13-*cis*-Retinoic Acid Alters Intracellular Serotonin, Increases 5-HT_{1A} Receptor, and Serotonin Reuptake Transporter Levels *In Vitro*

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In addition to their established role in nervous system development, vitamin A and related retinoids are emerging as regulators of adult brain function. Accutane (13-*cis*-retinoic acid, isotretinoin) treatment has been reported to increase depression in humans. Recently, we showed that chronic administration of 13-*cis*-retinoic acid (13-*cis*-RA) to adolescent male mice increased depression-related behaviors. Here, we have examined whether 13-*cis*-RA regulates components involved in serotonergic neurotransmission *in vitro*. We used the RN46A-B14 cell line, derived from rat embryonic raphe nuclei. This cell line synthesizes serotonin (5-hydroxytryptamine, 5-HT) and expresses the 5-HT_{1A} receptor and the serotonin reuptake transporter (SERT). Cells were treated with 0, 2.5, or 10 μ*M* 13-*cis*-RA for 48 or 96 hrs, and the levels of 5-HT; its metabolite, 5-hydroxyindoleacetic acid (5HIAA); 5-HT_{1A} receptor; and SERT were determined. Treatment with 13-*cis*-RA for 96 hrs increased the intracellular levels of 5-HT and tended to increase intracellular 5HIAA levels. Furthermore, 48 hrs of treatment with 2.5 and 10 μ*M* 13-*cis*-RA significantly increased 5-HT_{1A} protein to 168.5 ± 20.0% and 148.7 ± 2.2% of control respectively. SERT protein levels were significantly increased to 142.5 ± 11.1% and 119.2 ± 3.6% of control by 48 hrs of treatment with 2.5 and 10 μ*M* of 13-*cis*-RA respectively. Increases in both 5-HT_{1A} receptor and SERT proteins may lead to decreased serotonin availability at synapses. Such an effect of 13-*cis*-RA could contribute to the increased depression-related behaviors we have shown in mice.

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Key words: 13-*cis*-retinoic acid; depression; serotonin; 5-HT_{1A} receptor; SERT

Introduction

13-*cis*-Retinoic acid (13-*cis*-RA, isotretinoin), is the active ingredient in Accutane, a medication prescribed for severe acne. Administration of Accutane coincides with the onset of depressive symptoms in approximately 5% of patients [for review see (1)]. Although controversy exists as to whether or not 13-*cis*-RA induces depression in human patients (2–5), some case studies have reported that the onset of depressive symptoms occurred after commencement of treatment and were relieved following cessation of drug treatment (1). Additionally, there are case reports of recurrence of depressive symptoms after rechallenge with 13-*cis*-RA (1).

All-trans-retinoic acid (ATRA) is the endogenous ligand for retinoic acid receptors (RAR) and is synthesized from dietary vitamin A (retinol) (6). 13-*cis*-RA is a synthetic retinoid capable of either binding to RAR itself (7) or being isomerized to ATRA before RAR binding (8). RAR heterodimerize with retinoid X receptors (RXR), and together they bind to specific DNA sequences termed retinoic acid response elements (RARE) (6). Binding of ligand to the RAR initiates transcription (6), and this has led to the view that retinoids are primarily regulators of gene transcription, either directly or *via* intermediate genes. The expression of many neuronal genes is affected by ATRA, and a few have been reported to have functional RARE,

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suggesting that retinoids act as transcriptional regulators in neuronal cells [for review see (9)]. However, retinoids can also influence cellular function by altering protein levels *via* nontranscriptional mechanisms, such as increasing mRNA (10, 11) or protein stability (12).

Although the role of retinoids in development is widely studied, over the past few years, reports of 13-*cis*-RA actions in the adult brain have emerged. In human patients treated with 13-*cis*-RA, brain imaging studies have shown a decrease in orbital frontal cortex metabolism (13). In addition, in mice, 13-*cis*-RA administration has been shown to impair learning and memory (14) and increase depression-related behaviors (15). In the latter study, conducted by our research group, adolescent, DBA/2J mice that were treated with 1 mg/kg/day of 13-*cis*-RA, the same dose prescribed to human patients, exhibited significantly more immobility than vehicle-treated controls in both the tail suspension test and the forced swim test (15). Further analysis of data in the forced swim test revealed that the increase in immobility was accompanied by a decrease in swimming, but not climbing, behavior (15). For rodents, in the forced swim test, antidepressants that target the serotonergic system decrease immobility because of an increase in swimming time (16). In contrast, antidepressants that target the noradrenergic system, decrease immobility and increase time spent climbing (16).

Although the etiology of depression is still unknown, the serotonergic system has long been a target of antidepressant drugs (17). Serotonin (5-hydroxytryptamine, 5-HT) reuptake and metabolism are targets of antidepressants, with the net result being increased levels of 5-HT in the synaptic cleft. Intracellular levels of 5-HT are controlled by 5-HT synthesis from tryptophan, 5-HT degradation to 5-hydroxyindole acetic acid (5HIAA), vesicular packaging and release of 5-HT into the synapse, and reuptake of 5-HT into the cell by the serotonin reuptake transporter (SERT or 5-HTT). An additional regulator of 5-HT release is the somatodendritic serotonin receptor 1A (5-HT_{1A}). This autoreceptor is activated by 5-HT to inhibit firing of serotonergic raphe neurons (18). The role of 5-HT in human depression is supported by studies showing decreased plasma tryptophan levels, reduced 5HIAA levels, decreased 5-HT uptake and polymorphisms in 5-HT_{1A} receptor (19) and SERT (20–22).

The ability of 13-*cis*-RA to cause depression-related behavior in adolescent mice and the ability of retinoids to affect neuronal processes and organismal behavior led us to hypothesize that serotonergic function may be altered in the raphe nuclei in response to 13-*cis*-RA treatment. In this study, we used a cell line derived from rat raphe nuclei, RN46A-B14, as our model. These cells are serotonergic and express the 5-HT_{1A} receptor and SERT (23, 24). We show that treatment of RN46A-B14 cells with 13-*cis*-RA leads to increased intracellular 5-HT content. Intracellular 5HIAA content also tends to be increased. In addition, both the 5-HT_{1A} receptor and SERT are increased following 13-*cis*-RA treatment.

Materials and Methods

Tissue Culture. RN46A-B14 cells (a gift from Dr. Scott Whittemore, University of Louisville, Louisville, KY) were used to investigate the effects of 13-*cis*-RA on components of the serotonergic system. The RN46A parent cell line was isolated from embryonic Day 13 rat medullary raphe nuclei and immortalized with a SV40 large T antigen (25). The RN46A-B14 cell line is a stably transfected RN46A cell line that expresses full-length human brain–derived neurotrophic factor (BDNF), required for serotonergic differentiation (24). RN46A-B14 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% fetal bovine serum (FBS) and antibiotics (1000 units/ml penicillin and 1000 μg/ml streptomycin) at 33°C. Before differentiation, cells were plated at a density of 4×10^6 cells/plate in 100-mm dishes and allowed to grow for 2–3 days until reaching approximately 70% confluency. Cells were differentiated as described (24) by incubation at 39°C in DMEM/F12 medium containing 1% FBS, antibiotics (1000 units/ml penicillin and 1000 μg/ml streptomycin), 5 μg/ml insulin, 20 n*M* progesterone, 100 μ*M* putrescine, 1 μg/ml transferrin, and 1% ovalbumin (w/v). Medium was changed every day for the first 4 days, and on Day 4, 40 m*M* potassium chloride (KCl) were added. Depolarization with KCl enhances the serotoninergic phenotype of these cells (23, 25). Medium was then changed every 48 hrs and contained 40 m*M* KCl for the duration of the experiment. After 8 days at 39°C, differentiated cells were treated with 0, 2.5, or 10 μ*M* 13-*cis*-RA dissolved in an ethanol vehicle for 48 or 96 hrs. The Accutane package insert reports that the steady-state plasma concentration of 13-*cis*-RA ranges from a minimum (mean ± SD) of 352.32 ± 184.44 ng/ml (1.2 μ*M*) to a maximum of 731.98 ± 361.86 ng/ml (2.5 μ*M*). Thus, the 2.5 μ*M* concentration of 13-*cis*-RA was chosen to reflect the average steady-state concentration of 13-*cis*-RA, and the 10 μ*M* concentration of 13-*cis*-RA may be reached soon after 13-*cis*-RA administration. All cells, including control, were treated with an equal amount of vehicle. All retinoid manipulations were performed under subdued light.

High-Performance Liquid Chromatography (HPLC) Analysis of Intracellular 5-HT and 5HIAA Content. Isocratic HPLC with electrochemical detection was used to examine the effect of 13-*cis*-RA on 5-HT production in RN46A-B14 cells. Cells were grown and differentiated as described above. Although the medium contained 66 μ*M* tryptophan, to ensure adequate levels of 5-HT for detection by HPLC analysis after 48 or 96 hrs of 13-*cis*-RA treatment, cells were incubated with 10 μ*M* tryptophan and 10 μ*M* chlorgyline for 30 mins and then with 10 μ*M* fluoxetine for an additional 30 mins as described (23). Tryptophan is the precursor for 5-HT synthesis, chlorgyline inhibits monoamine oxidase A, and fluoxetine blocks SERT and, therefore, 5-HT entry into the cell, thereby allowing for detection of 5-HT. Cells were left

attached to the plate and washed twice with phosphate-buffered saline (PBS) and lysed by freezing at −80°C. The next day, 200 μl of e-pure water was added to the plate, the plate was scraped, and the lysed cells were harvested, transferred to an eppendorf tube, and centrifuged at 13,000 *g* for 10 mins. The supernatant was stored at −20°C until analysis. The protein content of each sample was determined using a portion of the supernatant and the BioRad (Hercules, CA) DC protein assay kit.

5-HT and 5HIAA levels in the lysate were determined in the College of Pharmacy Analytical Instrumentation Facility Core (University of Texas at Austin, Austin, TX) by HPLC electrochemical method modified from that of Bai *et al*. (26). Briefly, the samples (60–100 μl) were injected into the HPLC system, which consisted of a Shimadzu SCL-10A system controller and a LC-10AD pump equipped with a SIL-10A auto-sampler (Shimadzu, Columbia, MD), coupled to a four-channel CoulArray electrochemical detector (ESA Biosciences Inc., Chelmsford, MA). The isocratic mobile phase consisted of 4 m*M* citrate, 8 m*M* ammonium acetate, 20 mg/l EDTA, 120 μ*M* 1-octanesulfonic acid sodium salt (SOS) at pH 3.5, and 5% methanol. 5-HT and 5HIAA were separated by a 4.6 × 80 mm reverse-phase HR-80, 3-μm particle-size, 120-Å, column (ESA Biosciences) at a flow rate of 1 ml/min and analyzed by electrochemical detection using a CoulArray electrochemical detector (ESA Biosciences). The potential of Channels 1–4 of the CoulArray were set at −50, 0, 300, and 400 mV, respectively (Fig. 1A). Peak area (nC) of 5-HT or 5HIAA at the corresponding retention time on the chromatogram resulting from 300 mV was obtained using CoulArray for Windows, version 1.12 software (ESA Biosciences). The peak area was used to quantify the 5-HT or 5HIAA in each sample injected, based on the standard curve. The total 5-HT or 5HIAA concentration was corrected for sample volume loaded and normalized to protein content.

Northern Blot Analysis of 5-HT$_{1A}$ and SERT mRNA. For probe generation, total RNA was isolated from differentiated, vehicle control-treated RN46A-B14 cells or adult rat hippocampus with RNA Stat-60 (Tel-Test, Friendswood, TX). Two micrograms of RN46A-B14 or rat hippocampal total RNA were reverse-transcribed with oligo-dT primers and the Reverse Transcription System (Promega, Madison, WI) per manufacturer's instructions. Amplification of the 5-HT$_{1A}$ and GAPDH cDNA were performed using RN46A-B14 cDNA and the following primers: 5-HT$_{1A}$ forward primer, 5′-AGC ATC TCC GAC GTG ACC TTC AGC TAC CA-3′; reverse primer, 5′-GCT CCC TTC TTT TCC ACC TTC CTG ACA GT-3′, resulting in a 635-bp product (27). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 5′-CGT CTT CAC CAC CAT GGA GA-3′; reverse primer, 5′-CGG CCA TCA CGC CAC AGT TT-3′, resulting in a 260-bp product (28). Amplification of SERT cDNA was performed using hippocampal cDNA and the following primers. SERT forward primer, 5′-TGA CCA GCA GCA TGG AGA CC-3′; reverse primer, 5′-CCA CGG CAT AGC CAA TGA C-3′, resulting in a 304-bp product (29). 5-HT$_{1A}$, GAPDH, and SERT cDNA were amplified using Taq polymerase (New England Biolabs Inc., Ipswich, MA) per manufacturer's instructions under the following polymerase chain reaction (PCR) conditions: 95°C for 5 mins followed by 50 cycles of 95°C for 30 secs, 60°C for 30 secs, and 72°C for 30 secs, with a final extension period of 7 mins at 72°C. PCR reactions were then electrophoresed in a 1.8% agarose gel and products of the appropriate size were purified using the Qiagen Gel Extraction kit (Qiagen, Valencia, CA) per the manufacturer's instructions. All products were sequenced to confirm their identity.

Total RNA was isolated from cells differentiated and treated with 0, 2.5, or 10 μ*M* 13-*cis*-RA for 48 or 96 hrs as described above using RNA Stat-60 (Tel-Test). RNA was electrophoresed through 1.2% agarose/2.2 *M* formamide gels, transferred to nylon filters, and cross-linked to the filters with a UV-Stratalinker (Stratagene) as described (30). The cDNA probes for 5-HT$_{1A}$, SERT, and GAPDH were labeled with [α-^{32}P]dCTP using the Random Primed DNA Labeling Kit (Roche Applied Science, Indianapolis, IN) per the manufacturer's instructions. Membranes were prehybridized overnight at 42°C in hybridization solution [50% (w/v) deionized formamide/0.2% bovine serum albumin (BSA)/0.2% polyvinyl pyrrolidone/2% Ficoll (molecular weight 400,000 g/mole)/50 m*M* Tris·HCl, pH 7.5/0.1% sodium pyrophosphate/1% SDS/10% dextran sulfate/100 μg/ml salmon sperm DNA] and then hybridized overnight at 42°C in hybridization solution containing radiolabeled probe. After hybridization, the membranes were washed and processed as described previously (30). The membranes were then exposed to film for 24 hrs. All membranes were stripped and re-hybridized with GAPDH to control for loading differences. Autoradiographs were quantitated using a BioRad Gel Documentation System.

Western Blot Analysis of 5-HT$_{1A}$ and SERT Protein. The effect of 13-*cis*-RA on 5-HT$_{1A}$ and SERT protein levels in serotonergic cells was examined using semiquantitative Western blot analysis. RN46A-B14 cells were differentiated as described above and treated with 13-*cis*-RA for 48 or 96 hrs. For 5-HT$_{1A}$ and SERT analyses, cells were lysed in radioimmunoprecipitation (RIPA) buffer (150 m*M* NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 m*M* Tris, 1 mg/ml leupeptin, 1 m*M* dithiothreitol [DTT], 2 m*M* sodium orthovanadate, 1 mg/ml phenylmethylsulfonyl fluoride [PMSF], 1 mg/ml trypsin inhibitor, and 10 m*M* aprotinin) and equal amounts of protein (50 μg for 5-HT$_{1A}$ or 75 μg for SERT) were electrophoresed on 12% (5-HT$_{1A}$) or 10% (SERT) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Protein was quantitated using BioRad DC protein assay kit. After electrophoresis, protein was transferred to a nitrocellulose membrane.

For analysis of 5-HT$_{1A}$ levels, the blots were prehybridized with 5% milk in Tris-buffered saline with

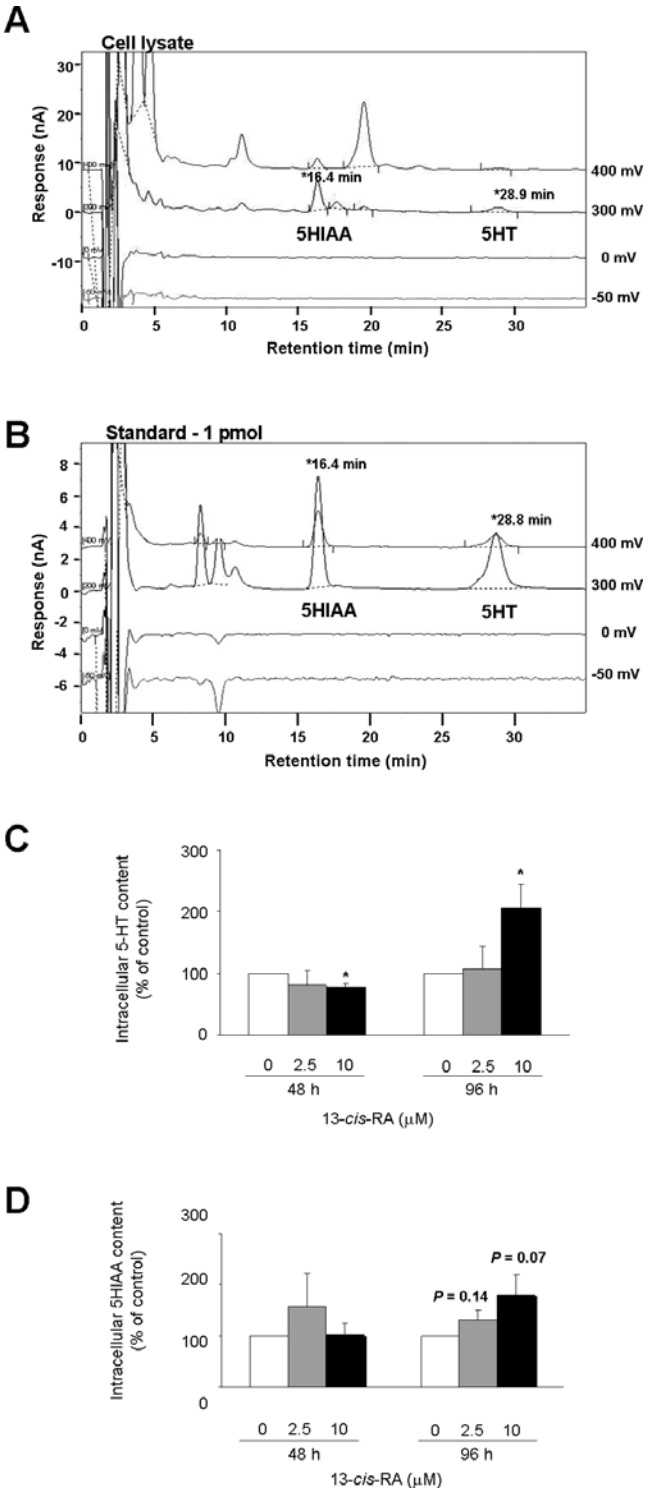

Figure 1. Effect of 13-*cis*-RA treatment on intracellular 5-HT and 5HIAA levels in cultured serotonergic cells. Representative HPLC tracing (A) for cell lysate and (B) 1 pmol standard. 5-HT and 5HIAA retention times are indicated. (C and D) Intracellular 5-HT and 5HIAA content. Cells were differentiated for 8 days before treatment with 0, 2.5, or 10 μ*M* 13-*cis*-RA for 48 or 96 hrs. Before harvesting, cells were incubated with 10 μ*M* tryptophan and 10 μ*M* chlorgyline for 30 mins, then 10 μ*M* fluoxetine was added for another 30 mins. Cells were lysed and 5-HT and 5HIAA content were detected electrochemically *via* HPLC. Results shown in (C) 5-HT and (D) 5HIAA are mean ± SEM for four separate experiments. Statistical analysis was performed using *t* tests comparing each 13-*cis*-RA concentration to control.

Tween-20 (TBST; 10 m*M* Tris, pH 8, 150 m*M* NaCl, and 0.5% Tween-20) for 1 hr at room temperature before overnight incubation at 4°C with 5-HT_{1A} polyclonal antibody (catalogue sc-10801; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:300 dilution. For analysis of SERT levels, blots were prehybridized with 5% milk in TBST before incubation with a 1:200 dilution of polyclonal anti-SERT antibody (catalogue sc-1458; Santa Cruz Biotechnology) in 1% BSA in TBST at 4°C overnight. Secondary antibodies were goat anti-rabbit (catalogue #0031460, Pierce, Rockford, Il) or bovine anti-goat (catalogue #sc-2350; Santa Cruz Biotechnology Inc.), for 5-HT_{1A} or SERT respectively, and were incubated in 5% milk for one hour at room temperature. Immunoreactivity was detected using the Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL). The membranes were then stripped and re-probed with polyclonal β-actin antibody (catalogue A2066; Sigma, St. Louis, MO) at a 1:10,000 dilution to control for differences in loading. Densitometry was performed using a BioRad Gel Documentation System.

RT-PCR Analysis of RAR/RXR Expression. RN46A-B14 cells were grown and differentiated for 8 days as described, and on the Day 8, total RNA was extracted from RN46A-B14 cells as described above. DNA was removed by DNase digestion and total RNA (1 μg) was reverse-transcribed with oligo-dT primers and the Reverse Transcription System (Promega) per manufacturer's instructions. cDNA was then subjected to 40 PCR cycles at a melting temperature (Tm) of 60°C, with the primers listed below. For adult rat dorsal raphe tissue, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). DNA was removed by means of a DNase digest and total RNA (0.25 μg) was reverse-transcribed using RT/ Platinum *Taq* Mix (Superscript One-Step RT-PCR with Platinum *Taq*, Invitrogen) per manufacturer's instructions, with the gene-specific primers listed. Amplification of RARα, RARβ, RARγ, RXRα, and RXRβ/γ cDNA were achieved using the following primers: RARα forward: 5′-CTG GAG ATG GAC GAT GCT GAG ACT-3′, reverse: 5′-CAC AGA TGA GGC AGA TGG CAC TGA-3′, resulting in a 54-bp product (31); RARβ forward: 5′-CAA AGC CTG CCT CAG TGG ATT CA-3′, reverse: 5′-AGT GGT AGC CCG ATG ACT TGT CCT-3′, resulting in a 178-bp product (31); RARγ forward: 5′-GGA ACT CAT CAC CAA GGT CAG CAA-3′, reverse: 5′-CGC TTC GCA AAC TCC ACA ATC TT-3′ resulting in a 175-bp product (31); RXRα forward: 5′-CTT TGA CAG GGT GCT AAC AGA GC-3′, reverse: 5′-ACG CTT CTA GTG ACG CAT ACA CC-3′, resulting in a 172-bp product (32); RXRβ/γ forward: 5′-AGG CAG GTT TGC CAA GCT TCT G-3′, reverse: 5′-GGA GTG TCT CCA ATG AGC TTG A-3′, resulting in a 102-bp product (33).

Statistical Analyses. Statistical analyses were performed using Excel (XP 2002; Microsoft, Redmond, WA). Two-tailed, Student's *t* tests were performed to test for

differences between vehicle control and 13-*cis*-RA treatments. Data are expressed as mean ± SEM, $n = 3$, unless otherwise indicated, and differences were considered significant at $P < 0.05$.

Results

Effect of 13-*cis*-RA Treatment on Intracellular 5-HT and 5HIAA Levels. HPLC was used to determine the effect of 13-*cis*-RA treatment on intracellular 5-HT levels in RN46A-B14 cells. The levels of 5HIAA, the primary metabolite of 5-HT, were also determined. 5-HT and 5HIAA peak retention times reflect their respective standards at 300 mV (Fig. 1A and B). We found that 48 hrs of 2.5 μ*M* 13-*cis*-RA treatment did not increase intracellular 5-HT (Fig. 1C) or 5HIAA (Fig. 1D) levels in RN46A-B14 cells. However, treatment with 10 μ*M* 13-*cis*-RA for 48 hrs decreased ($P = 0.01$) intracellular 5-HT levels to 77.8 ± 6.2% of control (Fig. 1C) but had no effect on 5HIAA (Fig. 1D). Extending treatment for 96 hrs with 10 μ*M* 13-*cis*-RA significantly increased the intracellular 5-HT concentration to 206.9 ± 37.1% of vehicle control ($P = 0.03$) (Fig. 1C). Both 2.5 and 10 μ*M* 13-*cis*-RA tended to increase intracellular 5HIAA levels to 132.4 ± 18.8% ($P = 0.14$) and 181.7 ± 38.1% of vehicle control ($P = 0.07$), respectively, after 96 hrs of treatment (Fig. 1D). These data indicate that prolonged treatment of RN46A-B14 cells with 13-*cis*-RA tends to increase intracellular 5-HT and 5HIAA content.

Effect of 13-*cis*-RA Treatment on 5-HT_{1A} mRNA and Protein Levels. Because retinoids are well known for their ability to induce gene transcription (6), Northern blot analysis was used to examine the effect of 13-*cis*-RA on 5-HT_{1A} mRNA levels in RN46A-B14 cells. 13-*cis*-RA treatment tended ($P = 0.06$) to slightly decrease 5-HT_{1A} mRNA levels (91.2 ± 3.8%) after 48 hrs of treatment with 2.5 μ*M* 13-*cis*-RA (Fig. 2A). 10 μ*M* 13-*cis*-RA treatment had no effect on 5-HT_{1A} mRNA levels after 48 hrs (93.9 ± 5.5% of control), and there were no differences in 5-HT_{1A} mRNA levels due to either 2.5 or 10 μ*M* 13-*cis*-RA treatment after 96 hrs (104.9 ± 5.6% and 104.9 ± 5.8% of vehicle control, respectively, Fig. 2A).

To determine the effect of 13-*cis*-RA on 5-HT_{1A} protein levels in RN46A-B14 cells, semiquantitative Western blotting was performed. Treatment of RN46A-B14 cells with 2.5 μ*M* and 10 μ*M* 13-*cis*-RA for 48 hrs significantly increased 5-HT_{1A} levels to 168.5 ± 20.0% and 148.7 ± 2.2% of control, respectively (Fig. 2B). This increase was maintained after 96 hrs of 13-*cis*-RA treatment. Thus, treatment of serotonergic cells with 13-*cis*-RA increases 5-HT_{1A} protein levels as early as 48 hrs after drug administration but does not affect 5-HT_{1A} mRNA levels.

Effect of 13-*cis*-RA Treatment on SERT mRNA and Protein Levels. Northern blot analysis was also used to determine the effect of 13-*cis*-RA treatment on SERT mRNA levels. Treatment of RN46A-B14 cells with 2.5 or 10 μ*M* 13-*cis*-RA for 48 hrs did not affect SERT mRNA levels (Fig. 3A). In contrast, 96 hrs of treatment with 10 μ*M* 13-*cis*-RA tended ($P = 0.10$) to increase SERT mRNA levels to 144.8 ± 20.7% of vehicle control. In addition to the increase in mRNA levels, semiquantitative Western blotting revealed that SERT protein levels were significantly increased after treatment with 2.5 μ*M* and 10 μ*M* 13-*cis*-RA for 48 hrs, reaching 142.5 ± 11.1% and 119.2 ± 3.6% of vehicle control, respectively (Fig. 3B). SERT protein levels tended to remain elevated after 96 hrs of treatment with 13-*cis*-RA (Fig. 3B). Therefore, treatment of serotonergic cells with 13-*cis*-RA leads to increased levels of SERT protein as early as 48 hrs after treatment and later to an increase in SERT mRNA.

RAR and RXR Expression. RAR and RXR mediate gene transcription in response to retinoic acid. Therefore, RT-PCR was used to examine RAR and RXR expression in RN46A-B14 cells and adult rat dorsal raphe tissue. RARα, β, and γ, as well as the RXRα and β/γ isoforms, were expressed in both RN46A-B14 cells and the raphe nuclei (Fig. 4), indicating that retinoid-mediated gene transcription via RAR/RXR/RARE can occur. In RN46A-B14 cells, the RARα expression level may be lower than RARβ and γ. However, this RT-PCR method is not quantitative.

Discussion

We show here that prolonged treatment with 13-*cis*-RA increases intracellular 5-HT and 5HIAA levels, although the effect of 13-*cis*-RA on 5HIAA levels was not significant. Additionally, 13-*cis*-RA treatment increased 5-HT_{1A} receptor and SERT protein levels, although it had little effect on their mRNA levels. Previously, we showed that 13-*cis*-RA administration induced depression-related behavior in adolescent male mice (15). Disturbances in the serotonergic system are known to be involved in depression, including altered 5-HT availability and changes in expression of the 5-HT_{1A} autoreceptor and SERT (22). An increase in expression of the 5-HT_{1A} autoreceptor and SERT levels in the raphe nuclei following 13-*cis*-RA treatment may lead to decreased serotonergic availability at the synapse and thus contribute to the increase in depression-related behavior observed *in vivo* (15).

We examined whether 13-*cis*-RA alters intracellular 5-HT levels or the levels of its metabolite, 5HIAA and saw that 13-*cis*-RA increased 5-HT and tended to increase 5HIAA *in vitro*. The increase in intracellular 5HIAA parallels the increase in intracellular 5-HT; thus, we suspect the increase in intracellular 5HIAA is due to the increase in intracellular 5-HT levels and occurred before chlorgyline treatment. Ferguson *et al*. (34) examined the effects of 13-*cis*-RA treatment on monoaminergic systems in adult rats. Although they found no effect on 5-HT or 5HIAA content in brain tissue homogenates of either hippocampus or frontal cortex, 5HIAA levels in the striatum were increased in male rats administered 13-*cis*-RA. These data indicate

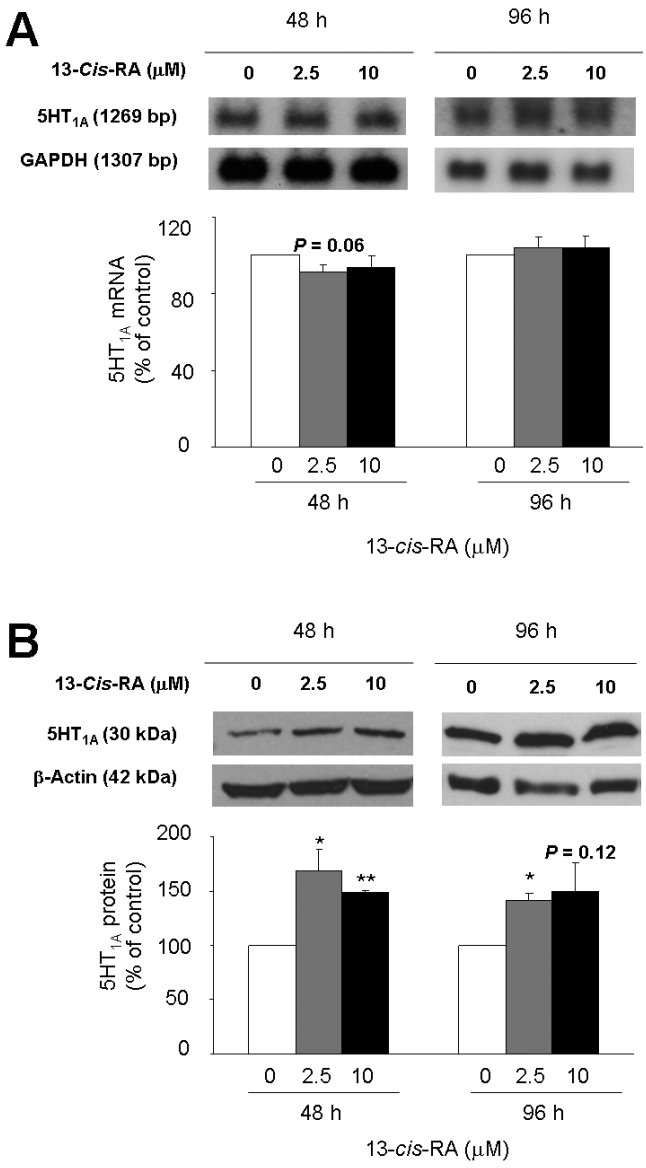

Figure 2. Effect of 13-*cis*-RA on 5-HT_{1A} mRNA and 5-HT_{1A} protein levels. Cells were differentiated for 8 days and then cultured with 0, 2.5, or 10 μ*M* 13-*cis*-RA for 48 or 96 hrs. (A) Total RNA was harvested and subjected to Northern blot analysis for 5-HT_{1A} and GAPDH mRNA (1269 and 1307 bp, respectively) as described in Materials and Methods. GAPDH was used to correct for loading differences. Northern blot analysis was repeated four separate times with similar results; a representative Northern blot is shown. (B) Total protein was harvested and subjected to Western blot analysis for 5-HT_{1A} and β-actin (30 and 42 kDa respectively) as described in Materials and Methods. β-Actin was used to correct for loading differences. Western blot analysis was performed three separate times with similar results; a representative Western blot is shown. Values reported are mean ± SEM. Statistical analysis was performed using *t* tests comparing each 13-*cis*-RA concentration to control. * $P < 0.05$, ** $P < 0.01$; significantly different from control.

that there are likely to be brain region–specific effects of 13-*cis*-RA on 5-HT/5HIAA levels. Ferguson *et al*. (34) did not examine the raphe nuclei, and thus, it remains possible that 13-*cis*-RA will cause an increase in 5-HT and 5HIAA in the raphe *in vivo*. Although we did not examine release, recycling, or reuptake of 5-HT in this study, we speculate that increased levels of intracellular 5-HT due to 13-*cis*-RA treatment could occur because of increased reuptake. In these experiments, we pretreated the cells with fluoxetine before HPLC analysis, this SERT inhibitor was not added until we had preloaded the cells with tryptophan for 5-HT synthesis. Therefore, greater 5-HT reuptake may be due to the ability of 13-*cis*-RA to increase SERT protein levels. Alternatively, increased intracellular 5-HT levels could result from increased 5-HT synthesis due to the effects of 13-*cis*-RA on the levels of synthetic enzymes such as tryptophan hydroxylase or amino acid decarboxylase.

Although retinoids are known for their ability to alter gene transcription when binding to RAR/RXR heterodimers on RARE, other roles for retinoids are becoming evident. ATRA has been shown to affect mRNA stability. Although the mechanism is not completely understood, ATRA can reduce tumor necrosis factor α (TNF-α) mRNA stability in a RXR-mediated manner in hepatocytes (10), increase keratin 19 mRNA stability in cultured keratinocytes (11), and increase protein stability in P19 cells (12). Adult raphe and RN46A-B14 cells express RARα/β/γ and RXRα/β/γ (Fig. 4) indicating presence of the cellular mechanisms for retinoid receptor-mediated gene transcription. We observed an increase in 5-HT_{1A} protein after 48 and 96 hrs treatment with 13-*cis*-RA without an increase in mRNA. It is possible that the increase in 5-HT_{1A} protein level in response to 13-*cis*-RA treatment is due to increased translation of 5-HT_{1A} mRNA or increased stability of the 5-HT_{1A} protein. In contrast, we observed an increase in SERT mRNA after 96 hrs of 13-*cis*-RA treatment. This may reflect an increase in stability of the mRNA, but given the delay in increase of mRNA with treatment, it is possible that the SERT gene is not directly regulated by 13-*cis*-RA. Instead, perhaps an intermediate gene, such as a transcription factor or a derepressor element that regulates SERT expression, is transcriptionally activated by 13-*cis*-RA. Because SERT protein, but not mRNA levels, are changed after 48 hrs of treatment, 13-*cis*-RA treatment, in RN46A-B14 cells, may initially increase SERT protein stability or mRNA translation. Increased transcription of early response genes that are transcription factors for SERT would then lead to the increase seen in SERT mRNA at 96 hrs and thus be the underlying cause of later elevated levels of SERT protein.

Functionally, the 5-HT_{1A} autoreceptor is involved in regulation of serotonergic neuron firing (18). Activation of 5-HT_{1A} autoreceptors residing on raphe nuclei by 5-HT results in reduced firing of the raphe nuclei and therefore the amount of serotonin in the synaptic cleft. Both increased and decreased expression of the 5-HT_{1A} receptor has been reported in depressed and suicidal patients (22). In addition, polymorphisms in the human 5-HT_{1A} promoter region have been linked to depression in some (19), but not all, patients (35). Interestingly, 5-HT_{1A}-null mice exhibit a decrease in immobility in both the forced swim test and the tail suspension test, consistent with an antidepressant-like effect

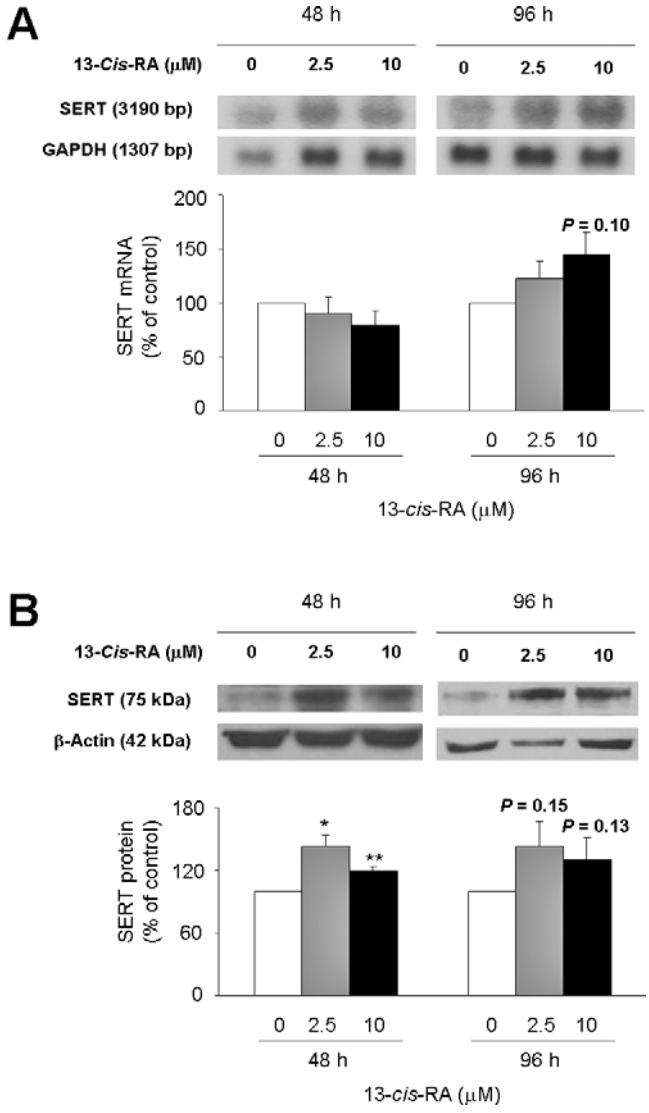

Figure 3. Effect of 13-*cis*-RA treatment on SERT mRNA and protein levels. Cells were differentiated for 8 days and then cultured with 0, 2.5, or 10 μ*M* 13-*cis*-RA for 48 or 96 hrs. (A) Total RNA was harvested for northern blot analysis of SERT and GAPDH mRNA (3190 and 1307 bp, respectively) as described in Materials and Methods. GAPDH was used to demonstrate equal loading. (B) Total protein was harvested and subjected to western blot analysis for SERT and β-actin (75 and 42 kDa respectively) as described in Materials and Methods. β-Actin was used to demonstrate equal loading. These experiments were performed three separate times with similar results; representative Western and Northern blots are shown. Results are mean ± SEM for three separate experiments. Statistical analysis was done using *t* tests comparing each 13-*cis*-RA concentration to control. * $P < 0.05$, ** $P < 0.01$; significantly different from control.

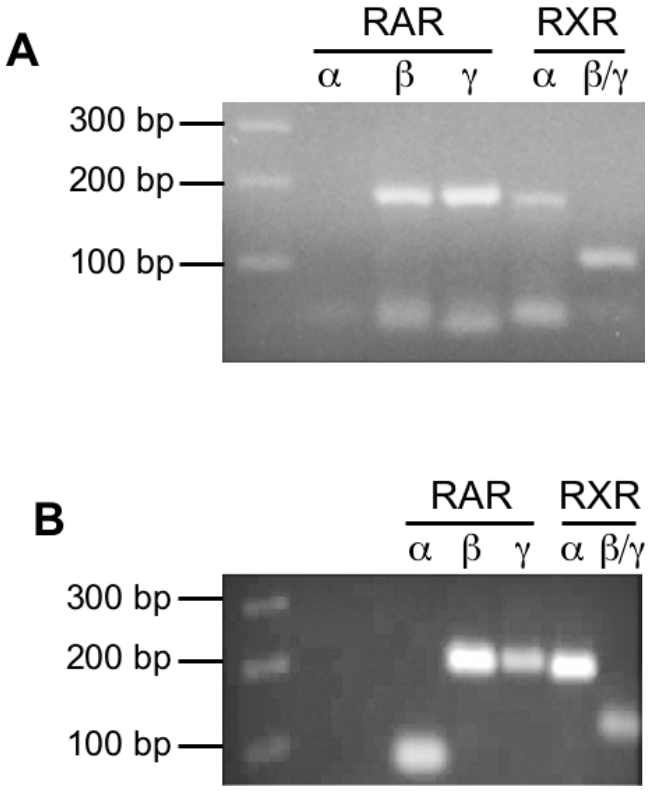

Figure 4. Expression of RAR and RXR in RN46A-B14 cells and rat raphe tissue. (A) RN46A-B14 cells were differentiated for 8 days, and total RNA was reverse transcribed. cDNA was then amplified with primers specific for RAR and RXR. (B) Total RNA from rat raphe tissue was reverse-transcribed in a one-step RT-PCR with the same primers for RAR and RXR. All isoforms of RAR and RXR were expressed in RN46A-B14 cells and rat raphe tissue. The RARα, RARβ, RARγ, PCR amplicons were 54, 178, and 175 bp in length. The RXRα and RXRβ/γ amplicons were 172 and 102 bp in length.

in the tail suspension test (36). In these knockout mice, there is an increase in basal firing of serotonergic neurons (37) that is accompanied by enhanced extracellular 5-HT release *in vivo* in hippocampus and frontal cortex (38) but not in striatum (39). Ultimately, increases in somatodendritic autoinhibitory 5-HT_{1A} levels may reduce serotonergic cell firing and thereby decrease serotonin signaling.

Abnormalities in SERT expression may also contribute to depression (22). SERT removes 5-HT from synaptic cleft to regulate serotonin signaling. Two functional polymorphisms in the human SERT gene are associated with increased vulnerability to depression and to affect the response to antidepressants (40). These polymorphisms of the SERT gene are associated with reduced SERT expression, reduced 5-HT reuptake rate (20, 41, 42) and increased susceptibility to depression (20, 21, 43). Also, SERT-null mice exhibit decreased immobility in the tail suspension test (44) and siRNA knockdown of SERT in adult mice reduced time spent immobile in the forced swim test (45). Interestingly, SERT knockout mice exhibit a gene dose-dependent decrease in SERT protein levels, a decrease in 5-HT uptake, and an increase in extracellular 5-HT levels (46–48). Because the function of SERT is to remove 5-HT from the synaptic cleft, both increases and decreases in SERT may disrupt serotonin signaling, affecting downstream neuronal targets, which may eventually result in depression.

Given that the roles of the presynaptic 5-HT_{1A} receptor and SERT are to regulate serotonin signaling, increases of

the 5-HT_{1A} presynaptic-receptor and SERT because of 13-*cis*-RA treatment may inhibit firing and reduce serotonin signaling from the raphe to other brain regions. Such impairment in serotonergic neurotransmission could contribute to the 13-*cis*-RA-induced increase in depression-related behaviors observed in mice.

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