Modulation of cholinergic locus expression by glucocorticoids and retinoic acid is cell-type specific

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Abstract Modulation of mRNA expression of choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT) by the glucocorticoid dexamethasone and by retinoic acid was examined in two neuronal cell lines: basal forebrain-derived SN56 and pheochromocytoma PC12. Dexamethasone up-regulated ChAT and VAChT in SN56 cells, while it had inhibitory effects on these genes in PC12 cells. Retinoic acid stimulated the cholinergic markers in both cell types, but in SN56 cells its effect was partially additive with that of dexamethasone, whereas it was much smaller and abrogated by dexamethasone in PC12 cells. Acetylcholine content correlated with these mRNA changes. The presence of a glucocorticoid response element consensus sequence in the VAChT/ChAT gene locus suggests direct transcriptional regulation by glucocorticoids.

Key words: Choline acetyltransferase; Vesicular acetylcholine transporter; Glucocorticoid; Retinoic acid; PC12 cell; SN56 cell

1. Introduction

Cholinergic neurotransmission is essential for many diverse functions of the nervous system, including the control of skeletal muscles, autonomic activity, the central control of movement, and learning and memory. Normal functioning of cholinergic neurons depends upon the expression of two proteins: choline acetyltransferase (ChAT) which synthesizes acetylcholine (ACh), and the vesicular ACh transporter (VAChT) which transports ACh into secretory vesicles. Of those two cholinergic markers, ChAT has been studied more extensively, and most information on the regulation of the cholinergic phenotype is derived from studies of ChAT activity and expression. ChAT expression is regulated during development and in the adult nervous system by several trophic factors (the best characterized being nerve growth factor), as well as by neurotransmitters, vitamins and hormones, including thyroid hormones [1–3], estradiol [4] and glucocorticoids [5]. Hormones belonging to the latter class are of particular interest because they are important for brain development as well as in behavioral responses to stress. Experiments in vivo or in primary cultures of brain neurons indicate that the regulation of the cholinergic phenotype by steroids is complex, involving both regulation of neuronal maturation [6,7], as well as modulation of ChAT itself. In most in vivo systems, exposure to glucocorticoids reduces ChAT activity [5,8,9]. However, other studies indicate that the response strongly depends on the administered dose, the stage of brain development and the brain region [10,11]. It has been reported that treatment with glucocorticoids significantly reduces ChAT activity and ACh content in cultured rat pheochromocytoma PC12 cells, which are derived from rat adrenal medulla and exhibit many properties of sympathetic neurons [12]. The non-neuronal production and release of ACh by pituitary cells, on the other hand, appears to be slightly up-regulated by glucocorticoids [13].

In contrast to ChAT, little is know about the regulation of VAChT expression. However, recent studies have shown that the region of the mammalian genome encoding ChAT and VAChT exhibits an unusual organization: the intronless VAChT open reading frame is entirely contained within the first intron of the ChAT gene [14,15]. Differential promoter use and alternative splicing result in a complex pattern of expression of this 'cholinergic locus' leading to the formation of many mRNA species that differ in the 5′ end and contain either the ChAT or VAChT coding region. The 5′-most exon is shared by some ChAT and VAChT mRNAs, but the most abundant classes of transcripts are initiated at separate ChAT- and VAChT-specific promoters. This genomic organization suggests that some regulatory elements could be shared between the two genes; however, the mechanisms regulating ChAT transcription are only partially understood (reviewed by Wu and Hersh [16]), and the regulation of VAChT expression is just beginning to be explored [17–19].

As a first step to investigate the molecular mechanisms of ChAT/VAChT regulation by steroid hormones, we examined ChAT and VAChT mRNA and ACh content in two neuronal cell lines treated with the synthetic glucocorticoid dexamethasone and with retinoic acid. Here we present evidence that cholinergic properties of these cells are regulated by glucocorticoids at the mRNA level and that the regulation by both glucocorticoids and retinoids is cell-type specific.

2. Materials and methods

2.1. Cell culture

Mouse septal neuron×neuroblastoma hybrids SN56 cells (a gift of Dr. Bruce Wainer) were created by fusing N18TG2 neuroblastoma cells with murine (strain C57BL/6) neurons from postnatal day 21 septa [20,21]. SN56 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin. Rat pheochromocytoma PC12 cells were grown in DMEM containing 5% FBS, 10% horse serum, and 50 μg/ml gen-

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Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GT, guanidinium isothiocyanate; RARα, retinoic acid receptor alpha; SSC, saline-sodium citrate; t-RA, all-*trans*-retinoic acid; VAChT, vesicular acetylcholine transporter

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tamicin. When the cells were grown in the presence of test compounds, i.e. glucocorticoids or retinoids, the medium was changed every 24 h.

2.2. Northern analysis

2.2.1. Extraction of RNA. Total RNA was extracted from SN56 cells using the acid guanidinium isothiocyanate (GT)-phenol-chloroform method [22,23]. Prior to lysis, cells were washed twice with phosphate-buffered saline and then dissolved in 4 M GT solution. Following lysis and phenol/chloroform extraction, RNA was precipitated with isopropanol, and the pellet was washed with 70% ethanol, dissolved in RNase-free water and stored at −80°C.

2.2.2. Northern blotting. RNA samples equalized for ribosomal RNA content (20 μg/lane) were size-fractionated on a 1% agarose gel containing 6% formaldehyde and transferred to a Hybond-N+ nylon membrane (Amersham). Hybridization was carried out in the Rapid-hyb solution (Amersham), according to manufacturer's instructions. DNA probes were labeled with [α-^{32}P]dCTP (New England Nuclear) to a specific activity of $1–2\times10^9$ cpm/μg DNA using Rediprime labeling kit (Amersham). Final washes were in 0.2×saline–sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 60°C for ChAT and at 65°C for VAChT. The blots were visualized and band intensities were quantified with a PhosphorImager 400E using ImageQuant NT software (Molecular Dynamics).

2.2.3. Probes. For the ChAT probe, we used a 1.32-kb cDNA fragment amplified by PCR from mouse brain cDNA (Clontech) with the following oligonucleotide primers:

Forward: 5′-CGGGATCCTGCCTCATCTCTGGTGT-3′
Reverse: 5′-ACGGGTCCATAACAGCAGAACA-3′

A 0.8-kb mouse VAChT cDNA probe located within the VAChT coding sequence was described previously [18].

2.3. Extraction and measurement of ACh

Cells were grown to subconfluence in 35-mm culture dishes. After the desired treatment, the medium was removed and the cells were incubated for 1 h at 37°C in a physiological salt solution (pH 7.4) supplemented with 5 μM choline and 15 μM neostigmine and consisting of the following (in mM): NaCl, 135; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 0.75; glucose, 10; HEPES, 10. The cells were washed once with ice-cold, choline-free physiological salt solution supplemented with 15 μM neostigmine, methanol was added, the cells were scraped into the methanol, and the suspension was transferred to a polypropylene tube. A solution of 15% 1 M formic acid and 85% acetone was added, the tubes were vortexed, and the extracts centrifuged to precipitate the protein. Both the protein pellets and the supernatant fluids, containing ACh, were dried under a vacuum. The ACh pellet was resuspended in HPLC mobile phase (28 mM sodium phosphate, pH 8.5, supplemented with Kathon CG Reagent), sonicated, and filtered (0.2 μm Nylon-66, Rainin Instrument). The content of ACh was determined by HPLC with an enzymatic reactor containing acetylcholinesterase and choline oxidase and an electrochemical detector using a commercial kit (Bioanalytical Systems) based on the method of Potter et al. [24]. Protein was determined by the method of Smith et al. [25].

3. Results and discussion

We measured the effect of a glucocorticoid receptor agonist, dexamethasone, on intracellular ACh levels and ChAT and VAChT mRNA in two neuronal cell lines: rat pheochromocytoma PC12 cells and the mouse septal cell line SN56. Consistent with a previous report [12], a 2-day treatment with 1 μM dexamethasone reduced ACh content in PC12 cells by 50% (Fig. 1A). All-*trans*-retinoic acid (t-RA, 1 μM) caused only a moderate (24%) increase in ACh content in these cells. This effect of t-RA was completely abrogated by dexamethasone, as shown by the combined treatment. In contrast, in septal SN56 cells, dexamethasone treatment nearly doubled intracellular ACh content (Fig. 1B). In agreement with our previous findings [26], t-RA tripled intracellular ACh concentration in these cells. Furthermore, the effect of dexamethasone in SN56 cells was partially additive with that of t-RA. Dexamethasone increased the ACh content of SN56 cells in a time- and concentration-dependent, saturable fashion (Fig. 2). The maximal effect of dexamethasone was observed after a 72 h treatment and was sustained until the last time point examined, 96 h (Fig. 2A). The saturating concentration of the hormone was between 10 nM and 100 nM (Fig. 2B). The concentration that resulted in a half-maximal effect (EC_{50}) was 6 nM.

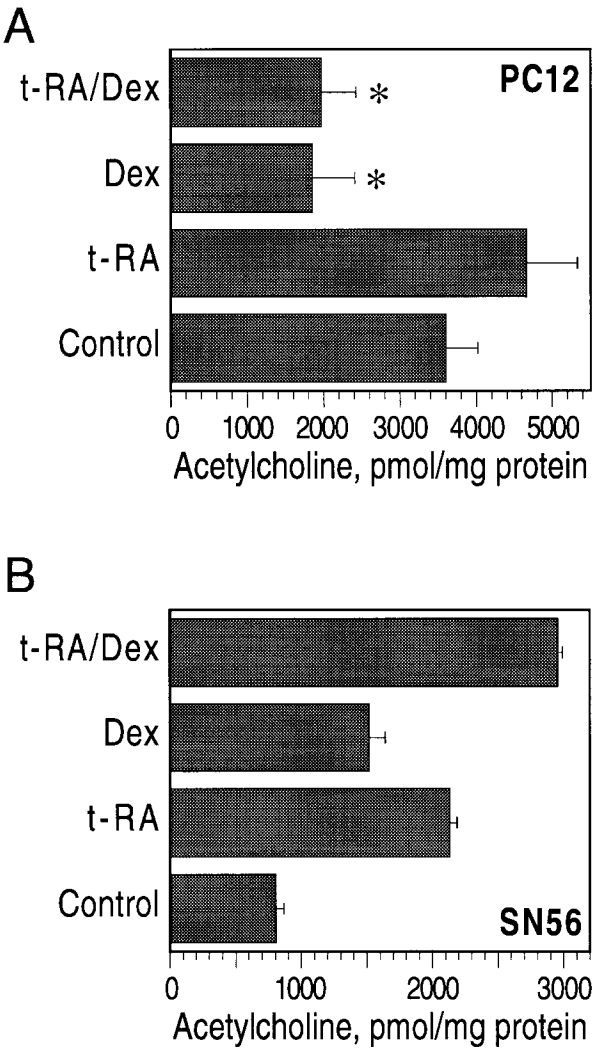

Fig. 1. Differential effects of dexamethasone on ACh content in (A) PC12 and (B) SN56 cells. Cells were treated for 48 h in the presence or absence of 1 μM retinoic acid, 1 μM dexamethasone, or both. Intracellular ACh was measured in cell extracts as described in Section 2. The results are presented as means ± SD. All comparisons between groups were statistically significant ($P<0.05$; Tukey test) except for the two groups indicated by *.

Northern blotting with cholinergic locus-specific probes demonstrated that the dexamethasone effect occurs at the mRNA level in both cell types (Figs. 3 and 4). The ChAT- and VAChT-specific probes were derived from the respective coding regions, and they recognize all known mRNA variants; however, the size differences between the variants are too small to be detected under our experimental conditions, and therefore ChAT and VAChT transcripts appear as single

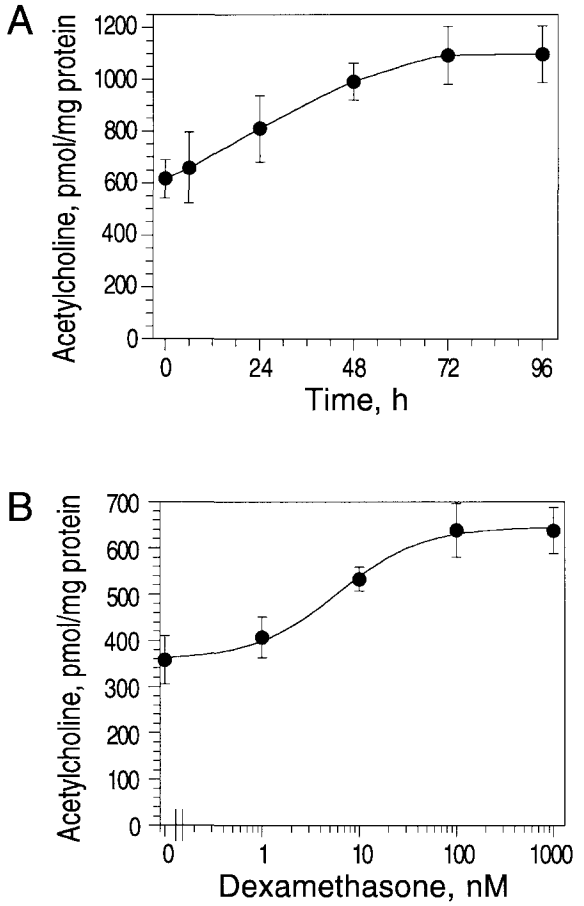

Fig. 2. Dexamethasone causes time- and concentration-dependent increases in intracellular ACh levels in SN56 cells. (A) SN56 cells were treated for the time periods indicated in the presence of 1 μM dexamethasone. ACh was measured in cell extracts as described in Section 2. (B) Cells were grown for 72 h in the presence of various concentrations of dexamethasone and ACh was measured as in (A).

bands. Analysis of band intensities from Northern blots showed that, in PC12 cells, dexamethasone reduced ChAT and VAChT mRNA levels by 50% and t-RA increased them by 30–40%. In dexamethasone-treated PC12 cells, t-RA had almost no effect on ChAT and VAChT mRNA (Fig. 3). In SN56 cells, the ChAT and VAChT mRNA levels were increased by dexamethasone by 80%, and 120%, respectively (Fig. 4). The effect of dexamethasone on ChAT/VAChT mRNA was additive with that of t-RA. These data are in agreement with the effects of these compounds on ACh content in both cell lines. The observation that, in both cell lines, the regulation of VAChT mRNA expression by dexamethasone parallels that of ChAT mRNA further supports previous findings that both genes are coordinately up-regulated by various extracellular stimuli [17–19]. The result of the glucocorticoid treatment of PC12 cells, however, provides the first demonstration of a coordinated reduction in mRNA levels of both genes.

Taken together, the data demonstrate that the regulation of expression of the cholinergic locus by dexamethasone and t-RA differs in SN56 and PC12 cells. Dexamethasone has opposite effects on ChAT/VAChT expression and ACh production in SN56 cells (up-regulation) and in PC12 cells (down-regulation). Retinoic acid has only a moderate effect on those markers in PC12 cells, and a substantial effect in SN56 cells. The molecular basis of these differences remains to be investigated. We have shown previously that the regulation of the cholinergic properties of SN56 cells by retinoids is mediated by the alpha subtype of the retinoic acid receptor (RARα) [18,26]. The simplest interpretation of the retinoic acid treatment data presented here is that the regulation of the cholinergic locus in PC12 cells also involves RARα, and the difference in the magnitude of the effect results from different availability of that receptor, or its co-activator, in the two cell lines. Steroid hormones also exert their biological actions by interacting with nuclear receptors which are transcription factors. The glucocorticoid receptor (GR) homodimer binds to the glucocorticoid response elements (GRE) in the promoters of responsive genes. Recently, Cervini et al. [27] cloned and sequenced the 5′ non-coding region of the rat VAChT gene and localized two VAChT-specific promoters, each comprising several transcription start sites. In that region there is a half-site of the consensus GRE present at 868 bp upstream from the VAChT translation initiation codon. It is not known whether this element is biologically active, and whether it can regulate both ChAT and VAChT transcription. In addition to their direct actions as transcription factors, both RAR and GR can participate in complex cross-talk between different regulatory pathways. For example, it has been shown that both RAR and GR interact strongly with the proteins (e.g. CBP/p300), that are required for activation of both CREB and AP-1 [28,29]. Additionally, GR has been shown to modulate gene expression by interfering with NF-κB signaling pathways [30,31], modulation of the expression of transcription factors including RAR [32] and the proteins of the C/EBP family [33], and direct binding to transcription factors (e.g. AP1, NF-κB) [34]. There are indeed indications that AP-1- and NF-κB-mediated transcription may contribute to the regulation of ChAT/VAChT by glucocorticoids. In vivo experiments involving both adrenalectomy and administration of dexamethasone showed that glucocorticoids interfere with both AP1 and NF-κB binding activity in rat brain [35,36]. There are putative AP1 and NF-κB sites in the promoter regions of both rodent and human cholinergic gene locus. It was shown in reporter gene assays that while dexamethasone did not enhance basal transcription from the human proximal ChAT promoter, it had an agonistic effect on Jun/Fos-induced promoter activity [37]. This effect, however, may strongly depend on the cell type, since nuclear receptors and AP-1 can act either synergistically or antagonistically depending on conformation and availability of specific protein components of the DNA binding complex [38]. On the other hand, Gotoh et al. [33] showed recently that glucocorticoid induction of the rat arginase gene is mediated through the transcription factor C/EBPβ, which binds to a cluster of response elements in the arginase enhancer. There are four perfect matches of the C/EBP consensus binding site (TT/GNNGNAAT/G) clustered in the murine proximal ChAT promoter region, and the rat distal ChAT/VAChT promoter also contains four perfect matches of this sequence. Thus, the regulation of the ChAT/VAChT locus by dexamethasone and retinoids may involve both direct transcriptional activation through their respective response elements, and modulation of other signal transduction pathways, with the final outcome dependent on the specific cellular environment.

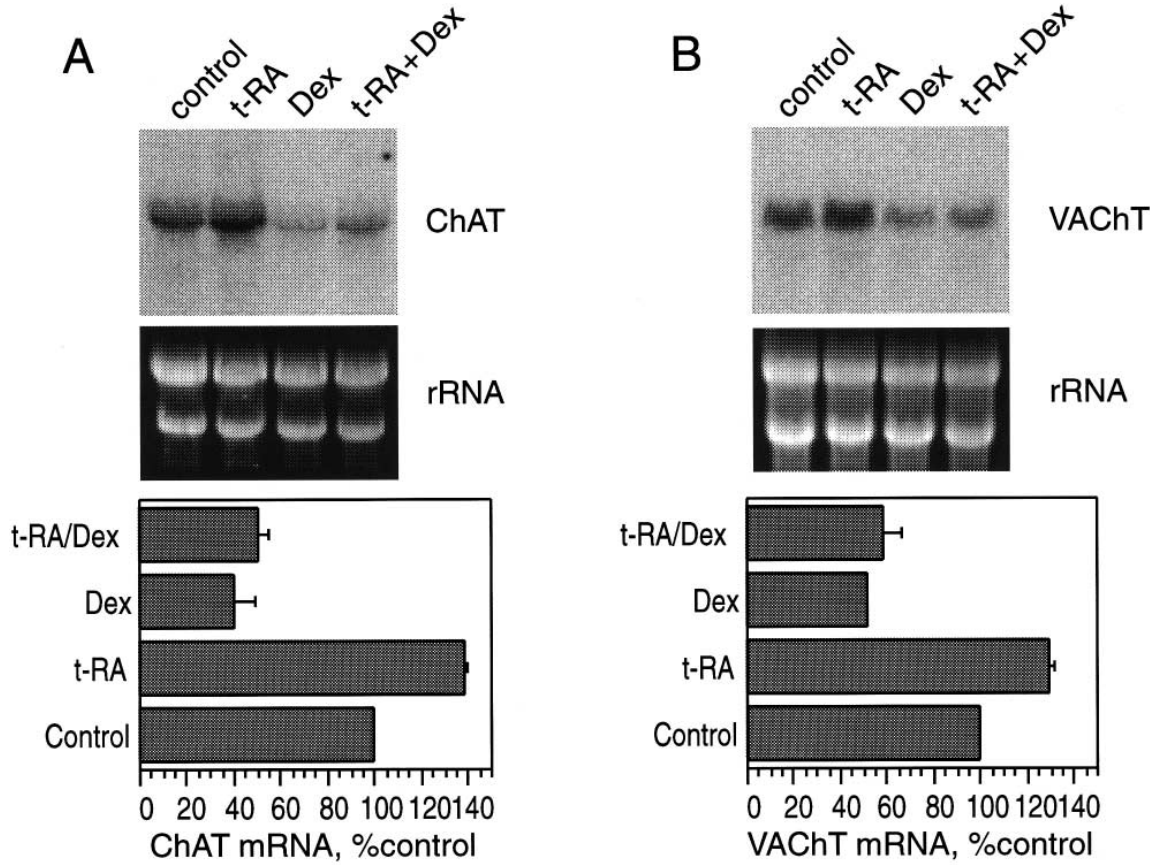

Fig. 3. Effect of dexamethasone on (A) ChAT and (B) VAChT mRNA levels in PC12 cells. Cells were treated as described in Fig 1. Total RNA (20 μg/lane) was transferred to a nylon membrane and probed with mouse ChAT and VAChT cDNAs. The hybridizing bands were visualized using a PhosphorImager 400E (upper panels) and band intensities were quantified with ImageQuant software (Molecular Dynamics). Ethidium bromide staining of rRNA bands (middle panels) demonstrates equal loading between lanes. The graphs in the lower panels present means ± range of band intensities from two experiments.

To our knowledge this is the first demonstration that the effects of glucocorticoids and retinoids on the mRNA of ChAT and VAChT are cell-type specific. If a similar cell-type dependence occurs in vivo, it would indicate that, in different populations of cholinergic neurons, corticosteroids either up-regulate or down-regulate cholinergic function. It is worth noting that PC12 cells may represent a special case in this regard as they are derived from adrenal chromaffin cells which are exposed to high concentrations of adrenal cortical hormones in vivo, and it is the action of these hormones which maintains their adrenergic phenotype [39].

In in vivo studies, administration of dexamethasone to rats altered ChAT activity in brain in a region-specific manner. Notably, hippocampal ChAT activity was elevated but that of the hypothalamus remained unchanged [40]. In contrast, ChAT activity of the gastrointestinal cholinergic system was reduced by the administration of corticosterone [41]. In a recent study by Hu et al. [8], subcutaneous injection of dexamethasone into rat pups resulted in a dramatic decrease of ChAT immunoreactivity in the caudate-putamen, with no change in other forebrain structures including septum. These in vivo experiments do not clarify whether the effects of glucocorticoids on cholinergic neurons are directly receptor-mediated, or indirect through an action on other cell types. Our data indicate that mechanisms exists for direct differential ChAT and VAChT mRNA expression in cholinergic cells of various origin. Since glucocorticoids are released by the adrenal glands in response to stress, our data suggest that stress may have a complex pattern of effects on cholinergic function.

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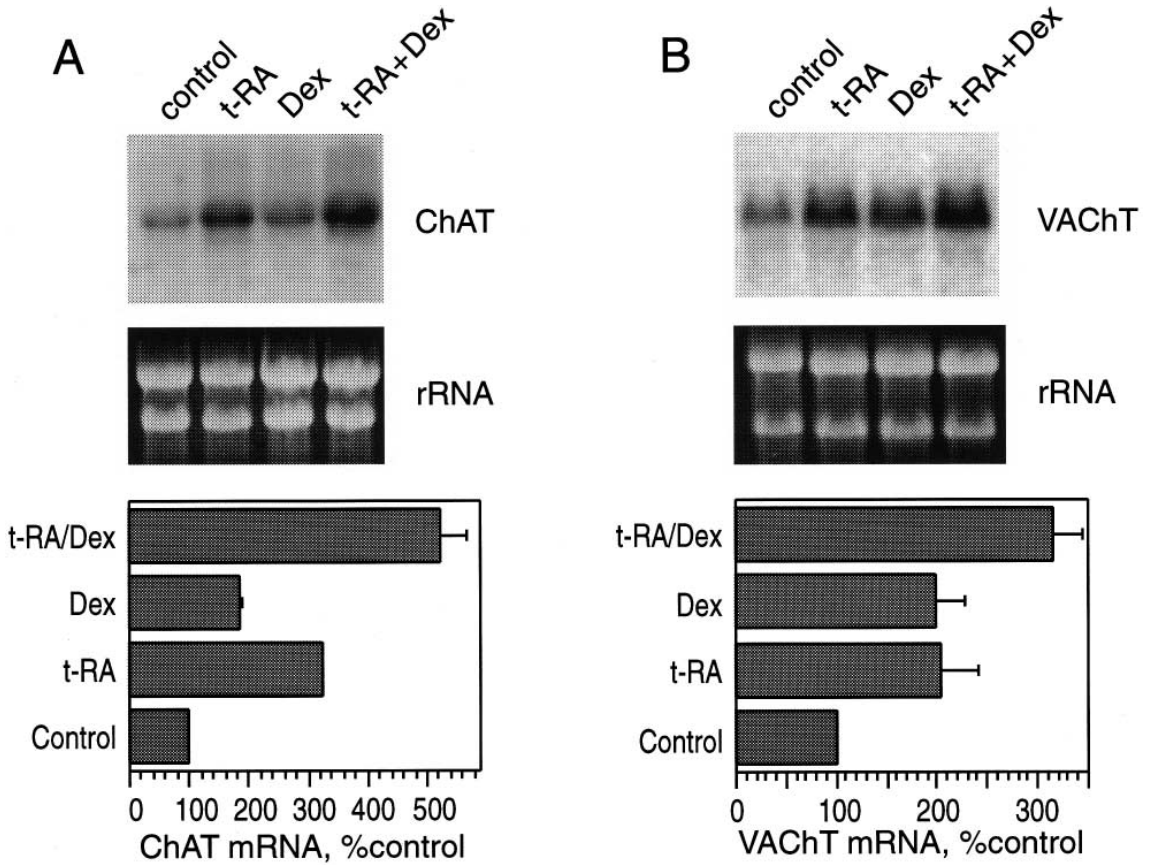

Fig. 4. Effect of dexamethasone on (A) ChAT and (B) VAChT mRNA levels in SN56 cells. Cell treatments and analyses of RNA were performed as described in Fig 3.

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