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Effects of neonatal dexamethasone and CpdA on the expression of genes for apoptosis regulator proteins in the neonatal hippocampus

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Abstract. Glucocorticoids (GC) are crucial regulators of homeostasis and function. Despite its negative side effects, glucocorticoid therapy in neonates is widely used antenatally for accelerating fetal lung maturation in cases of preterm birth. GC action is mediated via glucocorticoid receptors — ligand-activated transcription factors. Cell death and viability in the neonatal brain are regulated by many factors, but the glucocorticoid receptor signalling is high above them. The present work studies the changes in the expression of genes for apoptosis regulators with Bcl-2 homology (BH) domains (*Bcl-xL, Bax, Bim, Bok, Bid*) in the neonatal rat hippocampus after dexamethasone (DEX) and CpdA administration. CpdA is a dissociative ligand — glucocorticoid receptor modulator — that shifts glucocorticoid receptor (GR) activity toward transrepression. Ligands administration to P2 pups caused different patterns of timeline changes in the expression of the studied genes. We observed the first increase in the mRNA level of the genes which have glucocorticoid response element (GRE) (*Bcl-xL, Bim*) in their promoter 30 min after DEX administration. Activated GR action on cells in the neonatal hippocampus is complex and long-lasting; it could also contain receptor homo- and hetero-dimerisation. Using rat pheochromocytoma PC12 cells as a test system, we assessed GR-GR and GR-MR (mineralocorticoid receptor) dimerisation with proximity ligation assay (PLA) assay separately in the nucleus and cytoplasm after DEX and CpdA administration. An increase in GR-GR dimers in the cell nucleus was observed only after DEX administration. In the cell cytoplasm, we observed a gradual (DEX more than CpdA) increase in the number of both GR-GR and GR-MR dimers.

Keywords: glucocorticoid receptor, brain cell type markers, development, hippocampus, neocortex, apoptosis regulator protein, proximity ligation assay (PLA).

Introduction

High levels of glucocorticoids (GCs) resulting from hormonal therapy or early life stress severely affect the development of the brain (Lanshakov et al. 2016; Oitzl et al. 2010). It causes cell death of certain brain areas by indirect excitotoxicity (Lanshakov et al. 2016). During the neonatal period, elevated GC levels also cause behavioural abnormalities as well as impairment of learning and memory in the future (Holson et al. 1995; Nagano et al. 2008). The long-lasting neuro-behavioural consequences of early life exposures to stress or its hormones evidently occur due to the hormonal effects on the sensitive cells that express glucocorticoid receptors (GRs) (Shaburova, Lanshakov 2020; Shishkina et al. 2015a; 2015b). Among the key processes that GCs could affect in the neonatal brain are cell viability and apoptosis. GC acts via its receptors — intracellular ligand-activated transcription factors. Activated glucocorticoids receptors (GRs) could affect expression of the main apoptosis regulator proteins (Shishkina et al. 2015b). The BCL2 family of proteins is the hallmark of apoptosis regulation (van Delft, Huang 2006). This family has pro-apoptotic and anti-apoptotic members. Some of them possess glucocorticoid response element (GRE) in their promoter, some have nGRE or both, some do not seem to be regulated by GCs. Activated GR could affect transcription in several ways: by transactivation on positive +GRE, by transrepression on negative nGRE and by interaction (tethering) with other transcription factors, which has complex consequences for transcription (Surjit et al. 2011). Preferable pathways to affect transcription are determined mostly by the ligand. Several compounds that shift GR activity toward transrepression were recently discovered (Dezitter et al. 2014). Detailed molecular dynamics of classical and transrepression GR ligands on expression of Bcl apoptosis regulators in the neonatal brain is unclear and requires additional studies. To assess the influence on brain cell viability, we investigated the expression timeline of apoptosis regulatory genes with Bcl-2 homology (BH) domains — *Bcl-xL, Bax, Bim, Bok, Bid* — in the rat neonatal hippocampus after administration of dexamethasone (DEX) and CpdA — a GR modulator ligand that selectively activates GR-mediated transrepression. The negative glucocorticoid response element (nGRE) mediates DNA-dependent transrepression by GR and differs dramatically from classical activating response elements (GRE). Therefore, CpdA action on rat neonatal hippocampus could be completely different from DEX. In the process of GR transactivation, it dimerizes with itself. In transrepression, dimerization with mineralocorticoid receptor (MR) could happen. These two types of interaction could have an impact on GR genomic action. To assess GR-GR and GR-MR interaction after DEX and CpdA, the proximity ligation method (PLA) was used on PC12 cells.

Materials and methods

Animals and experimental design

All procedures were conducted in accordance with Directive 2010/63/EU of the EU and the Russian Ministry of Health regulations on Good Laboratory Practice (supplement to order No. 199n of 1 April 2016) and approved by the Animal Care Committee, Institute of Cytology and Genetics. All efforts were made to minimise animal suffering and to reduce the number of animals used. Pregnant Wistar rats were housed individually (22–24 °C, 12 hours light/dark cycle) with free access to food and water. The day of birth was considered as P0. DEX phosphate 0.2 mg/kg (KRKA, Slovenia), CpdA (sc-221677, Santa-Cruz) 0.2 mg/kg were injected subcutaneously in 25 µl saline (SAL); for the control group, the same volume of SAL was injected. For the gene expression experiments, there were 6–8 animals in the group. Pups were injected sequentially with a 7 min interval needed for further brain snap freezing. After injection, pups were returned to the mother. When the needed time had passed, hippocampi were dissected and snap freezed in liquid nitrogen.

Real-time RT-qPCR analysis

Total cellular RNA was isolated using a single-step acidic phenol extraction as previously described (Lanshakov et al. 2016). Reverse transcription was performed with MMLV Reverse Transcriptase (Sibenzyme) 1µg total RNA and oligo(dT) primer (Evrogen). All real-time PCR reactions were performed using the ABI ViiA7 system (Thermofisher scientific) and a standard two-stage cycle. Amplifications were done using the real time PCR Master Mix SYBR qPCRmix +LowROX (Evrogen) and primers from Table 1.

Immunohistochemistry

Immunohistochemistry was done as previously described (Drozd, Lanshakov 2020; Lanshakov et al. 2017; 2016; Menshanov et al. 2015) with antibodies from Table 2.

PLA staining

PC12 cells were cultivated using standard protocols. 12 hours before the experiments the cells were seeded on poly-l-lysine covered coverslips

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Table 1. Sequences and catalogue numbers of primers and taqman assays used in this study

Gene	Sequence or catalog number
Bid-F	agtcatccacaacattgccagg
Bid-R	gcaccctcagtccatctcatttcta
Bim-F	agagatacggatcgcacagg
Bim-R	gtcttccgcctctcggtaat
Bok-F	gaatttgtacgcaagaccctgg
Bok-R	gctgaccacacacttgagga
Bcl-xl	Rn00437783_m1(Thermofisher scientific)
Bax	Rn02532082_g1 (Thermofisher scientific)

Table 2. Antibody numbers, host species, dilutions used in the study for BCL-XL immunohistochemistry

Cat No.	Target	Host species	Dilution
sc-8392 (Santa-cruz)	BCL-XL	mouse	1:200
A10037 (Thermofisher scientific)	Mouse IgG	donkey anti mouse conjugated Alexa 568	1:400

in a 24-well plate at the density of 0.1×10^6. The following morning 100 μM DEX or CpdA in the culture medium was applied for 30 min. After that the cells were fixed with 4% buffered paraformaldehyde for 5 min. Then the coverslips were washed 2 times for 10 min in 1xPBS, following cell permeabilisation in 1xPBS containing 0.2% Triton X-100 (PBST). Nonspecific binding blocking was done with Duolink blocking solution for 1 h at 37 °C. Then the coverslips were incubated in respective primary antibody pairs (Table 3) overnight at 4 °C. Afterwards the coverslips were washed with PBST twice for 10 min. Duolink (DUO92102-1KT) probes and signal amplification were conducted according to the manufacturer's protocol. After the final washes for cytoplasm and nuclear counterstain, the cells were incubated with Alexa 488 conjugated phalloidin 20 μM in 1xPBS and 300 nM DAPI in 1x PBS, respectively. Afterwards cells were washed twice for 10 min in 1xPBS and mounted on glass slides with Mowiol.

Microscopy

All images were acquired using Zeiss LSM 780 confocal microscope using 488 nm, 561 nm and 405 nm lasers. Panoramic images of the whole brain were taken using the tile scan mode with the 20x objective. For the BCL-XL immunohistochemistry analysis, hippocampal non-confocal panoramic images were taken with the tile scan mode, the Plan-Apochromat 20x/0.8 M27 objective and a 200 μm pinhole. Then, fluorescence intensity of the red channel in the CA3 area was measured using the ZEN software in 10 images per animal. For the PLA image acquisition, the Plan-Apochromat 100x objective NA = 1.4 was used, and Z-stacks were taken with the pinhole set at 1AU. The PLA images were analysed using the Cell Profiler program. A software pipeline that counts Cy3 labelled PLA interaction signal separately in nucleus and cytoplasm was created. Total 20 cells per group were analysed in the PLA analysis.

Statistics

Gene expression data were analysed using the one-way ANOVA with Fisher's LSD post-hoc aposterior analysis. The PLA data were analysed using the one-way ANOVA with the Bonferroni multiple comparison test. Differences between means were estimated using the above analyses

Table 3. Antibody numbers, host species, dilutions used in the PLA assay staining

Cat No.	Target	Host species	Dilution
MABS1250 (Millipore)	MR	mouse	1:200
12041S (Cell Sign.)	GR	rabbit	1:200
sc-56851(Santa-cruz)	GR	mouse	1:200

Effects of the factors as well as differences between groups were considered significant at probability less than 0.05.

Results

Expression timeline of apoptosis regulator proteins with BH domains in the neonatal hippocampus after DEX and CpdA

To assess the GR impact on hippocampal cell viability, we studied the expression timeline of apoptosis regulators with BH domains after two different GR ligands administration to P2 rat pups: classical dexamethasone and CpdA. A glucocorticoid receptor modulator, CpdA is a "dissociating" GR ligand. It prevents GR dimerization and shifts GR activity towards transrepression. Expression of different apoptosis regulatory genes was investigated. Some of them are pro-apoptotic, others are anti-apoptotic. *Bim* and *Bcl-xL* have classical GRE in their promoters, while *Bcl-xL* also has several negative nGRE besides classical GRE (Fig. 1A). We did not observe any significant changes in the mRNA level for any studied genes after the SAL injection (all $p > 0.05$). These groups were merged for further statistical analysis. Interestingly, an elevation of the mRNA level of genes with GRE (*Bim, Bcl-xL*) was observed 30 minutes after the subcutaneous DEX injection [*Bim* — $F(6, 45) = 3.9962$, $p = 0.00273$; *Bcl-xL* — $F(6, 46) = 7.4192$, $p = 0.00001$] (Fig. 1B). The mRNA level of *Bid* proapoptosis regulator elevated 1 h after DEX administration; $F(6, 43) = 3.2729$, $p = 0.00969$ (Fig. 1B). The mRNA elevation of *Bok* proapoptosis regulator was observed 2 h after DEX administration; $F(6, 42) = 6.2119$, $p = 0.00010$ (Fig. 1B). This 2 h interval is greater than 30 min response time interval for the genes with GRE. We did not observe any changes in the *Bax* mRNA level after DEX administration. Not so much expression change was observed after CpdA administration. A small decline in the *Bid* mRNA level was observed 6 h after administration; $F(11, 83) = 1.2525$, $p = 0.05$. The *Bax* mRNA level did not change after DEX, but its small increase was observed 30 min after CpdA administration; $F(11, 84) = 3.0167$, $p = 0.00196$. Changes in the mRNA level were accompanied by changes in the protein levels. The BCL-XL protein level increased 1 h after DEX administration; $F(1, 8) = 23.393$, $p = 0.00129$ (Fig. 1C, D). Remarkably, intensive BCL-XL staining was observed in the hippocampal CA3 region where the Brain-derived neurotrophic factor (BDNF) expression site is (Fig. 1C).

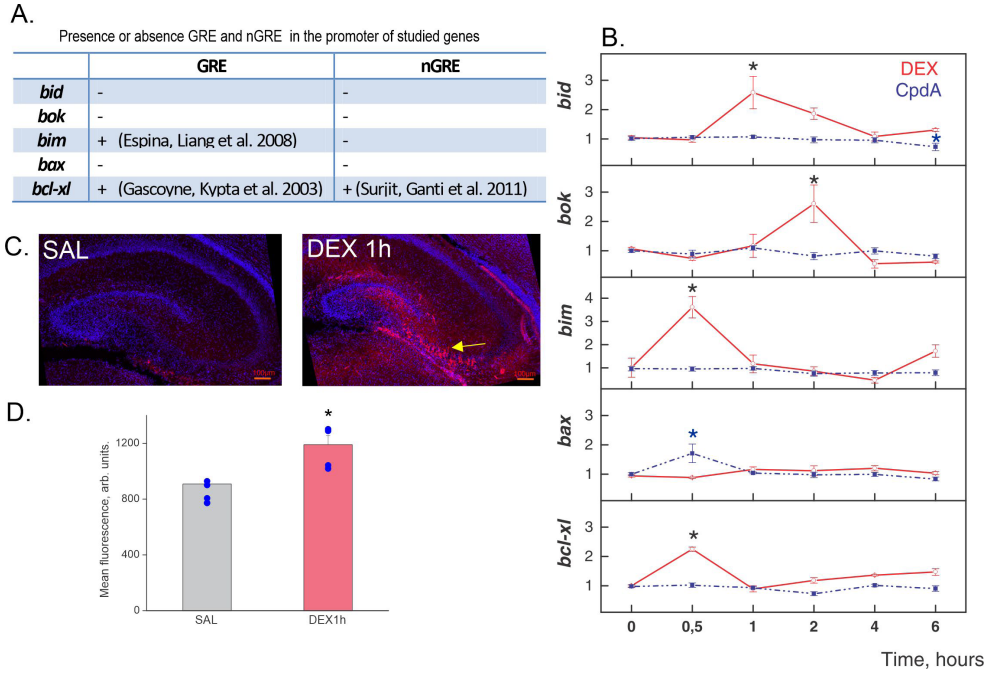

Fig. 1. Changes in the expression timeline of apoptosis regulators genes after DEX and CpdA administration.
(A) Presence or absence of GRE and nGRE in the promoters of the genes for apoptosis regulatory proteins.
(B) Changes in the expression timeline of the apoptosis regulatory proteins after DEX 0.2 mg/kg (red lines) and CpdA 0.2 mg/kg (blue lines) administration. Black asterisk for the group after DEX administration compared to the control group with SAL injection using the one-way ANOVA ($p < 0.05$); blue asterisk for the group after CpdA injection compared to the control group with SAL injection using the one-way ANOVA ($p < 0.05$).
(C) Representative microscopic images of Bcl-xL immunostaining on P2 rat hippocampi 1 h after DEX administration. Yellow arrow shows CA3 neurons with intensive Bcl-xL staining. Scale bar 100 μm.
(D) Quantification of Bcl-xL immunohistochemical signal in hippocampal CA3 area (n = 4–5), one-way ANOVA, $F(1, 8) = 23.393$, $p = 0.00129$, compared to the SAL control group

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Evaluation of GR-GR and GR-MR dimerization after DEX and CpdA

To assess the impact of the GR homo- and hetero-dimerization on transcription changes after DEX and CpdA, proximity ligation assay (PLA) was chosen. PLA is a method that allows in situ detection of protein interaction. It is based on the detection of proximity between two proteins with secondary antibodies conjugated with oligonucleotide (PLA probe). Two different PLA probes in close proximity could form a template for rolling circle polymerase, and so the signal is amplified (Fig. 2A). Fig. 2B shows representative confocal images where red dots are PLA signals labelled with Cy3. 30 minutes after GR ligands administration to the PC12 cells there was a significant elevation of GR-GR dimers in the cell nucleus only after DEX administration compared to the control group ($F(5, 1707) = 12{,}712$, $p < 0.05$; Fig. 2C). As is expected, no elevation of GR-GR dimers was observed after CpdA administration. Interestingly, after the CpdA administration the number of MR-GR heterodimers decreased (Fig. 2C), but after DEX we did not observe any changes in MR-GR dimers in the cell nucleus (Fig. 2C). In the cell cytoplasm there was a completely different situation. DEX administration produces the highest increase in both types of dimers: GR-GR and MR-GR ($F(5, 1062) = 13.496$, $p < 0.05$; Fig. 2D). CpdA applied to the culture media causes low elevation in the cell cytoplasm of both dimer types as well (Fig. 2D). It should be noted that with our Cell profiler pipeline, average PLA detected signals in the nucleus were higher than in the cytoplasm.

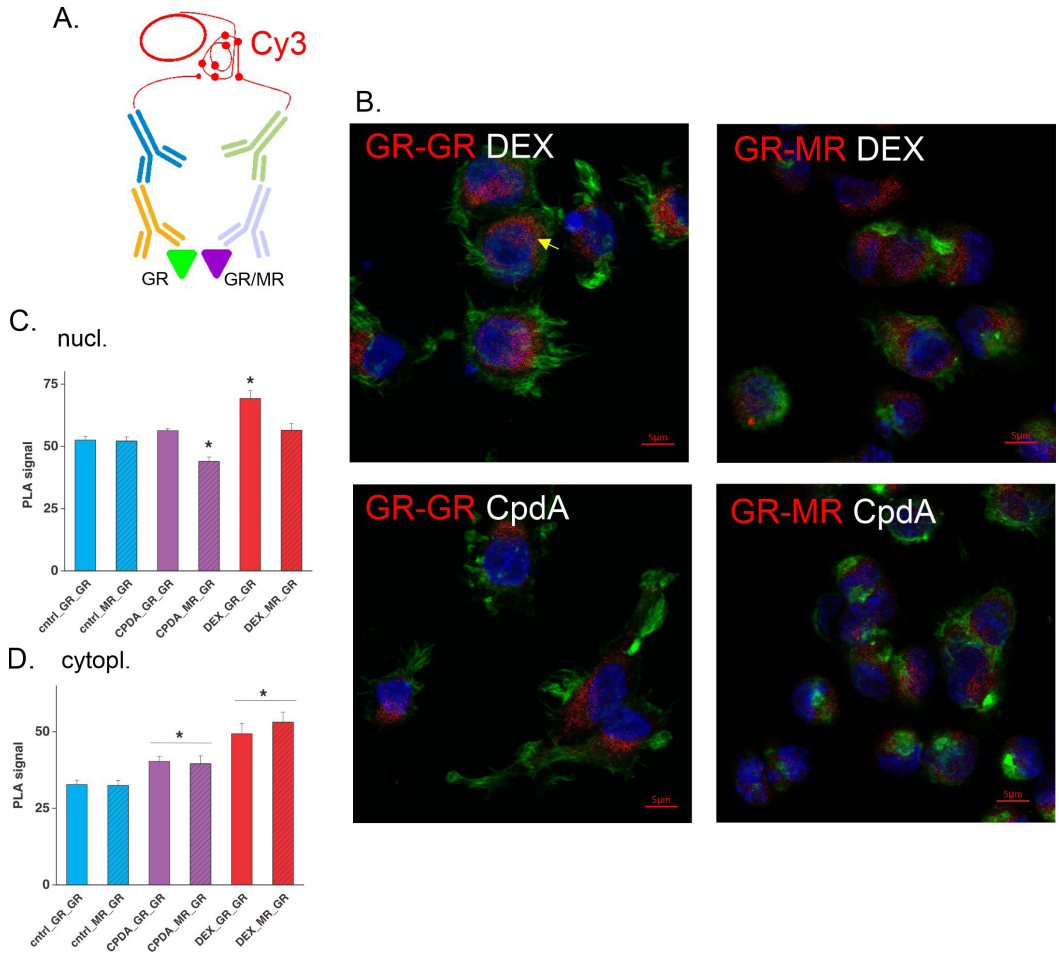

Fig. 2. Homo- (GR-GR) and hetero-dimerization (GR-MR) in the PC12 cells after DEX and CpdA administration. (**A**) Schematics of the method. Primary antibodies (AB) raised in different species binds to the target proteins, then secondary antibodies with conjugated nucleotides bind to respective primary AB. Afterwards, a template for rolling circle amplification polymerase is formed, and it makes concatamers. The fluorescently labelled probe binds to the concatamerased template. (**B**) Representative confocal images of PC12 cell after the PLA assay. Red — the PLA signal, Cy3 labelled; green — Alexa 488 phalloidin staining for counterstain cytoplasm; blue — nuclear counterstain DAPI staining. Scale bar 5 μm. (**C**) Evaluation of the GR-GR and GR-MR dimerization with the PLA assay on the PC12 as a test system. The PLA signal in the nucleus; one-way ANOVA, asterics — $p < 0.05$, compared to the untreated control group. (**D**) The PLA signal in the cytoplasm; one-way ANOVA, asterics — $p < 0.05$, compared to the untreated control group

Discussion

In the present work we studied expression of the main apoptotic regulatory proteins with BH domain in the neonatal hippocampus after administration of dexamethasone or a novel dissociative GR ligand — CpdA. Interestingly, the action of DEX on expression of genes possessing GRE in their promoters (*Bcl, Bim*) in vivo was observed 30 min after the DEX subcutaneous injection to P2 rat pups. It is similar to the GR action on genes expression in cell culture experiments (Reddy et al. 2009). This observation is consistent with the timeline tracking results for stress-induced GR activation in GRE luciferase reporter transgenic mice (Lee et al. 2016). Usually, for most of the genes an interval of 18–40 min is required for the gene expression response in cell culture (Reddy et al. 2009). Repression of the genes caused by activated GR in cell culture experiments begins later: in 44–53 min (Reddy et al. 2009). After a subcutaneous injection of the dissociative glucocorticoid modulator ligand (CpdA), we observed a small decline only in the *Bid* mRNA level 6 hours after the injection. That is much longer than the time needed for cells in culture. An increase in the *Bid* mRNA level was also observed 1 hour after DEX administration. These data could lead us to the conclusion that the *Bid* promoter could possibly possess GRE and nGRE as well; however, there have been no reports proving that yet. For the gene repression, much longer time might be needed for the mRNA level to decline, usually around 1–6 hours, but it depends on the particular mRNA stability or binding with RNA binding proteins like YTHDF2 that affect RNA stability (Du et al. 2016). Recent sci-fate and single cells transcriptomic studies revealed that glucocorticoids cause shifts of cells to a new transcriptional state that could take up to 10 hours (Cao et al. 2020). Overall, the investigated changes in the expression timeline in neonatal hippocampus after DEX had the opposite effect on the expression of BH3 apoptosis regulators. After DEX, an increase in the mRNA level of pro-apoptotic members — *Bid, Bim, Bok* — and anti-apoptotic *Bcl-xL* was observed. BCL-XL protein level increased in the neonatal hippocampus 1 h after DEX administration as well. Remarkably, BCL-XL elevated in the hippocampal CA3 region (Fig. 2C). BDNF is also more prominent in this hippocampal region. *Bcl-xL* and *Bdnf* are both regulated intensively by neuronal activity, stress and by GR's as well (Lanshakov et al. 2017; Shishkina et al. 2015b). Taking into account the same expression site in the hippocampus, a complex intersected mutual self-regulation net of *Bcl-xL* and *Bdnf* could be proposed.

As reported before, glucocorticoids could possibly cause cell death by indirect mechanisms like excitotoxicity in the neonatal brain (Lanshakov et al. 2016). Activated GR causes a glutamate release or transcription changes within the first hour and this, in turn, causes secondary changes in genes transcription. *Bok* is implicated in autophagy (Kalkat et al. 2013). The *Bok* mRNA level elevated 2 h after DEX administration, which is postponed from the direct events observed within the first hour. Considering time and absence of GRE in the promoter, it is likely that changes in the *Bok* mRNA level could be due to a secondary event that happened after the primary one activated by GR action.

GR homo- and hetero-dimerization can influence its genomic action. We analysed GR-GR homo- and GR-MR hetero-dimerization using proximity ligation assay after applying different ligands — DEX and CpdA. As is expected, in the cell nucleus, where the GR genomic action is happening, we observed a significant increase in GR-GR dimers only after DEX. The number of GR-MR heterodimers did not change after DEX and even went down after CpdA. In the cell cytoplasm, a gradual elevation of both dimer types was observed. The highest increase in the number of GR-MR dimers in the cytoplasm was observed after DEX. Fewer GR-MR dimers were detected after CpdA. Possibly, GR-MR dimers in the nucleus are minor to GR-GR ones and should be detected with more precise methods involving next generation sequencing. In the cytoplasm GR-MR heterodimers could be formed in the same number as GR-GR dimers, before transfer to the nucleus. It could be due to a possibly more dynamic dimers formation in the cytoplasm than in the nucleus (Tiwari et al. 2017).

Besides glucocorticoids, multiple other factors, such as neurotrophin BDNF and its precursor (Menshanov et al. 2015) as well as hypoxia (Bulygina et al. 2014) affect babies during labour and delivery (Alotaibi et al. 2015) and influence brain development. Nevertheless, the effect of glucocorticoids may be one of the most practically important ones, because these hormones and their synthetic analogues are widely used to prevent chronic lung disease in premature infants (Barrington 2001). Some effects of natural glucocorticoids may be positive for the developing nervous system and reduce apoptotic cell death (Kalinina et al. 2019). However, more often synthetic hormones induce cell loss, reduce neurogenesis and glial proliferation, attenuate dendrite formation, inhibit brain growth and cause abnormalities of brain structure and function (de Kloet et al. 2014; Reynolds 2013). These effects could be attributed to the direct action of the hormone on the GR-expressing cells as well as to

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the indirect effects of glucocorticoid that are mediated, for example, through glutamate excitotoxicity (Lanshakov et al. 2016). Nowadays, two potential approaches can be used to prevent some deleterious effects of glucocorticoids on the developing brain. The first approach is to block glutamate excitotoxicity with antagonists of glutamate receptors (Li et al. 2014). The second approach is to activate intracellular processes that interfere with the glucocorticoid action, such as that induced by mild hypoxia (Bulygina et al. 2014). However, both approaches are only suggestions and require further investigation.

Conclusion

In this work we showed that DEX administration to P2 rat pups leads to an increase in the mRNA level of various apoptosis regulatory genes with BH domain, both pro-apoptotic (*Bid, Bim, Bok)* and anti-apoptotic (*Bcl-xL).* We observed a rise in the mRNA level of genes with GRE 30 min after injection. After CpdA, a small increase in the *Bax* mRNA level was observed 30 min after injection and a small decrease in the *Bid* mRNA level was observed 6 hours after administration. Such discrepancies in action could be explained by different GR-GR and GR-MR dimer formation. On the PC12 cell line we showed that the number of GR-GR dimers in the cell nucleus increased only after DEX administration. In the cell cytoplasm, a gradual increase (DEX more than CpdA) in the number of both dimer types (GR-GR and GR-MR) was observed. Possibly, dimers formation after ligand administration goes faster in the cytoplasm than in the cell nucleus.

Conflict of interest

The authors declare no conflict of interests.

Author contributions

LDA — manuscript writing; LDA, KTS, SuEV — experimental design; LDA, KTS, SuEV, BVV — animal injections and specimen collection; LDA, KTS, SuEV — qRT-PCR; LDA, VVB — immunohistochemistry, confocal microscopy; LDA — cell culture and PLA staining, LTA — PLA samples microscopy and cell profiler analysis.

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