MicroRNA 18 and 124a Down-Regulate the Glucocorticoid Receptor: Implications for Glucocorticoid Responsiveness in the Brain

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Glucocorticoids (GCs) exert profound effects on a variety of physiological processes, including adaptation to stress, metabolism, immunity, and neuronal development. Cellular responsiveness to GCs depends on numerous factors, including the amount of the glucocorticoid receptor (GR) protein. We tested the hypothesis that micro-RNAs (miRs), a recently discovered group of non-coding RNAs involved in mRNA translation, might control GR activity by reducing GR protein levels in neuronal tissues. We tested a panel of five miRs consisting of 124aa, 328, 524, 22, and 18. We found that miRs 18 and 124a reduced GR-mediated events in addition to decreasing GR protein levels. miR reporter assays revealed binding of miR-124a to the 3′ untranslated region of GR. In correspondence, the activation of the GR-responsive gene glucocorticoid-induced leucine zipper was strongly impaired by miR-124a and -18 overexpression. Although miR-18 is expressed widely throughout the body, expression of miR-124a is restricted to the brain. Endogenous miR-124a up-regulation during neuronal differentiation of P19 cells was associated with a decreasing amount of GR protein levels and reduced activity of luciferase reporter constructs bearing GR 3′ untranslated regions. Furthermore, we show that miR-124a expression varies over time during the stress hyporesponsive period, a neonatal period when GC signaling is modulated. Our findings demonstrate a potential role for miRs in the regulation of cell type-specific responsiveness to GCs, as may occur during critical periods of neuronal development. Ultimately, our results may provide a better understanding of the etiology of stress-related diseases as well as the efficacy of GC therapy. (***Endocrinology*** **150: 2220–2228, 2009)**

Endogenous and synthetic glucocorticoids (GCs) exert profound effects on a wide range of physiological and developmental processes that are crucial for adaptation to stress and energy metabolism. GCs are widely used as immunosuppressant drugs because they inhibit immune cell proliferation via induction of apoptosis in T lymphocytes (for review, see Ref. 1). GCs also suppress cell growth and proliferation processes in the brain; several studies have documented reduced proliferation of granule cells after administration of hydrocortisone in neonates (2) as well as a reduced number of embryonic neuronal stem cells in offspring born from dams treated with dexamethasone (DEX) (3). Diminishing exposure of the developing brain to GCs may be one of the reasons that during early postnatal life in rodents, the adrenal GC output is strongly suppressed, leading to what is known as the stress hypo-responsive period (SHRP).

GCs act via binding to two types of intracellular nuclear receptors, *i.e.* the glucocorticoid receptor (GR) and the mineralocorticoid receptor. Upon binding of GCs, these receptors translocate to the nucleus where they act as transcription factors by regulating the transcriptional activity of specific target genes in a cell type-specific manner. Numerous factors have been shown to affect GC responsiveness by regulating GR activity. Such factors include GR coactivators and corepressors (4), GR splice variants (5–7), GR isoforms (8–10), and regulators of GR ret-

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Abbreviations: CB, Cerebellum; CMV, cytomegalovirus; DEX, dexamethasone; FC, frontal cortex; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; HIPP, hippocampus; HYP, hypothalamus; miR, microRNA; PIT, pituitary; PND, postnatal d; qPCR, quantitative PCR; RA, retinoic acid; RT, reverse transcriptase; SHRP, stress hypo-responsive period; UTR, untranslated region.

rograde transport to the nucleus (11). Moreover, the amount of receptor protein is an important determinant for the magnitude of GR-mediated responses (12–15). Transcriptional regulation via the use of several alternative promoters, mRNA degradation (6), regulation of translation (10), and control of protein breakdown are all known mechanisms that contribute to determine the amount of GR present in a given cell.

Micro-RNAs (miRs) constitute a recently discovered class of noncoding RNAs involved in mRNA stability and/or translation. Several miRs are expressed in a cell type-specific manner and, since their discovery in 2001, have been shown to play pivotal roles in diverse biological processes, including embryonic development, cancer, neuronal differentiation, and neuronal plasticity (16–18). miRs are transcribed mostly from unique genomic loci as pre-miR molecules. These are processed first into precursor miRs with imperfect stem loop-like structures, and then further into mature miRs of 21–23 nucleotides. One strand is subsequently incorporated in the RNA-inhibiting silencing complex and is believed to bind to 3′ untranslated regions (UTRs) of specific mRNA molecules. Binding of miRs may lead to translational repression or degradation of their targets, ultimately resulting in reduced protein levels.

In the current study, we tested the hypothesis that miRs might regulate GR activity by reducing GR protein levels. Using a combination of *in silico* prediction of miR binding sites, miR overexpression studies, mutagenesis of the GR 3′UTR, and expression studies of miRs, we identify miR-124a as a regulator of GR activity with a possible relevance for brain GC signaling.

Materials and Methods

Animals

Long-Evans rats were bred in our colony at Leiden University (Leiden, The Netherlands). Pregnant and lactating dams were kept at 20–22 C, 55 ± 15% humidity, on a 12-h light, 12-h dark cycle (lights on at 0800 h). Neonates were killed by decapitation according to the following time course: postnatal d (PND) 1, 4, 7, and 14, with the day of birth considered PND 0. The brain was removed quickly and dissected to extract the frontal cortex (FC), hippocampus (HIPP), hypothalamus (HYP), cerebellum (CB), and pituitary (PIT). Peripheral organs such as liver and adrenals were also collected. All tissues were snap frozen and stored at −80 C until further use. All experimental procedures were approved by the local committee on animal bioethics and welfare of the University of Leiden.

Cell culture

The maintenance of NS1 cells, a PC12 subclone, has recently been described (19). A549 and COS-1 cells were cultured in DMEM 4.5 g/liter glucose plus L-glutamine plus pyruvate, supplemented with 10% fetal bovine serum, and 100 U/ml penicillin, 100 μg/ml streptomycin. The cells were incubated at 37 C in humidified air/5% CO_2. Serum was charcoal stripped to remove endogenous cortisol. P19 cells were cultured in MEM-α with 7.5% heat-inactivated newborn calf serum and 2.5% heat-inactivated fetal bovine serum supplemented with L-glutamine, streptomycin, and penicillin. Differentiation of P19 cells was induced with all-trans retinoic acid (RA) (Sigma-Aldrich Corp., St. Louis, MO). Cells were grown for 4 d in culture medium containing 5×10^{-7} M RA with medium being refreshed every in 48 h.

Transfection

Cells were transfected by nucleofection. The Basic Nucleofector Kit (Amaxa Inc., Walkersville, MD) optimized for PC12 or A549 cells was used. Transfection was performed according to the manufacturer's protocol. One microgram of pMAX-GFP plasmid, 400 ng TAT3-LUC reporter plasmid (20), and 50 nM of the pre-miR precursor of interest were used. Pre-miR precursor miRs for miR 18, 22, 124a, 328 and 524 were purchased from Ambion (local reseller Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) Cells were seeded on collagen I-coated six-well plates with 10^6 cells per well in 2 ml steroid-stripped medium. Medium was refreshed 20 h after transfection.

All tissue culture supplies and media were from Invitrogen BV (Groningen, The Netherlands).

Luciferase reporter Glucocorticoid Responsive Element (GRE)-plasmid assays

Seventy-two hour after transfection, NS1 cells were incubated with increasing concentrations (10^{-11}, 5×10^{-10}, 10^{-9}, 5×10^{-9}, 10^{-8}, and 10^{-7} M) of the GR agonist DEX. After 3 h incubation, cells were rinsed with PBS and lysed using 200 ml/well passive lysis buffer (Promega Corp., Madison, WI). Firefly luciferase activities were determined with the luciferase reporter assay system (Promega) as described by the manufacturer's protocol and as described previously (15). Protein concentration of each sample was quantified by a Pierce protein assay (Pierce, Rockford, IL; local reseller Bio-Rad Laboratories, Veenendaal, The Netherlands).

Data are reported as fold activation of luciferase activity relative to the maximal-fold activation, which was achieved after stimulation of cells with 1×10^{-7} M DEX in the absence of miR overexpression. Half maximal effective concentration (EC_{50}) values were determined from a luciferase activity plot after curve fitting by GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Western blot analysis

NS1 cells transfected with 50 nM synthetic miR were harvested for protein extraction 72 h after transfection (n = 6). Western blot was performed as previously described (11, 19). Membranes were exposed to chemiluminescent film from Eastman Kodak Co. (Rochester, NY) for 5 and 30 sec. After digitalizing the film, ImageJ software from the National Institutes of Health (Bethesda, MD) was used to quantify band intensities.

Cloning of pMIR-REPORT construct

To generate reporter vectors bearing miR 18 and 124 binding sites, target sequences were cloned in a pMIR-REPORT vector (Ambion). A rat GR 3′UTR 1050-bp fragment bearing the predicted miR-18 site was isolated from cDNA of NS1 cells by PCR. Primers used to isolate this fragment were 5′-ATTCGAGCTC AGACTTTCAGTTGGCTGG-3′ (forward primer) and 5′-GGCCACCTTAAGTAGAAATCAGA-3′ (reverse primer) (Isogen Life Science, De Meern, The Netherlands). The PCR product was purified on agarose gel and isolated. The PCR fragment was first inserted into the pGEM-T easy vector (Promega) and subsequently subcloned in the *Hin*dIII/*Spe*I site of pmiR-Report. A second 580-bp PCR fragment, bearing the predicted miR-124a binding site, was generated using 5′-GACTGATTCAAGTACTAGTCATCGTCAAAAGGGAAGGGAAC-3′ and 5′-TCAGTGAACTGCGTACGCGTATGGACTTGGGCACGGTGGTTTAG-3′ as, respectively, forward and reverse primers. The 580-bp PCR fragment was cut with *Spe*I and Ml u1, and cloned in pMIR-report. Plasmid constructs were verified with automated DNA sequencing using an ABI 3600 sequencer (Applied Biosystems).

Site-directed mutagenesis of pMIR-REPORT construct

The predicted miR-124a binding site was mutated using 5′-TGCCTTACTAAGAAAGGTTCGGTTAAAGAAAGTTGAATTTAT-3′ and 5′-ATAAATTCAACTTTCTTTAACCGAACCTTTCTTAGTAAGGCA-3′ as forward and reverse primers (mutations are *underlined*).

Mutagenesis was performed using the QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

pMIR-REPORT luciferase assay

For the luciferase assay, Cos-1 cells were seeded in a 24-well plate at a density of 50,000 cells per well. Two hundred fifty nanograms of reporter construct were cotransfected with 50 nM synthetic miR 18 or 124a with Lipofectamine 2000 (Invitrogen BV) according to manufacturer's instructions. Twenty-four hours after transfection, cells were harvested in 100 ml Passive Lysis Buffer (Promega), and 20 ml lysate was used to measure the relative light unit (RLU) with a luciferase reporter assay kit (Promega). Five milliliters of lysate were used to measure the total amount of protein via the BCA method (Pierce; local reseller Bio-Rad laboratories, Veenendaal, The Netherlands).

miR quantification

Transfection and RNA isolation

NS1 cells were transfected (n = 6) with 50 nM synthetic miR-18, miR-124a (treatment group), or the antisense miR 22 (control group) as described previously. Seventy-two hours after transfection, cells were harvested, and the RNA was isolated using TRIZOL reagent (Invitrogen BV). TRIZOL extraction of total RNA was performed according to the manufacturer's specifications for cells on monolayer. The RNA concentration and purity were determined using a NanoDrop spectrophotometer (Nano Drop products, Wilmington, DE).

RT-qPCR (quantitative PCR)

Reverse-transcriptase (RT) reactions contained 10 ng total RNA samples, 50 nM stem-loop RT primer (miR-18 or RNU6B), 1× RT buffer, 0.25 mM each of deoxynucleotide triphosphates, 3.33 U/μl MultiScribe RT, and 0.25 U/μl ribonuclease inhibitor (all obtained from Applied Biosystems). The 15-μl reactions were incubated in a GeneAmp PCR system 9700 (Applied Biosystems) for 30 min at 16 C, 30 min at 42 C, and 5 min at 85 C. For standard curves, 7, 5, 3, 2, 1, and 0.5 nM synthetic miR-18 has been used instead of 10 ng total RNA.

Real-time PCR was performed using a standard TaqMan PCR kit protocol (Applied Biosystems) on a DNA Engine Opticon 2 (MJ Research Inc., South San Francisco, CA). The 20 μl PCR included 1.33 μl RT product, 1× TaqMan Universal PCR Master Mix, 0.2 μM TaqMan miR assay 1× with preformulated forward/reverse primer, and minor groove binding probe, which binds specifically to miR-18 cDNA or RNU6B. The reactions were incubated in a 96-well plate at 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec, and 60 C for 1 min. All reactions were performed in triplicate.

Data analysis

The intraexperimental variation was examined by taking the average of the mean comparative threshold values from RNA samples, which were processed in triplicate. The mean ± SD values were converted to a linear form using the term E^{-Ct}. Statistical analysis and nonlinear curve fitting were performed using the software GraphPad Prism version 4.03. Differences among groups were evaluated using parametric one-way ANOVA and Tukey's multiple comparison test. When required, single comparisons between two groups were made using the Student's *t* test. A *P* value less than 0.05 was considered significant.

DEX-induced glucocorticoid-induced leucine zipper (GILZ) expression in A549 cells after miR-18, -124, and -22 overexpression

A549 cells were seeded at 2×10^5 cells per well and transfected with 50 nM miR-18, -22, or -124 with oligofectamine. After 48 h, cells were exposed to 5×10^{-9} M DEX or to vehicle (ethanol), and 6 h later, cells were lysed using TRIZOL, and RNA was isolated. After cDNA synthesis, GILZ expression was determined using qPCR (LightCycler 2.0; Roche Applied Science, Indianapolis, IN), and primers 5′-AGCGTGGTGGCCCTAGACAACA-3′ (forward primer) and 5′-CAACCAGCTCACGAATCTGCTCCTT-3′ (reverse primer). Data were normalized using β-actin as a housekeeping gene, and primers 5′-GGACTTCGAGCAAGAGATGG-3′ (forward primer) and 5′-GCACTGTGTTGGCGTACAG-3′ (reverse primer).

Results

In silico identification of candidate miRs

To identify candidate miRs affecting GR activity via binding to its 3′UTR site, we followed an *in silico* approach (supplemental data 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Based on the maximum likelihood of binding, conservation, and accessibility, we selected five different miRs, including 18, 22, 124a, 328, and 524, for further experimental testing (supplemental Fig. 1).

miRs 18 and 124a reduce GR protein levels in neuroscreen cells

A hallmark of miRs' action is their ability to bind to the 3′UTR of specific target mRNAs, thereby inhibiting its translation, which ultimately leads to reduced protein levels. Therefore, to make a first sifting between potential relevant and nonrelevant miRs, we transfected synthetic miRs of miR 18, 22, 124, 328, and 524 in NS1 cells, and analyzed GR protein levels by Western blot analysis (Fig. 1). None of the miRs was predicted to target α-tubulin mRNA by any of the three software packages used (see *Materials and Methods*). In line with this prediction, no significant differences among the different groups were observed for α-tubulin, and, therefore, this housekeeping protein was used to normalize for technical variations. Using 100 nM miRs, we observed a significant ($P < 0.01$) reduction of GR protein levels for miR-18 (34.7 ± 5.1% reduction *vs.* mock transfected cells, n = 3) and miR-124a (42.1 ± 6.7% reduction *vs.* mock transfected cells, n = 3), whereas miRs 22, 328, and 524 did not reduce GR protein levels (Fig. 1).

Because miR transfection efficiency of NS1 cells was between 80 and 90%, we assume that the miR 18- and 124a-induced GR protein reduction is slightly higher in individual cells.

We concluded that miRs 18 and 124a are potentially relevant miRs to reduce GR protein levels, and miRs 22, 328, and 524 are not relevant. Therefore, we focused on miRs 18 and 124a in further experiments.

miRs 18 and 124a repress GR-dependent luciferase activity

To examine the potential effect of miRs 18 and 124a on GR transactivation properties, we constructed a dose-response curve for DEX in NS1 cells that were transfected with these miRs and the TAT3-LUC reporter plasmid, a well-known reporter construct to measure GR activity (20), miR-22 was included as a negative control.

In line with our Western blot analysis, miR-22 had no effect on GR transactivation properties. miRs 18 and 124a both significantly repressed GR activity as measured by the decrease in maximum induction of the TAT3 driven luciferase activity (Fig.

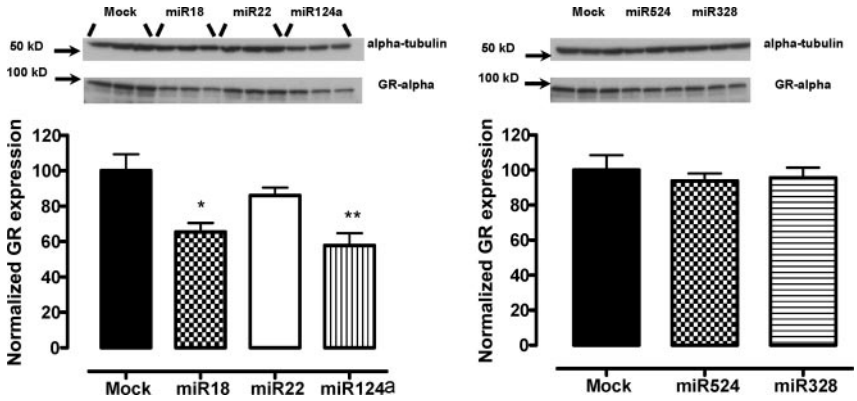

FIG. 1. miRs 18 and 124a down-regulate GR protein levels. Western blot analysis was used to measure steady-state GR protein levels. To normalize the data, the same membrane was also stained for α-tubulin. The experiment was performed with three biological samples and repeated three times with a similar outcome. A representative autoradiogram (n = 3) is shown. Overexpression of 100 nM miRs 124a and 18 (*left panel*) led to significantly decreased GR protein levels (*, $P < 0.05$; **, $P < 0.01$). Overexpression of miRs 22, 324, and 524 did not lead to significant reduction of GR protein levels (*right panel*).

2). Compared with nontreated cells, miR-18 repressed GR activity (38 ± 3.2% reduction *vs.* mock transfected cells, n = 3) and miR-124a slightly less (34.8 ± 5.2% *vs.* mock transfected cells, n = 3). Calculated EC_{50} values transformed to -log10 scale (pEC50) were not significantly different. In line with our Western blot analysis, these similar pEC50 values suggest that the repressive miR effects on GR activity are not due to reduced ligand affinity but are caused rather by a decrease in the number of receptor molecules (Fig. 2).

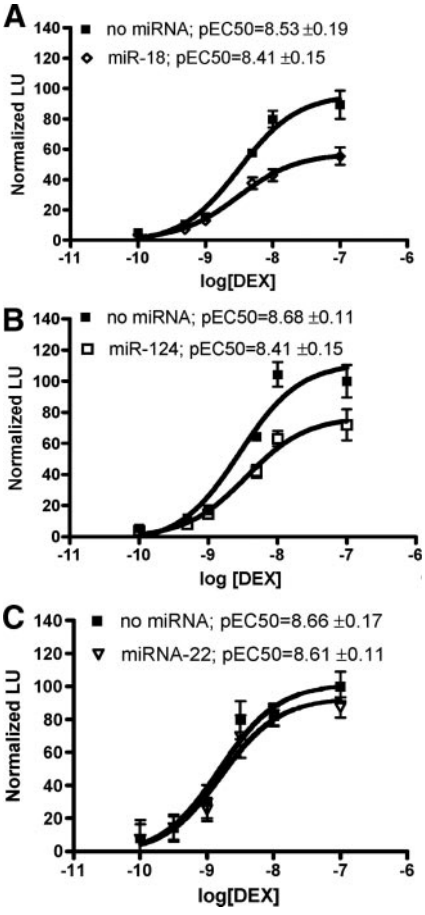

FIG. 2. miRs 18 and 124a repress efficacy of GR signaling. GR activity was measured 3 h after DEX exposure with or without 50 nM of each miR. A, miR-18. B, miR-124a. C, miR-22. DEX pEC50 values are given for each miR. Results are expressed in normalized light units (LU) as mean ± SEM of three independent biological samples assayed in triplicates.

miR-124a and -18 expression attenuates GR activation of GILZ expression

To investigate further the effect of miRs 124a and 18 on GR signaling, we overexpressed these miRs and miR-22 as a negative control in human A549 cells. We have used this cell line because it is known to express GR endogenously and to avoid cell line bias in our observations. To activate GRs we have exposed these cells to approximate EC_{50} values (5×10^{-9} M DEX for 6 h). Subsequently, cells were harvested to measure GILZ gene expression, a gene known to be induced by stress-like levels of GCs in A549 cells (21, 22). Induction of GILZ expression was not different between mock-transfected cells and miR-22 transfected cells (data not shown). DEX administration resulted in a 17-fold GILZ mRNA induction in miR-22 transfected cells compared with cells exposed to 0.1% ethanol. However, A549 cells transfected with miR-124a or miR18 exhibited significant ($P < 0.01$) reduction of this induction (69.1 ± 1.2% reduction *vs.* negative control, n = 3 and 71.6 ± 2.1% reduction *vs.* negative control, n = 3, respectively; Fig. 3). In individual cells, miR-induced repression of GR-activated GILZ expression is likely higher because the transfection efficiency of A549 cells was 80–90%. Overall, we conclude that miRs 124a and 18 attenuate GC-induced GILZ expression in A549 cells.

miR-124a binds to the GR 3′UTR

A single miR species is believed to affect several hundred different targets (23). Thus, it is possible that miRs 124a and 18

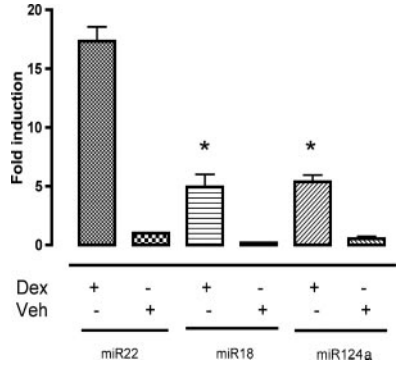

FIG. 3. miRs 124a and 18 repress GR-induced transcription of GILZ, a GR target gene. A549 cells were transfected with miR-22 (control), miR-18, or miR-124a and incubated with 5×10^{-9} M DEX for 3 h or with vehicle (Veh) (control). miRs 18 and 124a repressed DEX-induced GILZ induction (3.5 and 3.2-fold, respectively), compared with DEX-induced GILZ induction (17-fold) in the presence of miR-22. *Asterisks* indicate statistical significance ($P < 0.05$). Results are expressed as mean ± SEM of three independent biological samples assayed in triplicates.

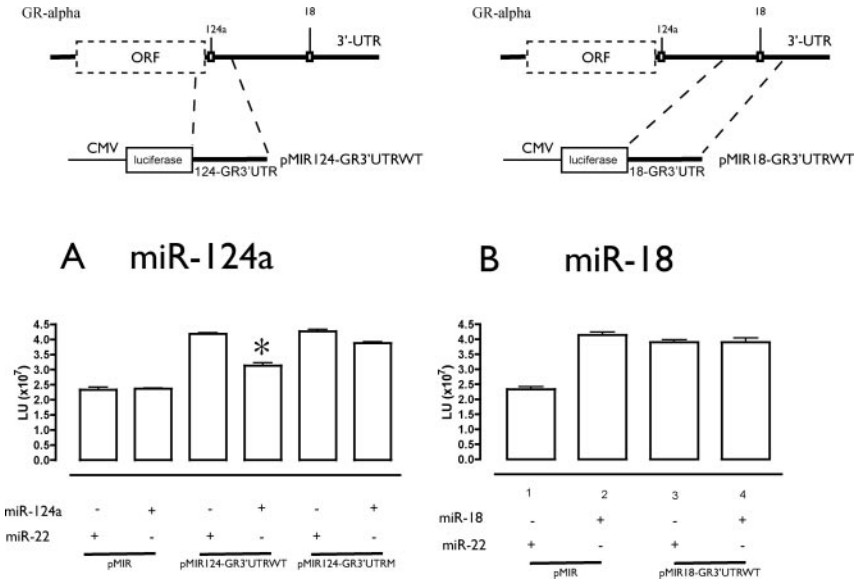

FIG. 4. Analysis of miRs 18 and 124a binding to predicted seed regions in the GR 3′UTR. A, Analysis of miR-124a binding to the GR 3′UTR. A549 cells were transfected with empty vector and miR-22 (negative control), empty vector plus miR124, pMIR124-GR3′UTRWT plus miR-22, pMIR124-GR3′UTRWT plus miR-124a, pMIR124-GR3′UTRM plus miR-22, and pMIR124-GR3′UTRM plus miR-124a. Note that miR-124a reduces luciferase activity by 25.0% compared with reporter constructs bearing mutated miR-124a seed regions. B, Analysis of miR-18 binding to the GR 3′UTR. A549 cells were transfected with empty vector plus miR-22 (negative control), empty vector plus miR-18, pMIR18-GR3′UTRWT plus miR-22, and pMIR18-GR3′UTRWT plus miR-18. Note that miR-18 overexpression leads to increased luciferase activity of the empty vector. *Asterisk* indicates statistical significance ($P < 0.01$). Results are expressed as mean ± SEM of three independent biological samples assayed in triplicates. LU, Light unit; ORF, open reading frame.

repress GR activity indirectly (*e.g.* by interacting with non-GR transcripts). To investigate this possibility, we cloned GR 3′UTR fragments containing putative miR binding sites downstream of the firefly luciferase gene that is under control of the cytomegalovirus (CMV) promoter in pMIR reporter plasmids. As negative controls we used similar reporter constructs bearing mutations in the predicted miR seed region. For miR-124a this resulted in reporter plasmids pMIR124-GR3′UTRWT and pMIR124-GR3′UTRM (negative control). As can be seen in Fig. 4A, overexpression of miR-124a did not affect expression of the reporter construct lacking 3′UTRs. Cloning GR124-3′UTR fragments led to increased luciferase activity compared with empty constructs. This may be due to the increased stability of the corresponding mRNA, a known function of 3′UTRs in general. Cotransfection of pMIR124-GR3′UTRWT with 50 nM miR-124a significantly ($P < 0.01$) repressed this luciferase activity (25.0 ± 4.4% reduction *vs.* pMIR124-GR3′UTRWT, n = 4; Fig. 4A). Mutations in the predicted miR-124a seed region abolished this repression, thus strongly indicating that miR124 binds to the predicted target site in the rat GR mRNA.

We performed similar experiments with miR-18 (Fig. 4B). Surprisingly, overexpression of miR-18 increased luciferase activity of empty reporter constructs, suggesting that miR-18 affects factors regulating CMV promoter-driven expression. As with GR124-3′UTRs, cloning of GR18-3′UTRs in pMIR (leading to pMIR18-GR3′UTRWT) led to an increase in luciferase activity (compare bars 1 and 3 in Fig. 4B). Subsequent miR-18 overexpression did not change luciferase signal (compare bars 3 and 4 in Fig. 4B), indicating that miR-18 cannot bind to pMIR18-GR3′UTRWT. Alternatively, repressive miR-18 effects might be masked by the stimulating effects miR-18 can have on CMV promoter-driven luciferase activity.

RA-induced differentiation of P19 cells is associated with up-regulation of miR-124a and GR protein down-regulation

To study possible associations of endogenous miR-124a up-regulation and GR protein levels, we have used P19 cells, an embryonic carcinoma cell line, and a well-established model to study neuronal differentiation (Fig. 5, A and B). Similarly to neuronal differentiation *in vivo* (24), miR-124a expression is highly up-regulated during RA-induced neuronal differentiation of P19 cells (25). In line with previous studies (25, 26), we found a 21-fold induction of miR-124a by 96 h exposure to RA, a factor known to induce neuronal differentiation of P19 cells (Fig. 5C). Interestingly, miR-124a up-regulation is associated with a 70% reduction of GR protein (Fig. 5D), suggesting an interaction between endogenous miR-124a and GR mRNA. To investigate further this interaction, we transfected P19 cells with pMIR124-GR3′UTRWT, pMIR124-GR3′UTRM, or empty plasmid vector (pMIR), and determined luciferase activity before and after RA-induced differentiation of P19 cells. In undifferentiated P19 cells, we did not observe a significant difference in luciferase activity between the different constructs (Fig. 5E). However, compared with pMIR, a significant ($P < 0.05$) 30% reduction in luciferase activity was found upon differentiation with pMIR124-GR3′UTRWT, whereas no significant reduction was seen with the negative control, pMIR124-GR3′UTRM (Fig. 5F).

Together, these results strongly suggest a role for miR-124a in the observed GR protein down-regulation during neuronal differentiation of P19 cells by a RNA interference-mediated mechanism.

In vivo expression levels of miRs 18 and 124a

Both miR-18 and -124a expressions have been reported during early brain development and neuronal differentiation (25, 27). In addition, early postnatal development is characterized by strong dynamic changes in the hypothalamus-pituitary-adrenal axis, known as the "stress hyporesponsive period" (28), and changes in GR protein levels in the brain. Therefore, to explore the relevance of our *in vitro* experiments, we analyzed endogenous miR expression levels at different postnatal times, *i.e.* PND 1, 4, 7, and 14 in the rat. We analyzed RNA from the HYP, HIPP, PIT, CB, FC, adrenal gland, and liver.

We found postnatal expression of miR-18 in different brain regions and at all time points studied (Fig. 6A). miR-18 not only exhibited differences in expression between different peripheral organs (adrenals and liver) and brain areas but also showed tem-

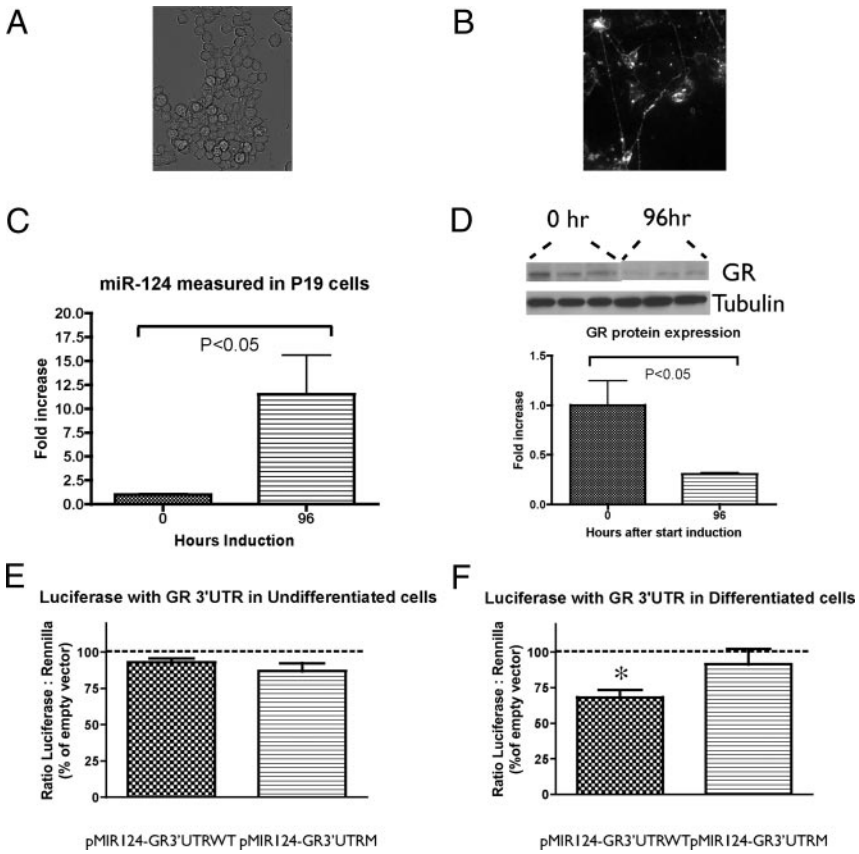

FIG. 5. Endogenous up-regulation of miR-124a in P19 cells is associated with reduced GR protein levels and reduced luciferase activity of constructs bearing GR 3′UTR. A, Representative picture of nondifferentiated P19 cells. B, Representative picture of RA-induced differentiation of P19 cells into a neuronal phenotype. C, miR-124a induction in P19 cells after 96 h (hr) exposure to RA. D, Reduction of GR protein levels after 96 h exposure to RA. A representative autoradiogram is shown (n = 3). E, Luciferase activity of pMIR124-GR3′UTRWT is not reduced compared with pMIR124-GR3′UTRM in nondifferentiated P19 cells. F, Luciferase activity of pMIR124-GR3′UTRWT is significantly reduced compared with pMIR124-GR3′UTRM in differentiated P19 cells after 96 h exposure to RA. *Asterisk* indicates statistical significance ($P < 0.05$). Results are expressed as mean ± SEM of three independent biological samples assayed in triplicates.

poral expression changes. For example, in the HIPP, high expression was found at PND 1 and 4, followed by a significant ($P < 0.01$) approximate 4-fold reduction at PND 7. At PND 14, miR-18 showed a significant increase compared with levels detected at PND 7. To assess the functional relevance of the miR measured *in vivo*, we compared miR-18 expression levels with those found in cells after transfection with an effective amount of miR-18. In transfected cells, miR-18 levels were five to seven times higher compared with CB (PND 4) or HIPP (PND 4), respectively (Fig. 6B).

We found a marked tissue-dependent differential expression of miR-124a (Fig. 6C). miR-124a is expressed at relatively high levels in all the brain areas (HYP, HIPP, CB, and FC), when compared with nonbrain tissues (PIT, adrenal, and liver). These findings are in line with previous studies that characterized miR-124a as a brain-specific miR (for review, see Ref. 16). At PND 1, different brain regions appeared to express similar levels of miR-124a, with somewhat higher levels in the FC. As with miR-18, temporal changes in miR-124a expression were observed in the HIPP with an abrupt increase at PND 7 ($P < 0.05$), followed by a significant decrease at PND 14 ($P < 0.01$). A similar pattern was observed in the FC, but not in the other brain areas (Fig. 6C). We further compared expression of miR-124a observed in transfected cells with brain tissue *in vivo* levels. Overall, we found that *in vivo*, expression levels of miR-124a were significantly higher ($P < 0.01$) than levels measured in transfected cells as early as PND 1 but also at later time points in all brain areas studied (Fig. 6D). These findings indicate that a substantial amount of miR-124a is expressed in brain cells that potentially could affect GR activity in the early phases of brain development.

Discussion

The current study tested the hypothesis that miRs can regulate GR responsiveness by reducing GR protein levels. *In silico* prediction revealed that miRs 18 and 124a may bind GR mRNA and decrease GR activity. These miRs were tested for their ability to alter translational activity of GR and reduce GR protein levels in cell cultures *in vitro*. We report that: 1) miR-18 and -124a overexpression reduced GR protein levels, 2) miR-18 and -124a overexpression attenuated GR-mediated transactivation, 3) miR-18 and -124a overexpression reduced the induction of the well-known GR-target gene GILZ, 4) miR-124a is able to bind to the predicted seed region in the GR 3′UTR, 5) *in vivo* expression levels of miR-124a in brain tissues is higher than what is necessary *in vitro* to reduce GR protein level, and 6) neuronal differentiation of P19 cells is associated with strong miR-124a up-regulation and down-regulation of GR protein levels. Together, the current data provide strong evidence to support our hypothesis.

miR-18 and GR translational activity and protein levels

miR-18 was found to reduce GR translational activity but did not affect EC_{50} values, suggesting reduced GR protein levels. In support of these findings, and in line with a recent paper describing inhibiting effects of miR-18 on GR mRNA translation (29), GR protein levels were reduced by miR-18. However, our luciferase-GR 3′UTR reporter assay data suggest an indirect effect on GR transcriptional activity because miR-18 did not repress luciferase activity. Alternatively, the predicted miR-18 binding site may be unavailable for miR-18 by differential tertiary RNA folding of the luciferase-GR 3′UTR, which might be different from folding of the GR mRNA *in vivo*.

miR-18 has been expressed in the developing brain (27). Our qPCR analyses indicated a 5- to 7-fold lower miR-18 expression level in different brain areas at different postnatal time points when compared with miR-18 levels observed in transfected cells

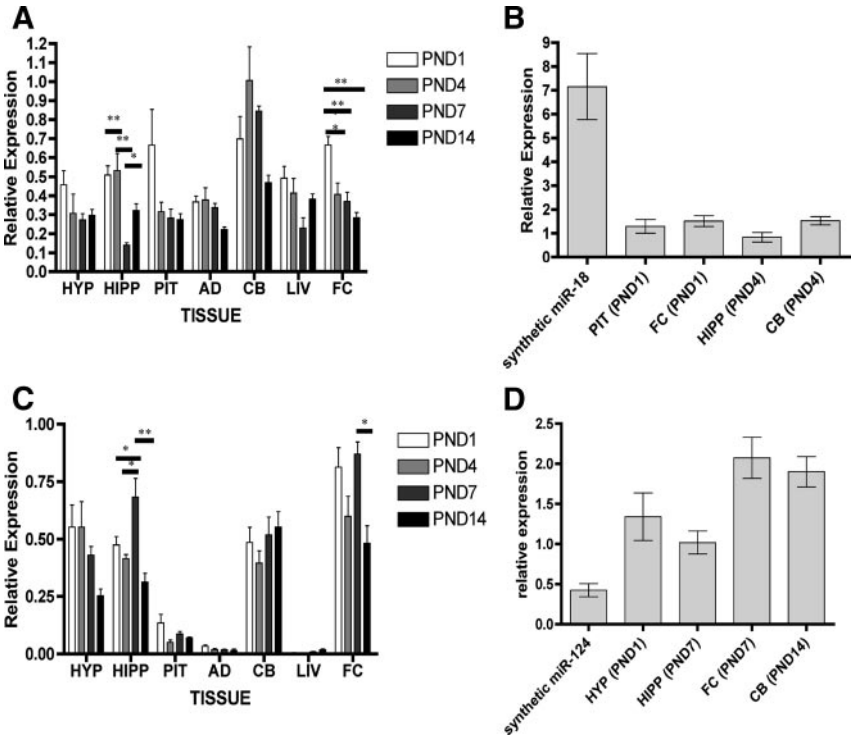

FIG. 6. miR-18 and 124a expression during postnatal development. Values were obtained by real-time qPCR analysis using snoRNA202 as an internal control. The statistical test used for comparison was one way-ANOVA with Tukey's *post hoc* (with n = 4 or 5). *Asterisks* indicate statistical significance: *, $P < 0.05$; **, $P < 0.01$. Results are expressed as mean ± SEM. A, miR-18 expression during postnatal development. miR-18 expression in the HIPP at PND 7 is significantly lower compared with PND 1 and 4. miR-18 is significantly higher at PND 1 compared with PND 4, 7, and 14 in the FC. B, *In vivo* miR-18 expression levels compared with miR-18 levels in transfected cells (synthetic miR-18). C, miR-124a expression during postnatal development. miR-124a expression in the HIPP at PND 7 is significantly higher compared with PND 1 and 4, and with PND 14. miR-124a is significantly higher at PND 7 compared with PND 14 in the FC. D, *In vivo* miR-124a expression levels is higher compared with miR-124a levels in transfected cells (synthetic miR-124a). For further details, see *Results*. AD, Adrenals; LIV, liver.

in vitro. Given that miR-18 expression is cell type specific (25, 30) and that the postnatal brain areas studied contain many different cell types, we estimate that (low) miR-18 expression levels found in the brain are likely to be a considerable underestimation of the expression levels in particular cell types. Moreover, DNA microarray studies showed highest miR-18 expression at early embryonic developing mammalian brain development [embryonic d 10 (27)]. Therefore, our expression data do not preclude a potential miR-18 effect on GR activity at specific time points or in specific neuronal cell types.

Although not directly related to our hypothesis on brain GR, it is interesting to note that miR cluster 17–92 up-regulation, including miR-18, has been causally related to small cell lung cancer (31, 32). In small cell lung cancer, reduced GR levels have been associated with GC resistance, which can be reversed by GR overexpression (33). Together with the present data, these findings suggest a mechanism in which miR-18 up-regulation might cause GR protein down-regulation, resulting in GC resistance of small cell lung cancer cells. However, this hypothesis remains to be tested.

miR-124a and GR translational activity and protein levels

miR-124a was found to reduce GR protein levels and interfere with GR translational activity *in vitro*. We show that luciferase activity produced by luciferase-GR 3′UTR reporter constructs was repressed but not when the miR-124a predicted seed region was mutated. In long 3′UTRs, as is the case in the mammalian GR mRNA (>4 kb), miR target sites tend to cluster near the start or near the end of the 3′UTR (34). In line with this, the predicted target site of miR-124a is close (within 20 nucleotides) to the start of the GR 3′UTR. Altogether, these data clearly show that the GR mRNA is a genuine and direct miR-124a target *in vitro*, and given our results with P19 cells and miR-124a expression in the brain, most likely also *in vivo*.

We found miR-124a to be specifically expressed in brain tissue at relatively high levels, which is in agreement with previous studies (35). Although it is difficult to directly compare quantification of transfected cell line RNA with that of brain tissue RNA, our estimation is that *in vivo* miR-124 expression levels exceed those found in our miR-124-transfected cell lines (known to effectively repress GR translation and reduce GR protein levels). Therefore, we suggest that amounts found in the brain could be effective on GR translational activity and GR protein levels.

Interestingly, miR-124a has been proposed to repress nonneuronal target genes, thereby promoting a neuronal phenotype (36). Its targets include small C-terminal domain phosphatase mRNA, an antineuronal factor expressed in nonneuronal tissues (37), RE1 silencing factor (38), a transcriptional repressor of neuronal target genes, including the miR-124a gene (39), and PTBP1, a general repressor of alternative splicing in nonneuronal cells (40). The repression of nonneuronal factors by miR-124a correlates with findings in the current literature on the role of GR in neuronal differentiation during development. For example, administration of GCs to pregnant rats decreases the proliferation of neural stem cells (3). In the dentate gyrus of the adult HIPP, administration of GCs inhibits neuronal differentiation of progenitor cells, and pharmacological experimentation suggests that these events are mediated by GR (41–43). Thus, proper neuronal differentiation of progenitor cells may require narrow limits of GC responsiveness, and our data suggest that miR-124a action on GR may be implicated. These findings are also important in light of the body of evidence showing that GCs play a crucial role in growth and differentiation of the developing brain (41, 42), and for epidemiological findings showing long-lasting alterations of physiological and neural functions in individuals treated with the GC analog DEX, such as prematurely new born infants.

Dynamic expression of miRs 18 and 124a during PND 1-14

Temporal variation in GR levels may complement the altered adrenal responsiveness, and ensuing low GC exposure of the

neonate brain during the SHRP. During this period (PND 4-14), neonates do not respond to mild stressors with an elevation in GC levels (28, 44). Interestingly, specific patterns of expression were observed at the onset of the SHRP in the HIPP. Although miR-18 displays a significant decrease in levels of expression from PND 4–7, the opposite pattern was observed for miR-124a. During development the GR protein level in the hippocampal CA3 cell field shows a down-regulation around PND 7 (45). Therefore, it would be of interest in future studies to examine the causal relationship between the changes in miR-124 and -18 expression with GR protein levels in HIPP, an area involved in learning and memory processes undergoing active growth and differentiation during the first 2 wk of life.

Implications for GC responsiveness

GR mRNA and GR protein levels can be regulated by different mechanisms. At the transcriptional level, GR mRNA is regulated by numerous factors, including coactivators and corepressors (46). At the protein level, GR isoforms can be generated in a cell type-specific manner, and each of these GR isoforms may target specific sets of genes (9, 10, 47). Moreover, posttranslational modifications, such as phosphorylation, can affect GR activity (48, 49). Our data indicate that another class of molecules, the miRs, can also affect GR activity via regulation at the protein level. Because several miRs, like miR-124a, are expressed in a cell type-specific manner, they may be part of the mechanism underlying regulation of GC responsiveness in specific tissue and cell type.

Although correlative, our current findings also suggest that miRs may play a role during development by altering responsiveness to GCs in the HIPP and other brain areas. Finally, the modulation of GR translation activity and GR protein production by miRs may, in a more general manner, underlie changes in GC responsiveness, a fundamental factor in the etiology of stress-related disorders and GC resistance.

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