gene expression in the adrenal is glucocorticoid dependent. It was previously reported that glucocorticoid regulates Penk gene expression in the adrenal (10, 16, 17). In the present study, we investigated the effect of hypox on the levels of c-*fos*, PPenk, and TH mRNA levels and on the MTZ induction of these mRNA levels. As shown in Table 1, the steady state levels of adrenal PPenk mRNA in saline-treated rats were significantly decreased after hypox, compared with sham surgery, whereas the levels of c-*fos* and TH mRNA were not significantly altered. In saline-treated rats, the steady state levels of PPenk mRNA in the hypox adrenal were restored to the levels in sham-operated animals by ACTH treatment, a reliable agent for the replacement of glucocorticoids in hypox animals (10). Total cellular RNA per gland in the hypox adrenals was much lower, compared with sham-operated adrenal, and was completely restored by ACTH treatment (Table 1).

MTZ induction of adrenal c-*fos*, Penk, and TH gene expression is differentially affected by ACTH and hypox treatments. After hypox, a procedure that depletes serum and adrenal glucocorticoids (10, 16), both the absolute amount of the increase and the percentage of change in the MTZ induction of PPenk mRNA were significantly attenuated (Table 1; Fig.

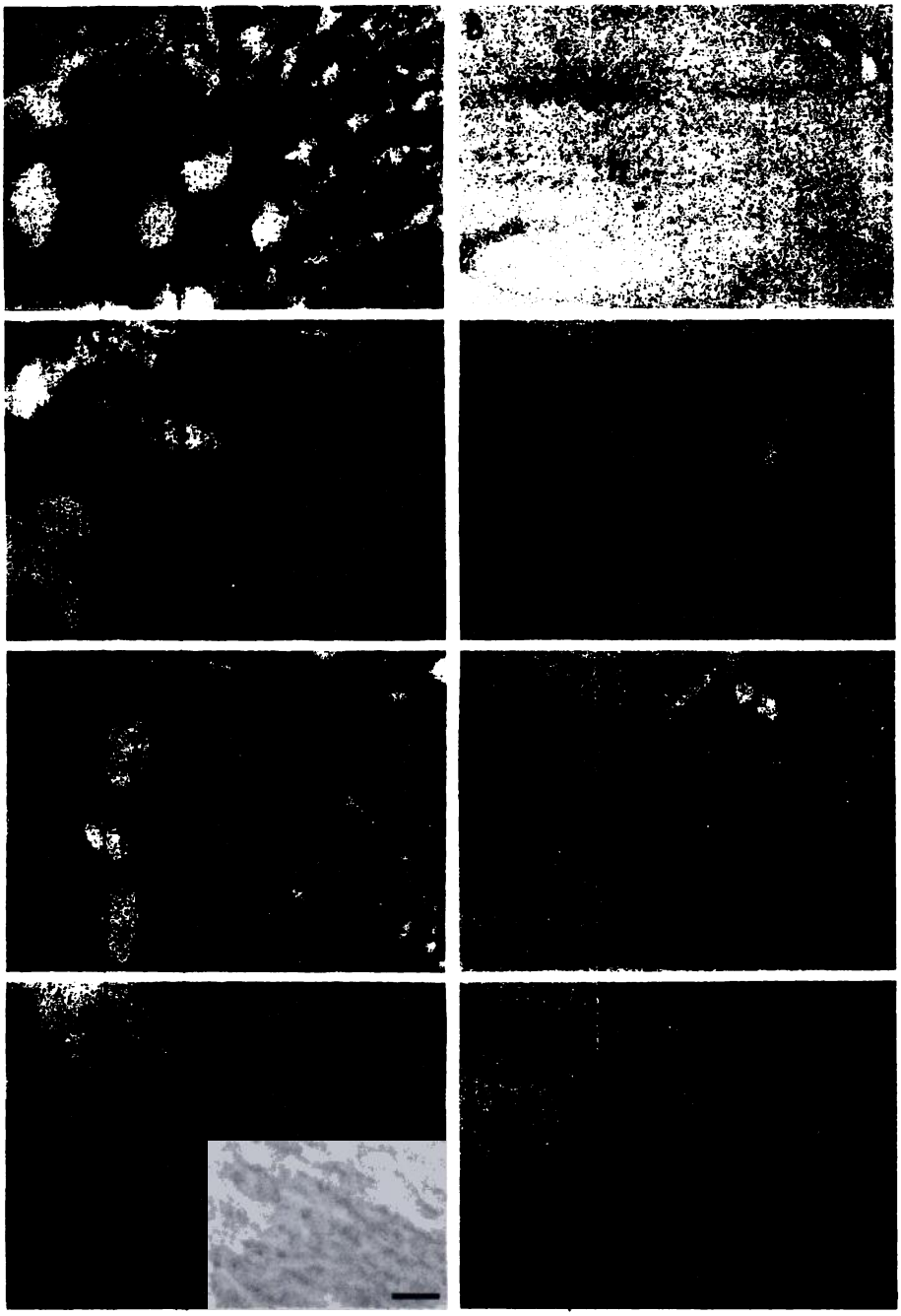

Fig. 3. Immunocytochemical analysis of the MTZ induction of Fos-LI and its modulation by chronic ACTH treatment in rat adrenal (*left*) and hippocampus (*right*). Fos-LI was determined 3 hr after saline (A and B) or MTZ (70 mg/kg, subcutaneously) (C and D) treatment. After 8 days of ACTH treatment (4 units/day, subcutaneously), tissue was obtained 3 hr after saline (E and F) or MTZ (G and H) treatment. *Arrowheads* in C, Fos-LI in adrenal medulla. *Scale bar* in G (for the *left column*), 50 μm; *scale bar* in H (for the *right column*), 100 μm. *ac*, adrenal cortex; *am*, adrenal medulla; *gcl*, granule cell layer; *h*, hilus.

5B). In contrast, the ability of MTZ to increase c-*fos* and TH mRNAs (expressed as a percentage of control) was not significantly altered (Fig. 5, A and C). Based on the variances obtained from the c-*fos* mRNA values of the sham-MTZ group (Fig. 5A), a power calculation indicates that more than doubling the sample size would not allow detection of a group difference of 25% with a 90% power ($\alpha = 0.05$, $\beta = 0.10$). However, ACTH treatment blocked the MTZ induction of c-*fos* mRNA in sham-operated adrenal and attenuated this effect in hypox adrenal (Fig. 5A), whereas both PPenk and TH mRNAs were increased nearly 3-fold (Fig. 5, B and C). In sham-operated rat adrenal, ACTH treatment did not alter the absolute amount of PPenk mRNA induced by MTZ (Table 1), whereas the percentage of induction of PPenk mRNA by MTZ was attenuated from 5-fold to nearly 3-fold (Fig. 5B). This apparent attenuation resulted from the contribution of ACTH alone, which slightly but significantly increased the PPenk mRNA levels, so that the calculated percentage of change after MTZ administration was less than after saline treatment (compare Table 1 and Fig. 5B). In hypox rat adrenals, ACTH treatment partially restored the MTZ effect on PPenk mRNA, when expressed as either absolute amount or percentage of change above that seen in hypox rats given MTZ (Table 1; Fig. 5B).

To further explore the unexpected effect of ACTH on the MTZ induction of c-*fos* mRNA, the levels of Fos-LI were assessed by immunocytochemistry. As shown in Fig. 3, the induction of Fos-LI by MTZ was also completely blocked in the adrenal by ACTH treatment, without a significant change in the MTZ induction of Fos-LI in the granule cell layer of the hippocampus. This effect of ACTH on c-*fos* mRNA was also

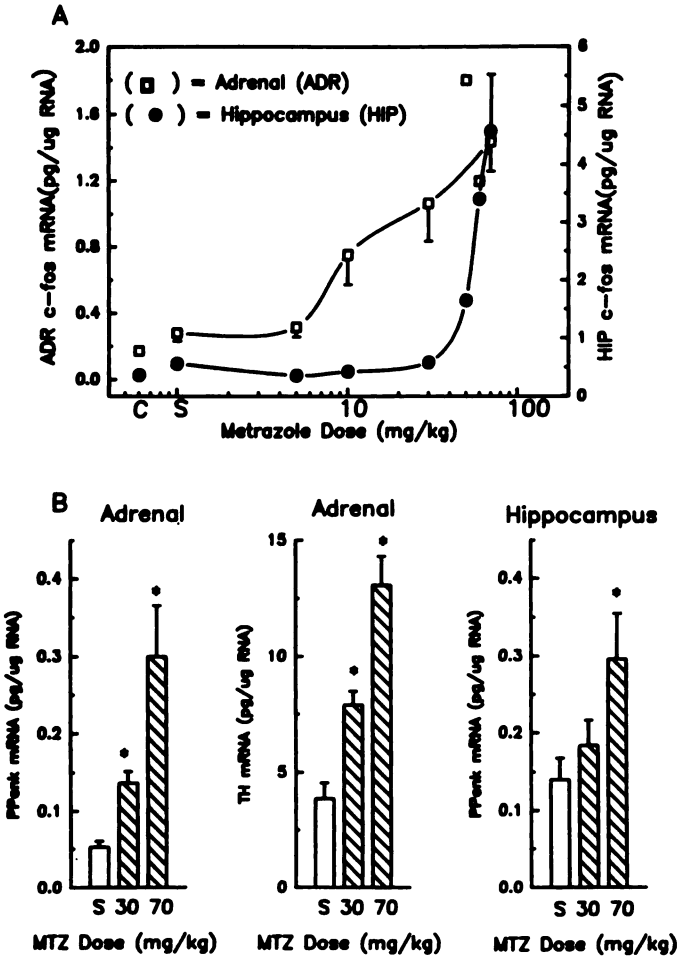

Fig. 4. A, Dose-response curves for MTZ induction of c-*fos* mRNA in rat adrenal and hippocampus. Total cellular RNA was obtained 1 hr after MTZ or saline administration and the levels of c-*fos* mRNA and total cellular RNA were determined by solution hybridization. *ADR*, adrenal; *HIP*, hippocampus; *C*, untreated control; *S*, saline-treated animal. Number of animals per group averaged seven, except for the untreated control group and the groups receiving 50 and 60 mg/kg doses of MTZ, for which values were obtained from a pooled sample derived from three animals. B, Differential induction of adrenal and hippocampal mRNAs as a function of the dose of MTZ. The levels of PPenk and TH mRNAs were determined 6 hr after MTZ administration (30 or 70 mg/kg, subcutaneously). The number of animals per group was five or six. *, $p < 0.05$, compared with saline-treated (*S*) group (Student-Newman-Keuls test).

seen after a single dose. ACTH significantly reduced the MTZ induction of c-*fos* mRNA in the adrenal when ACTH pretreatment occurred 2 hr before MTZ administration (Fig. 6A). The ability of a single dose of ACTH to block MTZ induction of adrenal c-*fos* did not occur in the hippocampus (Fig. 6B).

Discussion

MTZ-induced seizures have been used previously as a model for the study of stimulus-transcription coupling in the CNS (19, 20) and as a model for the study of stress (40). Anticonvulsants block both the MTZ-induced convulsions and the induction of c-*fos* in the CNS (41) and in the adrenal.[2] Peripheral ganglionic blockade using a combination of the cholinergic antagonists chlorisondamine and methylatropine does not prevent the MTZ-induced convulsions but blocks adrenal c-*fos* induction.[2] Thus, the effects of MTZ on the rat adrenal appear to be mainly mediated by an increase in splanchnic nerve transsynaptic activity. In the present study, we have used the MTZ-induced convulsion model to study the regulation of c-*fos*, Penk, and TH gene expression and the relationship between expression of these genes in the rat adrenal and hippocampus. The present results, using a solution hybridization procedure for the quantitation of mRNA levels, extend the previous observations (20) obtained in mice, showing that MTZ sequentially increases the levels of hippocampal c-*fos* and PPenk mRNAs, to rats. Moreover, our results indicate that MTZ treatment sequentially elevates the levels of c-*fos*, PPenk, and TH mRNAs in the rat adrenal as well as hippocampus. In addition, we have demonstrated the MTZ dose-response curves, the time course profiles for the alteration of these mRNAs, and the modification of this inductive effect by hypox or ACTH treatment. The results obtained indicate circumstances in which the MTZ induction of c-*fos* and, subsequently, Penk and TH gene expression can be dissociated.

The MTZ-induced increase in the steady state levels of c-*fos*, PPenk, and TH mRNAs could be due to either transcriptional or nontranscriptional (e.g., stabilization) events, and our experiments do not directly address this issue. However, there is evidence to indicate that these genes are regulated at the transcriptional level by various stimuli (21, 42, 43). The transient elevation of c-*fos* mRNA after MTZ treatment suggests that the degradation of c-*fos* mRNA was not significantly altered. Furthermore, because the apparent half-life of rat adrenal PPenk mRNA is about 10 hr (11), even the complete inhibition of PPenk mRNA degradation by MTZ should result in less than doubling of the levels of this mRNA at 6 hr after MTZ administration, rather than the 5-fold increase we observed (Fig. 1A). Taken together, these data support the activation by MTZ of c-*fos* and Penk gene transcription as the primary event, although a definitive conclusion requires direct information on transcription rates.

To date there is a great deal of evidence to support a sequential model, wherein c-*fos* functions as a third messenger to activate the Penk and TH genes by binding at an AP-1 site (19–21). Putative AP-1 sites have been identified in the promoter regions of Penk and TH genes (20, 32, 44–47). By use of a transactivation assay, Sonnenberg *et al.* (20) reported that the Penk gene was activated after either c-*fos* or c-*jun* alone or c-*fos* and c-*jun* together were co-transfected with the Penk gene in F9 cells. Stachowiak (32) reported the activation by c-*fos* and c-*jun* of TH gene expression with a similar assay in the SH-5YSY cell line (a neural crest-derived cell line), and Yoon and Chikaraishi (45) have shown by use of a transactivation assay that the AP-1 motif of the rat TH gene is required for the basal and tissue-specific expression of the TH gene. Recently, La Gamma *et al.* (46) and Koistinaho (48) showed by use of gel shift assays that cholinergic agonist treatments increased the levels of AP-1 proteins and the AP-1 (Fos/Jun) proteins bound in the Penk gene in rat adrenal medulla, and the same treatments also increased the levels of PPenk mRNA in this tissue. Our present sequential MTZ induction of c-*fos*, Penk, and TH gene expression in rat hippocampus and adrenal is consistent with the previous report by Sonnenberg *et al.* (20) that showed a sequential MTZ induction of c-*fos* and Penk gene expression in the mouse hippocampus and that by Icard-Liepkalns *et al.* (30) that showed a sequential reserpine activation of c-*fos* and TH gene expression in the rat adrenal. Furthermore, the present results (Fig. 3) and previous experiments (2, 20, 30, 31) have demonstrated that the expression of

[2] Y-S. Zhu and C. E. Inturrisi. *Mol. Brain Res.*, in press (1993).

TABLE 1

Effects of hypox and ACTH treatment on the basal and MTZ-induced levels of c-*fos*, PPenk, and TH mRNAs in the rat adrenal

The numbers in parentheses indicate the sample size. A logarithmic transformation of raw data and statistical analysis by one-way analysis of variance and *post hoc* Student-Newman-Keuls multiple range test on eight treatment combinations were performed as described in Materials and Methods.

Treatment	Sham-operated adrenal				Hypox adrenal			
	Total RNA	mRNAs			Total RNA	mRNAs		
		c-*fos*	PPenk	TH		c-*fos*	PPenk	TH
	μg/gland	pg/gland			μg/gland	pg/gland		
Saline	148 ± 12 (5)	67 ± 8 (8)	23 ± 1 (5)	361 ± 47 (8)	30 ± 3 (5)[a]	51 ± 3 (9)	11 ± 1 (2)[a,b]	279 ± 40 (9)
MTZ	108 ± 16 (6)	301 ± 36 (11)[c]	113 ± 11 (6)[c]	828 ± 65 (6)[c]	21 ± 2 (5)[a,c]	179 ± 14 (6)[a,c]	25 ± 3 (5)[a,c]	519 ± 28 (5)[a,c]
ACTH + saline	241 ± 18 (6)[c]	84 ± 6 (5)	35 ± 3 (6)[c]	313 ± 21 (6)	161 ± 18 (5)[a,c]	83 ± 10 (5)	18 ± 2 (2)[a–c]	314 ± 29 (5)
ACTH + MTZ	193 ± 22 (5)[d]	62 ± 7 (15)[d]	97 ± 17 (5)[e]	905 ± 58 (5)[e]	128 ± 8 (6)[a,d]	140 ± 13 (10)[e]	64 ± 5 (6)[a,d,e]	708 ± 21 (6)[a,d,e]

[a] $p < 0.05$, compared with the same treatment group in sham adrenal.
[b] Mean values ± standard error from two pooled samples; each pooled sample contains five animals.
[c] $p < 0.05$, compared with parallel saline-treated group.
[d] $p < 0.05$, compared with MTZ group.
[e] $p < 0.05$, compared with ACTH plus saline group.

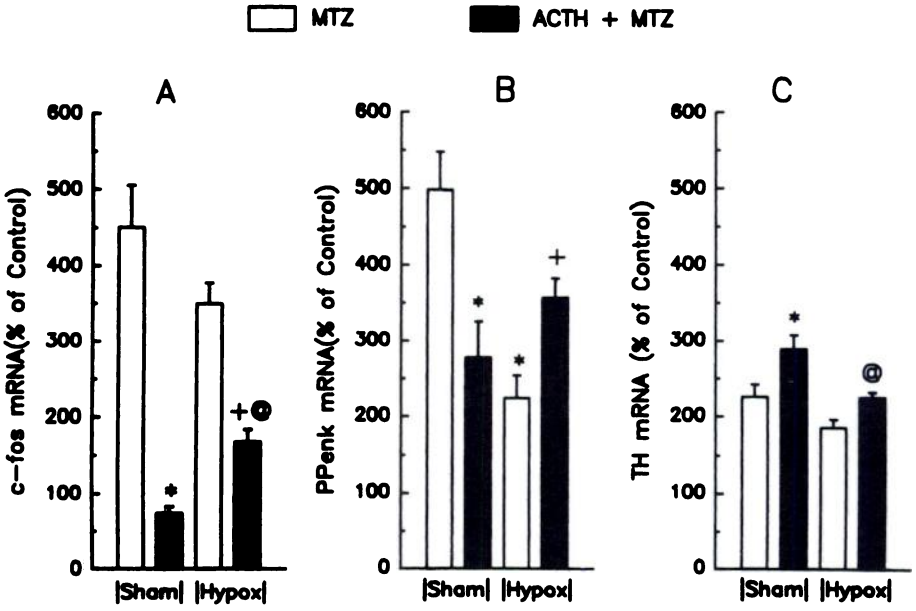

Fig. 5. Effect of hypox and ACTH treatment on MTZ induction of c-*fos* (A), PPenk (B), and TH (C) mRNAs in rat adrenal. Five days after hypox, treatment with ACTH (4 units/rat/day) was initiated. Thirteen days after hypox or sham operation, the rats were challenged with MTZ (70 mg/kg, subcutaneously) or saline. Adrenal levels of c-*fos* mRNA were determined 1 hr after MTZ treatment and the PPenk and TH mRNA levels were determined 6 hr after MTZ treatment. The percentage was calculated by comparison with a parallel saline-treated group (taken as 100%). The number of animals per group is given in Table 1. For statistical analysis, a logarithmic transformation of the raw data was performed as described in Materials and Methods. *, $p < 0.05$, compared with MTZ group for sham-operated adrenal; +, $p < 0.05$, compared with MTZ group for hypox adrenal; @, $p < 0.05$, compared with the same treatment for sham-operated adrenal (Student-Newman-Keuls test).

adrenal c-*fos*, Penk, and TH genes is co-localized in the medullary cells, whereas the expression of c-*fos* and Penk genes is co-localized in the hippocampus. In addition, MTZ induction of the levels of c-*fos*, PPenk, and TH mRNAs was a function of the dose of MTZ (Fig. 4). Taken together, these results support the hypothesis that *fos* may function *in vivo* as a transcription factor to activate Penk and TH genes. However, the question addressed by our study was whether there are *in vivo* conditions where changes in c-*fos* mRNA can be dissociated from the changes in PPenk and/or TH mRNAs in adrenal or hippocampus.

By the use of physiological and pharmacological manipulations in intact animals, we were able to obtain results to support the hypothesis that the MTZ induction of adrenal c-*fos*, Penk, and TH gene expression can be dissociated. The most dramatic example is the demonstration that ACTH treatment completely blocked the MTZ induction of c-*fos* mRNA and Fos-LI, whereas the MTZ induction of PPenk and TH mRNAs was increased nearly 3-fold in the rat adrenal (Figs. 3 and 5; Table 1). In hypox animals, MTZ induction of PPenk mRNA was reduced 3-fold, whereas c-*fos* and TH mRNAs were not significantly altered (Fig. 5). Thus, in the adrenal PPenk and TH mRNA levels can increase in ACTH-treated rats after MTZ treatment without a preceding increase in c-*fos* mRNA. Because our antiserum also recognizes FRAs, these results suggest that ACTH also blocks the MTZ induction of these proteins, which are part of the AP-1 nucleoprotein complex (20, 38). However, we did not measure the induction of c-*jun* in these studies. c-*jun* can form homodimers, which can transactivate Penk constructs *in vitro* (20). Therefore, our observations relate to c-*fos* and FRAs but do not exclude c-*jun* and other *jun*-related AP-1 factors as contributing to the MTZ induction of Penk mRNA levels.

In hypox rats, MTZ can increase c-*fos* and TH mRNA levels without a corresponding increase in PPenk mRNA. Furthermore, we have observed that trifluoperazine inhibits the MTZ induction of c-*fos* mRNA and potentiates the MTZ induction of PPenk mRNA by MTZ in the rat hippocampus and adrenal.[2] Additionally, Yin and co-workers (49, 50) showed in C6 glioma cells that glucocorticoids decreased the c-*fos* mRNA levels and increased the PPenk mRNA levels, whereas endothelin increased the c-*fos* and c-*jun* mRNA levels and decreased the PPenk mRNA levels.

The mechanism of ACTH blockade of MTZ induction of c-

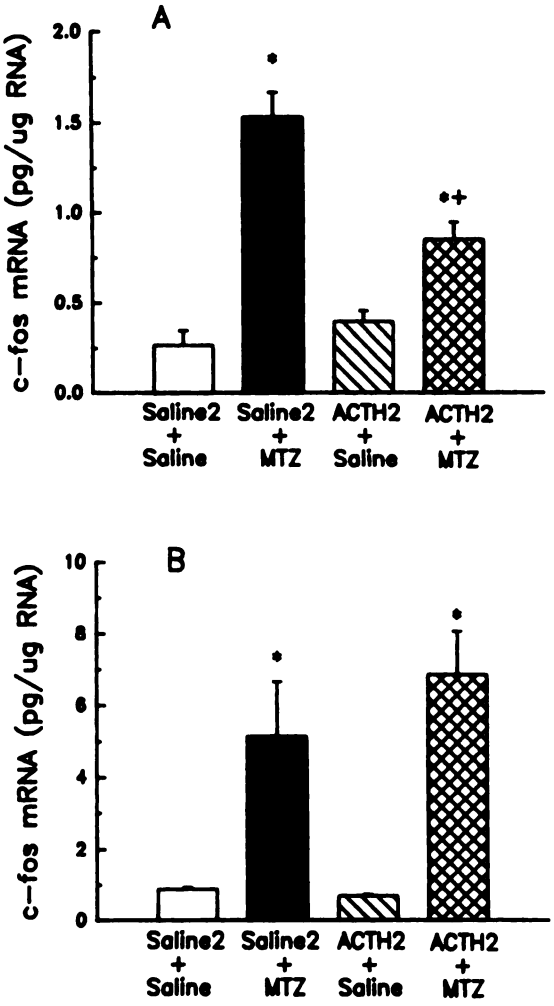

Fig. 6. Effect of a single dose of ACTH given 2 hr before MTZ on the MTZ induction of c-*fos* mRNA in rat adrenal (A) and hippocampus (B). The levels of c-*fos* mRNA were determined in animals that had been pretreated with saline (*Saline2*) or ACTH (4 units/rat, subcutaneously) (*ACTH2*) 2 hr before a challenge with saline or MTZ (70 mg/kg, subcutaneously). The tissues were collected 1 hr after the challenge. The number of animals was four or five per group. *, $p < 0.05$, compared with corresponding saline-treated control group; +, $p < 0.05$, compared with corresponding saline- plus MTZ-treated group (Student-Newman-Keuls test).

fos mRNA and Fos-LI in rat adrenal is unknown. ACTH did not alter the convulsive potency of MTZ. Interestingly, concurrent measurements showed that MTZ induction of c-*fos* in the hippocampus was not altered by ACTH treatment. Thus, this effect of ACTH may be tissue specific. It has been suggested that c-*fos* mRNA levels are subject to autoinhibition by Fos protein (51, 52). However, because ACTH treatment blocked both adrenal c-*fos* mRNA and Fos-LI (Figs. 3 and 6; Table 1), this mechanism appears unlikely. A single dose of ACTH can increase the plasma concentration of adrenocortical steroids, and chronic ACTH treatment produces morphological changes in the adrenal cortex that favor the biosynthesis of adrenocortical hormones in rats (53, 54). The relatively high concentration of adrenocortical steroids and their metabolites may block the MTZ induction of c-*fos* gene expression by some as yet undefined process. Because the MTZ induction of c-*fos* mRNA is not significantly reduced in hypox rats (Fig. 5A), neither the pituitary nor circulating levels of ACTH and glucocorticoids are required for this effect. In hypox rats given ACTH, the MTZ induction of c-*fos* mRNA is attenuated but present (Table 1; Fig. 5A). If we assume that ACTH restored circulating glucocorticoids in hypox rats, then by exclusion the pituitary is not required for the MTZ induction of c-*fos*. However, in sham-operated rats with an intact pituitary, ACTH blocked MTZ induction of c-*fos* mRNA (Figs. 5A and 6A) and Fos-LI (Fig. 3). Thus, it would appear that ACTH administration induces the pituitary to release a substance that blocks MTZ induction of c-*fos* in the adrenal by an unknown mechanism. Alternately, elevated ACTH levels may interact with a circulating pituitary factor at the level of the adrenal to prevent the MTZ-induced stimulus-signal transduction event that is manifest as an increase in c-*fos* mRNA.

It is established that in rat adrenal both the basal level and the denervation-induced increase of Penk gene expression are glucocorticoid dependent (10, 16, 17, 55). To these observations we add the requirement for glucocorticoid for MTZ induction of adrenal PPenk mRNA (Fig. 5B). These effects are assumed to result from the interaction of the steroid-glucocorticoid receptor complex with glucocorticoid response elements (56). Putative glucocorticoid response elements have been identified by sequence scanning both in the 5′ upstream region and in intron A of the rat Penk gene (16, 17, 47, 57). In contrast, neither the basal levels nor the MTZ-induced levels of adrenal c-*fos* and TH gene expression appear to be dependent on glucocorticoids. These results suggest that, although the genes are co-localized in the same medullary cells, the expression of adrenal c-*fos*, Penk, and TH genes is differentially regulated in hypox rats and in animals treated with ACTH.

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