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Both insufficient and excessive glucocorticoid receptor-mediated signaling impair neuronal migration

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Abstract

Glucocorticoids (GCs) are a class of steroid hormones that regulate numerous physiological events in the human body. Clinically, glucocorticoids are used for anti-inflammatory and immunosuppressive actions via binding with glucocorticoid receptors (GRs). Emerging evidence has also indicated that inappropriate GC and GR levels are detrimental for brain development and eventually lead to severe neurological diseases. However, the roles of GC/GR signaling in brain development are not fully understood. Here, we showed that stable GR expression levels were critical for brain development, because both GR knockdown and overexpression severely impaired neuronal migration. Further studies showed that the multipolar–bipolar transition and leading process development were interrupted in GR-knockdown and GR-overexpressing neurons. To elucidate the underlying mechanism, we screened the protein levels of downstream molecules and identified RhoA as a factor negatively regulated by the GR. Restoration of the RhoA protein level partially rescued the neuronal migration defects in the GR-knockdown and GR-overexpressing neurons, indicating that RhoA played a major role in GR-mediated neuronal migration. These data suggest that an appropriate level of GC/GR signaling is essential for precise control of neuronal migration.

Key Words

- ▶ neocortex
- ▶ neuronal migration
- ▶ GR
- ▶ glucocorticoids
- ▶ RhoA

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Introduction

Glucocorticoids (GCs) have profound influences on many physiological functions, including growth, metabolism, development, behaviors and stress reactions (Blodgett *et al.* 1956, Munck *et al.* 1984, Haskett 1985). They also exert anti-inflammatory and immunosuppressive effects and thus are used as therapeutic agents (Coutinho & Chapman 2011). The major actions of GC are mediated by the glucocorticoid receptor (GR), which is a transcriptional regulator that is ubiquitously expressed in mammalian cells. Three GR isoforms are generated by alternative splicing. GR α is the main receptor of GCs,

whereas GR β and GR γ function as regulators of GR α activity (Oakley & Cidlowski 2013). Once bound to GCs, activated cytoplasmic GRs translocate to the nucleus and regulate gene expression through either direct interaction with specific promoter sequences or protein–protein interactions with other transcription factors (Mitre-Aguilar *et al.* 2015). Although GC/GR signaling-mediated gene expression is clinically significant, excessive exposure to this signal can also lead to severe side effects.

Clinical and epidemiological studies have demonstrated that early adversity-triggered GC elevation

or exposure to excessive GCs is detrimental for brain development and function. For instance, maternal stress-triggered GC elevation changes the fetal brain structure, leading to attention and learning deficits in adulthood (Weinstock 2008). Prenatal stress is highly associated with increased anxiety disorders in adolescence and an increased risk of psychiatric disorders in adulthood (Phillips *et al.* 2005, Fineberg *et al.* 2016). Antenatal GC exposure reduces the whole cortex convolution index and leads to a smaller cortical surface area in infants (Modi *et al.* 2001). In addition, decreased GR expression is involved in schizophrenia, which is a developmental disease characterized by early developmental defects followed by progressive clinical symptoms (Webster *et al.* 2002). Studies with mouse models have also confirmed that aberrant GR expression significantly affects neurogenesis, synaptic plasticity and behavioral responses (Erdmann *et al.* 2008, Garabedian *et al.* 2017). However, research attention has been focused on exploring GC/GR-related neurological disorders, and thus, the physiological roles of GCs/GRs in brain development, especially early brain development, are not fully understood.

Previous study has shown that gestational exposure to excessive GCs suppresses neuronal migration (Fukumoto *et al.* 2009), which is a key step in establishing correct cortical lamination. Cortical neurons originating from the ventricular zone (VZ) and subventricular zone (SVZ) follow an inside-out pattern to reach their proper cortical locations in the cortical plate (CP) (Nadarajah *et al.* 2001, Kriegstein & Noctor 2004). Disturbed neuronal migration is associated with a variety of cognitive diseases, such as mental retardation and schizophrenia (Gleeson & Walsh 2000, McManus & Golden 2005, Muraki & Tanigaki 2015). The expression levels of multiple molecules critical for cell migration have been shown to be regulated by GC/GR signaling, including RhoA (Rubenstein *et al.* 2003), L-Caldesmon (Mayanagi *et al.* 2008) and ERK1/2 (Kassel *et al.* 2001, Osterlund *et al.* 2014). However, GR-mediated gene expression exhibits cell type specificity and differences in GC sensitivity (Whirledge & DeFranco 2018). Therefore, it is of interest to study the role of GC/GR signaling in neuronal migration and the underlying mechanism.

Here, we studied the role of GR in neuronal migration using *in utero* electroporation. Both GR knockdown and overexpression impaired neuronal migration by interrupting the multipolar-bipolar transition. We also found that *RhoA* expression was negatively regulated by the GR. Restoration of the RhoA level partially rescued the neuronal migration defects caused by GR

knockdown or overexpression. Thus, GR-mediated transcriptional regulation is critical for brain development.

Materials and methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Northeast Normal University, China. All mice were bred on the C57BL/6 background. Pregnant mice used for *in utero* electroporation were prepared by mating females with males overnight. Briefly, we bred 2 females (>8 weeks) and 1 male (>8 weeks) per cage overnight (20 h to 8 h, 12 h in total) and then separated the male from the females the next morning. Thereafter, the female mice were raised in isolated cages for 2 weeks before we checked the status of the female mice. Visually pregnant mice were used for *in utero* electroporation at the indicated time point. Otherwise, the female mice were subjected to a new round of mating. Noon on the day after breeding was considered embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0).

Plasmids

For the RNAi experiments, GR small hairpin RNAs (shRNAs) were generated using BLOCK-iTTM RNAi Designer (Invitrogen) and inserted into the pSUPER vector. The targeting regions of the GR shRNAs are shown below:

GR shRNA187, 5'-GCAGCAGAGGATTCTCCTT-3' and GR shRNA417, 5'-CAGACTTTCGGCTTCTGGA-3'.

The RhoA shRNAs were also generated using BLOCK-iTTM RNAi Designer and inserted into the PLL3.7 vector. The targeting regions of the RhoA shRNAs are listed below:

RhoA shRNA99, 5'-GGATGCGTTCTTGAGCAATCG-3' and RhoA shRNA1442, 5'-GCTTTCCTTGTTAACAGACTC-3'.

The GFP-hGR plasmid was previously constructed by Carey *et al.* (1996) and purchased from Addgene.

Antibodies and drugs

The following primary antibodies were used: rabbit polyclonal anti-GR (sc-1004, 1:100), mouse monoclonal anti-GSK3 α/β (sc-7291, 1:400) and rabbit polyclonal anti-Caldesmon (sc-15374, 1:200) from Santa Cruz Biotechnology; mouse monoclonal anti-glucocorticoid

receptor (BuGR2) (ab2768, 1:500), chicken polyclonal anti-MAP2 (ab5392, 1:2000), chicken polyclonal anti-GFP (ab13970, 1:1000), mouse monoclonal anti-RhoA (ab54835, 1:200) and mouse monoclonal anti-class III β -tubulin (Tuj1) (ab7751, 1:1000) from Abcam; mouse monoclonal anti-GAPDH (HC301, 1:5000) and mouse monoclonal anti- α tubulin (HC101, 1:5000) from Transgene; and rabbit polyclonal phospho-GSK3 β (Ser9) (#9336, 1:1000), rabbit monoclonal anti-phospho-NF- κ B p65 (Ser536) (#3033, 1:1000), mouse monoclonal anti-NF- κ B p65 (#6956, 1:1000), rabbit polyclonal anti-pERK1/2 (#9101, 1:1000) and mouse monoclonal anti-ERK1/2 (#4696, 1:1000) from Cell Signal. Dexamethasone (DEX, D4902) and mifepristone (MIF, M8046) were purchased from Sigma-Aldrich.

Immunoblotting

Brain tissues or cultured cells were homogenized in optimized radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM NaF, 1 mM Na₃VO₄ and a protease inhibitor cocktail). The lysates were separated by SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated with diluted primary antibody overnight at 4°C, followed by incubation with the appropriate HRP-conjugated secondary antibody. The ECL Prime Western Blot Detection reagent (GE Healthcare) was used to visualize the chemiluminescent signals. Tanon-5200 (Tiangen (Beijing) Biotech Co., Ltd.) was used to detect the ECL signal at appropriate exposure time points. The ImageJ (NIH) software was used to quantify the band intensity, and the value was normalized to the α -tubulin or GAPDH expression level in each sample. The pp65, pERK and pGSK3 β expression levels were normalized to those of p65, ERK and GSK3 β , respectively.

Cell culture and transfection

HEK293T and NLT cells (a normal gonadotropin-releasing hormone (GnRH) neuronal cell line transfected with the large T antigen) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin-streptomycin. The HEK293T cells were transfected with GFP or GFP-hGR using polyetherimide (PEI), and the NLT cells were transfected with a scrambled shRNA or

Figure 1

GR expression pattern in the developing neocortex. (A) Immunoblotting of GR in cerebral cortex lysates at the indicated developmental stages. (B) Quantitative results from A. The data are from three independent experiments. (C) Immunostaining of GR in E15.5 brain sections. Scale bar: 100 μ m. (D) Immunoblotting of GR in cultured cortical neurons. (E) Quantitative results from D. The data are from three independent experiments.

GR shRNA 417 using an Amaxa electroporation protocol. Cortical neurons were cultured as previously described (Yu *et al.* 2015).

Real-time PCR

Total mRNA was extracted from NLT or HEK293T cells using the TRIzol reagent (Invitrogen). Complementary DNA was synthesized using the TranScript One-Step gDNA Removal and cDNA Synthesis SuperMix (AT-311-03, TransGen Biotech, Beijing, China) according to the manufacturer's protocol. Real-time PCR was performed with SYBR Premix Ex Taq™ II (RR820A, Takara) using the ABI StepOnePlus Real-Time PCR System. Each sample was analyzed in triplicate, and the mRNA levels were normalized to the GAPDH mRNA level using the $2^{-\Delta\Delta CT}$ method. Melting curves were automatically recorded and analyzed for each reaction. The following primers were used:

Figure 2

GR knockdown impaired neuronal migration in the developing neocortex. (A) NLT cells were transfected with a scrambled or GR shRNA for 48 h. Cell lysates were extracted for GR immunoblotting to examine the knockdown efficiency of the GR shRNAs. (B) Quantification of the GR shRNA knockdown efficiency. The data are from three independent experiments. Each shRNA group was compared with the scrambled shRNA group using Student's *t*-test. (C) Representative images of mouse cortical sections electroporated with the indicated plasmids at E15.5 and examined at E18.5. Scale bars: 100 μm. (D) Quantification of the GFP+ cell distribution in the E18.5 brain sections. One-way ANOVA followed by the LSD *post hoc* test was used for the statistical analysis. The brain sections were from at least three embryos of independent litters. (E) Representative images of neurons that electroporated with indicated plasmids at P7. The red arrow heads indicated the mis-located neurons. (F) Quantification of GFP+ neurons that located in layers IV-VI and WM (white matter). Student's *t*-test was used for statistical analysis. The brain sections were from at least three embryos from independent litters. (G, H and I) Quantitative analysis of the soma perimeter (G), number of dendrite branches (H), and total lengths of all branches (I) of the GFP+ neurons. The numbers of brain sections or cells scored in each quantification result are indicated on the graphs. All data are shown as the mean ± S.E.M. ns, no significant difference; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

RhoA (*Homo sapiens*), forward 5'-CTGGTGATTGTTGGTGATGG-3'; reverse 5'-GCGATCATAATCTTCCTGCC-3'. GAPDH (*Homo sapiens*), forward 5'-ATGACCCCTTCATTGACCTCA-3'; reverse, 5'-GAGATGATGACCTTTTGGCT-3'. RhoA (*Mus musculus*), forward 5'-GTGATTGTTGGTGATGGAGC-3'; reverse 5'-CTCGTGGCCATCTCAAAAAC-3'. GAPDH (*Mus musculus*), forward 5'-TGTGTCCGTCGTGGATCTGA-3'; reverse 5'-CCTGCTTACCACCTTCTTGA-3'.

In utero electroporation

In utero electroporation was performed as previously described with modifications (Kawauchi *et al.* 2003,

Yu *et al.* 2012). Briefly, plasmids were microinjected into the lateral cerebral ventricle of E15.5 mouse embryos with a glass micropipette. Then, a 37V square-wave pulse was delivered 5 times through the ECM-830 (BTX, Holliston, MA, USA). The GR knockdown/overexpression plasmid and the corresponding control plasmid were microinjected into different embryos from the same litter. The EGFP-expressing plasmid containing the cytomegalovirus (CMV) early enhancer element and the chicken β-actin (CAG) promoter was co-transfected to show neuronal morphology. Then, the embryos were allowed to develop to E18.5 or P7 for brain isolation. At least three electroporated embryos from three pregnant

mice were subjected to statistical analysis. For each electroporated embryo, three coronal sections around the somatosensory cortex were quantified for the statistical analysis.

Immunostaining

The brain tissues were fixed with 4% PFA/PBS overnight at 4°C and dehydrated in 30% sucrose in PBS for 2 days. Brain sections were prepared with a freezing microtome at a 16 µm thickness and preserved at -80°C. For immunostaining, frozen sections were washed with PBS,

and antigen retrieval was performed in 0.01M sodium citrate buffer (pH 6.0) at 98°C for 5 min. Then, the sections were blocked with 2% BSA in 0.2% Triton X-100/PBS for 1 h, followed by incubation with primary antibodies overnight at 4°C. After washing in PBS supplemented with 0.1% Tween-20, the brain sections were incubated with appropriate fluorochrome-conjugated secondary antibodies for 1 h.

Imaging and quantification

The immunostained sections were observed under an Olympus FV1000 View confocal microscope with the FV1000-ASW 3.0 Viewer software or under a Zeiss LSM 780 confocal microscope with the ZEN 2012 software. Only the brightness, contrast and color balance were optimized after imaging. The numbers of EGFP+ cells in different regions of the neocortex were counted using the ImageJ software. Bipolar cells were defined as cells with one or two processes, and multipolar cells were defined as cells with three or more processes. All counting and measurements were performed in a blinded fashion. The statistical analysis was performed using the SPSS 17.0 software. The unpaired two-tailed Student's *t*-test following the normality distribution test ($P > 0.05$) was used for comparisons between two groups. One-way ANOVA followed by the LSD *post hoc* test was used for comparisons between three or more groups. The data are presented as the mean ± standard error of the mean (S.E.M.). P values ≤ 0.05 are considered statistically significant.

Figure 3

GR knockdown impaired the multipolar-bipolar transition of migrating neurons. (A) Representative images (left panels) of electroporated neurons in the lolZ in the E18.5 cortex. The cell morphology (right panels) was delineated based on GFP staining. (B) Quantification of process numbers of GFP+ cells in the lolZ. (C) Representative images of electroporated neurons in the upIZ in the E18.5 cortex. The cell morphology (right panels) was delineated based on GFP staining. (D) Percentages of GFP+ neurons exhibiting bipolar or multipolar morphology in the upIZ of the E18.5 mouse cortex. (E) Representative images of transfected neurons in the CP in the E18.5 cortex. The cell morphology (right panels) was delineated based on GFP staining. (F) Quantification of the lengths of the leading processes for GFP+ cells in the CP. The numbers of brain sections or cells counted in these experiments are indicated on the graphs. The brain sections and cells were from at least three embryos from independent litters. All data are shown as the mean ± S.E.M. Student's *t*-test was used for the statistical analysis for all data in this figure. ns, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

The GR is stably expressed in the developing neocortex

To study the role of GRs during brain development, first we analyzed the GR expression pattern in the developing neocortex. Western blotting showed that GR expression was maintained at a relatively stable level from E13.5 to P7 (Fig. 1A and B), which was consistent with a previous report (Meaney *et al.* 1985). Next, we performed immunostaining to examine the GR distribution in the cerebral cortex at E15.5. GR showed strong positive staining in the upper intermediate zone (upIZ) and CP and relatively weak positive staining in the lower intermediate zone (loIZ) (Fig. 1C), suggesting that GRs might play

Figure 4

GR overexpression delayed neuronal migration in the developing neocortex. (A) Immunoblotting for GFP in GFP-hGR or GFP plasmid-transfected cell lysates. (B) Representative images of mouse cortical sections electroporated with the indicated plasmids at E15.5 and examined at E18.5. Scale bars: 100 μ m. (C) Quantification of the GFP+ cell distribution in the E18.5 brain sections. The brain sections were from at least three embryos from independent litters. (D) Representative images of neurons transfected with the indicated plasmids at P7. (E, F and G) Quantitative analysis of the soma perimeter (E), number of dendrite branches (F) and total lengths of all branches (G) of the GFP+ neurons. The numbers of brain sections or cells used in these experiments are indicated on the graphs. All data are shown as the mean $\pm$ s.e.m. Student's *t*-test was used for the statistical analysis for all data in this figure. ns, no significant difference; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

important roles in post-mitotic neurons in the developing neocortex (Jossin & Cooper 2011). Costaining for the GR and MAP2, which is a neuron dendritic process marker, revealed that the GR was mainly distributed in the nucleus in the upIZ and in both the nucleus and cytoplasm in the CP (Fig. 1C). In addition, we examined the GR protein levels in cultured neurons, which were relatively constant based on Western blotting (Fig. 1D and E).

GR is required for neuronal migration in the neocortex

Since the GR was strongly expressed in post-mitotic neurons and was previously implied to affect neuronal migration, we focused on neuronal migration to investigate the roles of GRs in the developing neocortex. RNA interference (RNAi) was applied to knockdown GR expression. The immunoblotting results showed that GR shRNA417 had a better knockdown efficiency than GR shRNA187 in NLT cells (Fig. 2A and B). Thus, GR shRNA417 was used for all subsequent experiments. GR shRNA417 and the scrambled shRNA were electroporated into the E15.5 cerebral cortex, and then the electroporated brains were

examined at E18.5, which was a suitable time window for the neuronal migration assay. The brain sections were stained with an anti-GFP antibody, and the distribution of GFP+ cells was quantified (Fig. 2C). The quantitative results showed that GR knockdown led to a slight increase in GFP+ cells in the VZ/SVZ, a major increase in GFP+ cells in the IZ, and a significant decrease in GFP+ cells in the CP (Fig. 2D). These defects were fully rescued by coexpression of human GR (hGR), which confirmed the knockdown specificity of GR shRNA417. These results suggested that the GR played an important role in neuronal migration.

To study whether the migration defects in GR-knockdown neurons were temporary effects, we performed *in utero* electroporation at E15.5 and examined the brain slices at P7. In contrast to the control group in which almost all GFP+ cells were located at layers II/III, more than 10% of GR-deficient neurons were located in deeper layers, revealing that GR knockdown disrupted the final positions of these neurons (Fig. 2E and F). Additionally, we analyzed the dendrite morphology of the GFP+ neurons. Although the soma perimeter was not affected (Fig. 2G), the branch numbers and total dendrite

lengths were significantly decreased in the GR-knockdown neurons even though they were localized at proper laminar positions (Fig. 2H and I). These results suggested that GR knockdown impaired neuronal migration and dendritic arborization.

GR knockdown disrupts the multipolar-bipolar transition of migrating neurons

During neuronal migration, postmitotic neurons possess a multipolar morphology in the loIZ and then establish a bipolar morphology in the upIZ. The multipolar–bipolar transition of migrating neurons is critical for promoting neuronal migration. Since we observed an abnormal

accumulation of neurons in the IZ, the morphology of these neurons was analyzed to assess whether GR knockdown affected the multipolar-bipolar transition. The number of processes of GFP+ cells in the loIZ was comparable between the GR knockdown and control groups (Fig. 3A and B), indicating that GR knockdown had little impact on neurogenesis. In the upIZ, most of the control neurons had a long leading process pointing towards the pial surface and thus exhibited typical bipolar morphology. Conversely, the GR-knockdown neurons had multiple short processes and exhibited multipolar morphology (Fig. 3C). The statistical analysis showed that GR knockdown significantly increased the proportion of multipolar neurons and decreased the proportion of bipolar neurons in the upIZ (Fig. 3D). To explore whether GR knockdown generated a sustained morphological defect, we analyzed the morphology of GFP+ neurons in the CP. Unlike the control neurons, which exhibited a typical bipolar morphology with a long leading process towards the pia, the GR-deficient neurons exhibited an abnormal bipolar morphology with a significantly shorter leading process (Fig. 3E and F). These data suggested that the GR was critical for the multipolar–bipolar transition.

GR overexpression temporarily impairs neuronal migration

As mentioned previously, excessive GC exposure also leads to brain malformation. Therefore, we further investigated the role of GR when overexpressed (Fig. 4A). We found that GFP-hGR overexpression also impaired neuronal migration (Fig. 4B). The statistical analysis showed that GR overexpression led to a slight increase in GFP+ cells in the VZ/SVZ, a significant increase in GFP+ cells in the IZ, and a significant decrease in GFP+ cells in the CP (Fig. 4C), which was similar to the effects of GR knockdown. Then, we examined the final positions of GFP-hGR-overexpressing neurons at P7. All electroporated neurons were located at layers II/III in both the GFP and GFP-hGR groups (Fig. 4D). These data suggested that GR overexpression only temporarily impaired neuronal migration without interrupting their proper localization. In addition, we analyzed the dendrite morphology of GR-overexpressing neurons. Although the soma perimeter was comparable between the GFP and GFP-hGR groups (Fig. 4E), the numbers of branches and the total dendrite lengths were both decreased by GR overexpression (Fig. 4F and G).

Figure 5

GR overexpression impaired the development of leading processes. (A) Representative images of transfected neurons in the loIZ in the E18.5 cortex. The cell morphology (right panels) was delineated based on GFP staining. (B) Quantification of process numbers of GFP+ cells in the loIZ. (C) Representative images of transfected neurons in the upIZ in the E18.5 cortex. White arrow heads indicate neurons with poorly developed neurites. The cell morphology (right panels) was delineated based on GFP staining. (D) Percentages of GFP+ neurons that exhibited bipolar or multipolar morphology in the upIZ of the E18.5 mouse cortex. (E) Representative images of transfected neurons in the CP in the E18.5 cortex. The cell morphology (right panels) was delineated based on GFP staining. (F) Quantification of the lengths of the leading processes for the GFP+ cells in the CP. The numbers of brain sections and cells counted in these experiments are indicated on the graphs. The brain sections and cells were from at least three embryos from independent litters. All data are shown as the mean $\pm$ S.E.M. Student's *t*-test was used for the statistical analysis for all data in this figure. ns, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

GR overexpression damages the bipolar morphology of migrating neurons

To explore how GR overexpression slowed down neuronal migration, we analyzed the morphology of GFP+ neurons in the loIZ and upIZ. As shown in Fig. 5A and B, the process number of GFP+ cells in the loIZ was comparable between the GFP and GFP-hGR groups, suggesting that GR overexpression had little effect on neuritogenesis. However, in the upIZ, the GFP-hGR-transfected neurons exhibited an aberrant morphology with poorly developed processes (Fig. 5C). Substantially more multipolar neurons were found in the GFP-hGR group than in the control group (Fig. 5D). In the CP, the lengths of the leading processes were decreased in the GFP-hGR-transfected neurons compared with those of the control neurons (Fig. 5E and F). These data suggested that GR overexpression damaged the bipolar morphology of migrating neurons.

GR negatively regulates *RhoA* expression

Intriguingly, both GR knockdown and overexpression impaired neuronal migration. Since GR functions as a ligand-dependent transcription factor, we analyzed changes in downstream target genes. Based on the literature, genes regulated by GRs can exert a wide range of effects on cell adhesion, cytoskeleton reorganization and signaling cascades. Thus, first we screened the protein levels of RhoA, L-Caldesmon, p65 and ERK1/2, which were reported to be regulated by GRs and were implicated previously in neuronal migration (Table 1). In GR shRNA417-transfected NLT cells, the RhoA level was increased and the L-Caldesmon level was decreased (Fig. 6A and B), whereas the protein levels of the other candidates were comparable to those of the scrambled shRNA group. On the other hand, in the GFP-hGR-overexpressing HEK293 cells, only the RhoA level was significantly decreased, whereas the protein levels of the other candidates did not change (Fig. 6C and D). To further confirm that the change in RhoA resulted from regulation of gene expression, qPCR analysis was applied to examine the *RhoA* mRNA levels in the previously mentioned cells. Consistent with the immunoblotting results, GR knockdown increased the *RhoA* mRNA level and GR overexpression decreased *RhoA* transcription (Fig. 6E and F). Moreover, immunoblotting of NLT cells treated with dexamethasone (DEX, an agonist of GR) or mifepristone (MIF, an antagonist of GR) confirmed that RhoA was negatively regulated by GC/GR signaling

(Fig. 6G and H). Altogether, the GR levels had major impact on *RhoA* expression.

Restoration of the *RhoA* level partially rescues the neuronal migration defect caused by GR manipulation

RhoA is a well-studied cytoskeleton regulator that has been shown to impair neuronal migration in both knockdown and overexpression conditions (Ota *et al.* 2014, Tang *et al.* 2014). Since the effects of RhoA were similar to those of GR, we tested whether restoration of the RhoA level could rescue the neuronal migration defects caused by GR knockdown. Because GR negatively regulated *RhoA* expression, we co-electroporated GR shRNA417 with RhoA shRNA1442, which efficiently suppressed RhoA expression in NLT cells (Fig. 7A and B), at E15.5 and examined the brain sections at E18.5. The results showed that coexpression of RhoA shRNA1442 partially rescued the migration defects caused by GR shRNA417, as shown by the substantial increase in the number of GFP+ cells in the CP and decrease in the number of GFP+ cells in the IZ compared to those of the GR-knockdown group (Fig. 7C and D). Similarly, when RhoA was coexpressed together with GFP-GR, the delay in neuronal migration caused by GFP-GR was significantly ameliorated (Fig. 7E and F). Taken together, these findings demonstrated that restoration of the RhoA level partially rescued the neuronal migration deficits caused by GR modulation.

Discussion

Although GCs are used for the treatment of many inflammatory, allergic, immunologic and malignant disorders (Liu *et al.* 2013), growing evidence suggests that inappropriate GC or GR levels are tightly associated with neurodevelopmental disorders (Modi *et al.* 2001, Webster *et al.* 2002, Phillips *et al.* 2005, Weinstock 2008, Fineberg *et al.* 2016, Garabedian *et al.* 2017). In this study, we showed that GRs were maintained at relatively stable levels during early development of the neocortex (Fig. 1A and B). Both GR knockdown and overexpression interrupted the multipolar–bipolar transition, which in turn impaired neuronal migration (Figs 2C, D and 4B and C). In detail, GR knockdown suppressed the multipolar–bipolar morphologic transition (Fig. 3C and D), whereas GR overexpression damaged the development of leading process (Fig. 5C and D). Consistent with our findings, previous studies showed that excessive exposure to DEX,

Table 1 Summary of target genes or downstream effectors involved in GR-mediated neuronal migration.

Target genes or downstream effectors	Regulation	Downstream effector	Main findings in neuronal migration and morphogenesis	References
L-Caldesmon	UP	Myosin II	Caldesmon regulates neuronal radial migration by negatively controlling Myosin II function	Mayanagi <i>et al.</i> (2008), Fukumoto <i>et al.</i> (2009)
I κ B α	UP	NF κ B	I κ B α mediates the NGF signaling that controls dendrite morphology in hippocampal neurons via activation of NF κ B	Deroo & Archer (2001), Chacón <i>et al.</i> (2010), Gutierrez & Davies (2011)
MKP1	UP	ERK1/2	G β 2 regulates proper neuronal migration during neocortex development by activating the ERK1/2 signaling pathway	Kassel <i>et al.</i> (2001), Guo <i>et al.</i> (2017)
RhoA	DOWN		Negative regulation of RhoA by Mst3 phosphorylation or Gmp is important for radial migration	Rubenstein <i>et al.</i> (2003), Ota <i>et al.</i> (2014), Tang <i>et al.</i> (2014)
GSK3 β	Suppression	Phospho-GSK3 β (Ser9)	Gsk3-deleted neurons exhibited striking migration failure in cortex and Ser9 phosphorylation of GSK3 β is required for neuronal migration	Yun <i>et al.</i> (2009), Asada & Sanada (2010), Morgan-Smith <i>et al.</i> (2014)

Figure 6

RhoA expression was negatively regulated by the GR. (A) Lysates of NLT cells electroporated with the scrambled or GR shRNA were prepared 48 h after cell transfection and then immunoblotted with the indicated antibodies. (B) Quantification results from A. The data are from three independent experiments. Student's *t*-test was used for the statistical analysis. (C) Lysates of HEK293T cells transfected with GFP or GFP-GR were prepared 48 h after cell transfection and then immunoblotted with the indicated antibodies. (D) Quantification results from C. The data are from three independent experiments. Student's *t*-test was used for the statistical analysis. (E) Real-time PCR analysis of the *RhoA* mRNA levels in NLT cells was performed 24 h after electroporation with the scrambled or GR shRNA. The results represent three independent tests performed in triplicate each time. Student's *t*-test was used for the statistical analysis. (F) Real-time PCR analysis of the *RhoA* mRNA level in HEK293T cells was performed 24 h after transfection with the GFP or GFP-GR plasmid. The results represent three independent tests performed in triplicate each time. Student's *t*-test was used for the statistical analysis. (G and H) Immunoblotting and quantification of the RhoA levels in NLT cells treated with different DEX or MIF concentrations for 24 h. One-way ANOVA followed by the LSD *post hoc* test was used for the statistical analysis. The numbers of experiments performed are indicated on the graphs. All data are shown as the mean $\pm$ S.E.M. ns, no significant difference; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Figure 7

RhoA was involved in GR-mediated neuronal migration. (A) NLT cells were transfected with a scrambled or RhoA shRNA for 48 h. Cell lysates were extracted for RhoA immunoblotting to detect the knockdown efficiency of the RhoA shRNAs. (B) Quantification results from A. The data are from three independent experiments. Each shRNA group was compared with the scrambled shRNA group using Student's *t*-test. (C) Representative images of mouse cortical sections electroporated with the indicated plasmids at E15.5 and examined at E18.5. Scale bars: 100 μ m. (D) Quantification of the GFP+ cell distribution in the E18.5 brain sections. One-way ANOVA followed by the LSD *post hoc* test was used for the statistical analysis. The brain sections were from at least three embryos from independent litters. (E) Representative images of mouse cortical sections electroporated with the indicated plasmids at E15.5 and examined at E18.5. Scale bars: 100 μ m. (F) Quantification of the GFP+ cell distribution in the E18.5 brain sections. One-way ANOVA followed by the LSD *post hoc* test was used for the statistical analysis. The brain sections were from at least three embryos from independent litters. (G) Working model of GR-mediated neuronal migration. The numbers of brain sections or cells used in these experiments are indicated on the graphs. All data are shown as the mean $\pm$ s.e.m. ns, no significant difference; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

which is a synthetic glucocorticoid that activates the GRs, suppressed neuronal radial migration (Mayanagi *et al.* 2008, Fukumoto *et al.* 2009). Based on our results, the effects of GR knockdown were more severe, because more than 10% of the GR-knockdown neurons failed to reach their proper laminar positions (Fig. 2F). In addition to the neuronal migration defects, inappropriate GR levels also led to malformation of dendrites at later developmental stages (Figs 2E, H, I and 4D, F and G). These results reveal that precise transcriptional regulation by GRs is essential to promote proper neuronal migration and dendritic arborization.

GRs belong to the nuclear hormone receptor superfamily and regulate gene transcription directly or indirectly. To elucidate the mechanism by which GRs regulated neuronal migration and morphogenesis, we screened candidate genes regulated by GRs that were involved in neuronal migration. Among the tested genes, *RhoA* expression was shown to have the most significant change and was negatively associated with the GR level (Fig. 6A, B, C, D, E and F). Previous studies revealed that both *RhoA* overexpression and knockdown interrupted neuronal migration (Ota *et al.* 2014, Tang *et al.* 2014), supporting the speculation that GRs mediated neuronal

migration via regulating *RhoA* expression. In the rescue experiments, we observed that restoration of *RhoA* expression partially rescued the neuronal migration deficits in GR-knockdown and GR-overexpressing neurons (Fig. 7C, D, E and F). These data suggested that *RhoA* was a major effector of the ability of the GR to regulate neuronal migration, but other downstream effectors could also be involved. Indeed, we found that L-Caldesmon expression was also decreased by GR knockdown (Fig. 6A and B). In addition to the genomic pathway, GC/GR signaling mediated nongenomic pathway effects. We showed that the GSK3 β phosphorylation level but not its expression level was negatively regulated by the GR level, whereas the ERK1/2 phosphorylation level was unchanged (Fig. 6A, B, C and D). Similar to *RhoA*, both high and low phosphorylated GSK3 β levels were detrimental to neuronal migration (Asada & Sanada 2010, Morgan-Smith *et al.* 2014). Therefore, multiple genes and downstream molecules should be responsible for GR-mediated neuronal migration, whereas *RhoA* apparently plays a major role.

An appropriate GR level is important not only for early brain development but also for development of other tissues. Taking the epidermis as an example, GR transgenic mice showed patches of thinning or absent skin as well as epidermal desquamation, whereas GR-knockdown mice died perinatally and exhibited an incompetent skin barrier and an abnormal epidermal ultrastructure (Perez *et al.* 2001, Perez 2011, Bollag & Isales 2013). Considering the complexity and significance of GR-mediated genomic and non-genomic pathway, the changes of GR levels in either direction may eventually cause severe consequence for embryonic development. Currently, GR agonist are clinically used for treatment of allergic diseases and autoimmune diseases, while GR antagonist is considered as a potential treatment for depression. However, these drugs should be used with extreme caution in clinic, especially for gravidas and infants.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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