ORIGINAL ARTICLE

Dexamethasone decreases insulin-like growth factor-I and -II via a glucocorticoid receptor dependent mechanism in developing rat brain

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Abstract

OBJECTIVES: Dexamethasone (Dex) causes neurodegeneration in developing brain. Insulin-like growth factor-I (IGF-I) and -II (IGF-II) are potent neurotrophic and differentiation factors and play key roles in the regulation of growth and development of CNS. Current project evaluated the effects of Dex on IGF-I and -II in developing rat brains.

MATERIAL AND METHODS: Sprague-Dawley rat pups in each litter were divided into vehicle (n=230) or Dex-treated (n=234) groups. Rat pups in the Dex group received one of the 3 different regimens of i.p. Dex: tapering doses (DexTD) on postnatal days (PD) 3 to PD 6 or repeated doses on PD 4 to PD 6 or single dose on PD 6. To quantify the glucocorticoid receptor (GR) blockade effect, rat pups in the DexTD group on PD 3 and 5 received vehicle or RU486 (GR blocker, 60 mg/kg) s.c., twenty minutes prior to Dex treatment.

RESULTS: Dex decreased the gain of body and brain weight while RU486 inhibited these effects. RU486 also prevented the DexTD-induced increase in caspase-3 activity and reduction in IGF-I and -II proteins. Compared to the vehicle, the expression of mRNA of IGF-I and -II decreased at 24 h after DexTD treatment, while RU486 prevented this decrease on IGF-II but not IGF-I.

CONCLUSIONS: Our findings indicate that Dex via GR decreases IGF-I and -II and causes neurodegeneration in the neonatal rat brain.

Abbreviations:
BDNF   - brain derived growth factor
Dex   - dexamethasone
DexTD   - dexamethasone tapering doses
DexRD   - dexamethasone repeat doses
DexSD   - dexamethasone single dose
GC   - glucocorticoid
GR   - glucocorticoid receptor
IGF-I   - insulin-like growth factor-I

INTRODUCTION

Clinical observations implicate an abnormal glucocorticoid (GC) concentration or profile in the pathogenesis of psychiatric disorders such as depression and post-traumatic stress disorder as well as chronic neurodegenerative diseases such as Alzheimer's disease (Abraham *et al.* 2001). A great deal of research has evaluated the role of GCs on neurodegeneration particularly the effects of chronic stress on hippocampus

(Reagan & McEwen 1997). Studies to evaluate GCs' neurotoxicity in newborns are very important because the use of dexamethasone (Dex) in premature infants to prevent and/or treat bronchopulmonary dysplasia (BPD) adversely affects neuro-cognitive development (Yeh *et al.* 2004) and causes cerebral palsy (Shinwell *et al.* 2000; O'Shea *et al.* 2007). Animal studies show that Dex not only impairs brain function, fetal growth and neurodevelopment (Flagel *et al.* 2002; Neal *et al.* 2004) but also reduces life expectancy and affects social behavior adversely (Kamphuis *et al.* 2007; Kamphuis *et al.* 2004). Mechanisms of these effects are unclear and likely multi-factorial, and may include apoptosis. Dex-induced inhibition of trophic growth factors is also suspected to be involved in its neurotoxicity (Baud 2004). Trophic growth factors like nerve growth factor (NGF) and brain derived growth factor (BDNF) play an important role in the CNS during development and in adulthood (Riva *et al.* 1995). NGF and BDNF can influence neuronal survival and play a role in the maintenance of cell homeostasis (Riva *et al.* 1995). Lack of functional NGF and BDNF genes results in severe neuronal deficits and an early postnatal death (Conover & Yancopoulos 1997) while heterozygous BDNF knockout (+/−) mice exhibit impaired spatial learning (Linnarsson *et al.* 1997).

In addition to those NGFs, the insulin-like growth factor (IGF) System, specifically IGF-I, has pleiotropic functions in the brain in an endocrine, autocrine and paracrine fashion (Russo *et al.* 2005). IGF-I and its receptor IGF-IR mRNA expression is detectable in many brain regions (Rotwein *et al.* 1988; Bondy *et al.* 1992). IGF-I null mice have reduced brain size, altered brain structure and alteration in myelination process (Conover & Yancopoulos 1997; Cheng *et al.* 2003; Ye *et al.* 2002). Knockout of the IGF-IR gene produced severe intrauterine growth restriction (IUGR), with a small brain with CNS hypomyelination, loss of hippocampal granule and striatal parvalbumin-containing neurons (Beck *et al.* 1995). Systemic IGF-I is not readily transported through the blood-brain barrier, thus local production of IGF-I is considered the primary source of the ligand for brain cells (Russo *et al.* 2005). IGF-II mRNA is also expressed in the CNS, with the highest level of expression found in myelin sheath, and also in all non-neuronal structures, such as leptomeninges, microvasculature, and choroid plexus, that enable diffusion of growth factors to their sites of activity (Stylianopoulou *et al.* 1988). IGF-II stimulates proliferation of both neuronal and glial cells, outgrowth of neurite, and acts as a survival factor for a variety of neuronal cell types (Beilharz *et al.* 1995). Previous *in vitro* and *in vivo* studies have shown that Dex down-regulates NGF, BDNF and IGF-I mRNA expression in the adult brain (Riva *et al.* 1995; Vellucci *et al.* 2001; Ye *et al.* 1997; Woods *et al.* 1999). Additionally, Ye *et al.* (1997) showed that 6 h after a single dose of Dex IGF-I-luciferase fusion gene activity decreased in several brain regions in P7

transgenic mouse. Thus, limited information is available about the effects of Dex on these trophic growth factors in the developing brain. Flagel *et al.* (2002) have developed a neonatal rat model to study the long-term effects on the developing CNS of a clinically relevant, prolonged tapering course of neonatal Dex administration in human premature infants. Using a similar model, we previously showed that Dex induces apoptosis and neurodegeneration in newborn rats (Feng *et al.* 2009). In the current project using a similar model we investigated whether Dex affects NGF, BDNF, IGF-I and IGF-II in the developing rat brain.

METHODS

Animals

The protocol was approved by the University of Mississippi institutional committee on animal use. Gestationally timed pregnant Sprague-Dawley rats (Charles Rivers, Wilmington, MA, USA) were housed in our animal unit and maintained in accordance with the National Institutes of Health guidelines. All animals were exposed to 12:12 light-dark cycles at 21–23 °C, and allowed access to food and water ad libitum.

Drug Treatment

The day of birth was designated as postnatal day 1 (PD1). A total of 48 litters were used in the present study. Each litter was sexed, culled to a maximum of 12 pups and randomly divided into the vehicle (veh, n=230) or Dex treated groups (DexTD, n=234). All pups were removed from their mother and treated between the hours of 11:00 and 13:00 by i.p. injection. Pups in the Dex group received one of the three different regimens of Dex (dexamethasone phosphate, American Reagent, Inc., Shirley, NY, USA). Rat pups in the Dex tapering doses (DexTD) group received tapering doses of Dex on postnatal day 3 to 6 (0.5, 0.25, 0.125 and 0.06 mg/kg, respectively). Pups in the repeated doses group (DexRD) received 0.5 or 1 mg/kg/day of Dex on PD 4, PD5, and PD 6. Pups in the single dose group (DexSD) received 0.03, 0.06, 0.125, 0.25 or 0.5 mg/kg of Dex on PD6. Pups in the corresponding vehicle group received equivalent volumes of saline. To quantify the effect of glucocorticoid receptor (GR) blockade, rat pups in the tapering doses group on PD 3 and 5 received vehicle or RU486 (GR blocker, 60 mg/kg) (Sigma-Aldrich Co., St. Louis, MO, USA) s.c., twenty minutes prior to Dex treatment. These doses and modes of administration were chosen from previous studies (Flagel et al., 2002; Kanagawa et al., 2006, Feng et al., 2009). The pups were killed by cervical dislocation at different time points after treatment; after removing the cerebellum, medulla oblongata and pons, the cerebrums were dissected, weighed and brain cortices were stored by appropriate methods for real-time RT-PCR, and ELISA experiments. Although evidence suggests that sex is not a factor affecting brain weight or the neurological exam following neonatal

Dex exposure at PD7 (Neal et al., 2004, Feng et al., 2009), we still divided male and female pups in similar numbers in Dex and vehicle groups. Due to the small number of animals, all data from male and female pups were combined for analysis.

Measurement of caspase-3 activity

Caspase-3 activity was measured in vehicle and DexTD groups as described previously (Bhatt *et al.* 2013). Briefly, the brain cortices collected at 6 and 12 h as well as 1, 3 and 7 days after the last dose of treatment from the pups were homogenized and caspase-3 activity was measured using a commercially available assay kit (Calbiochem, Inc., Diego, CA, USA), following the manufacturer's instructions. Caspase-3 activity was determined colorimetrically with a microplate reader at 405 nm. A p-nitroanilide (pNA) calibration curve was established to quantify units of caspase-3 activity.

RNA isolation and cDNA preparation

Total RNA was extracted from the brain cortices collected at 1 day after the last dose of treatment using TRIzol Reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA integrity was assessed by ethidium bromide staining of the nucleic acids after agarose gel electrophoresis (data not shown). Total RNA was treated with DNase I (Invitrogen) to remove contaminating genomic DNA. Total RNA (2.5 µg) was used to synthesize cDNA using 0.5 µg oligo(dT)$_{15}$ primer (Promega, Madison, WI , USA) and Super-Script III reverse transcriptase (Invitrogen) following the manufacturer's instructions.

Real-time reverse transcription (RT)-PCR

Analysis of the expression levels for NGF, BDNF, IGF-I and IGF-II mRNA were performed by real-time RT-PCR on an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad). Primers used for the amplification for IGF-I, -II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA have been published previously. Primers for BDNF and NGF were designed using Primer Express (Applied Biosystems). The sequences of primers are: IGF-I forward: 5'-GTGGACGCTCTTCAGTTCGT-3'; IGF-I reverse: 5'-GGAAGCAACACTCATCCACAATG-3'; IGF-II forward: 5'-TCGATGTTGGTGCTTCTCATCTC-3'; IGF-II reverse: 5'-CTCCCCTCCGCACAGAGTCT-3'; (Lacroix-Fralish *et al.* 2006); BDNF forward: 5'-CATTCTTTCCCTCCCTCCTC-3' ; BDNF reverse: 5'-ATATTTCAAAGCGCGCAACT-3'; NGF forward: 5'-TGACTCCAAGCACTGGAACTCAT-3'; NGF reverse: 5'-GTTTGTCGTCTGTTGTCAACGC-3'; and GAPDH, forward: 5'-AAGATGGTGAAGGTCGGTGT-3'; reverse: 5'-GTTGATGGCAACAATGTCCAC-3'. Melting curves of all samples were always performed as controls for specificity. PCR cycles consisted of an initial denaturation step at 94 °C for 3 min,

followed by 40 cycles of 45 s at 94 °C, 45 s at 58 °C, and 30 s at 72 °C. PCR amplification of the housekeeping gene, GAPDH, was used as a control of equal loading and to allow normalization between samples. PCR products were confirmed as a single band using gel electrophoresis. For quantitative analysis, we evaluated the difference in cycle threshold (ΔC_t) between Dex and vehicle treated animals by $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). C_t values were the means of triplicate replicates. Experiments were repeated three times. Efficiency of real time RT-PCR was determined by serial dilutions of cDNA. Amplification efficiencies of primer sets ranged from 91% to 108%. The coefficient of determination across different dilution factors was >0.99 in all the experiments, indicating the high efficiency of the real time RT-PCR.

ELISA

Sample homogenates preparation and assays were performed as recommended by the manufacturer. Brain cortices collected at different time points after treatment from the pups in vehicle and Dex groups were used. The total protein concentration in the supernatant was determined by the method of Bradford (1976). The protein levels of IGF-I and IGF-II were measured using the mouse IGF-I and mouse IGF-II immunoassay kits from R&D Systems (Minneapolis, MN, USA) which are designed to eliminate interference by soluble receptors, binding proteins and other factors present in biological samples. The protein levels of NGF and BDNF were measured using the NGF and BDNF Emax ImmunoAssay systems from Promege Co. (Madison, WI, USA). All the results were normalized and expressed as pg per mg of total protein.

Statistical analysis

All data were expressed as mean ± S.E.M. Differences between two groups were analyzed using Student's *t*-test. Multiple comparisons were performed using one-way ANOVA with Holm-Sidak *post hoc* test. These analyses were performed using SigmaStat software package. Differences were considered significant at $p<0.05$.

RESULTS

Dex decreased the gain of body and brain weight while RU486 inhibited these effects

Neonatal DexTD exposure significantly decreased the gain of body (Figure 1*A*, $p<0.01$) and brain weight (Figure 1*B*, $p<0.01$) compared with the corresponding vehicle group. This data validates the reliability of the Dex treatment model used in a previous study (Feng *et al.* 2009). In a separate experiment, treatment with RU486 significantly inhibited the decrease of gain of body and brain weight (Figure 1*C, D*, $p<0.01$ vs. vehicle, $p<0.01$ vs. DMSO + DexTD). We further tested the effect of RU486+saline alone on body and brain weight.
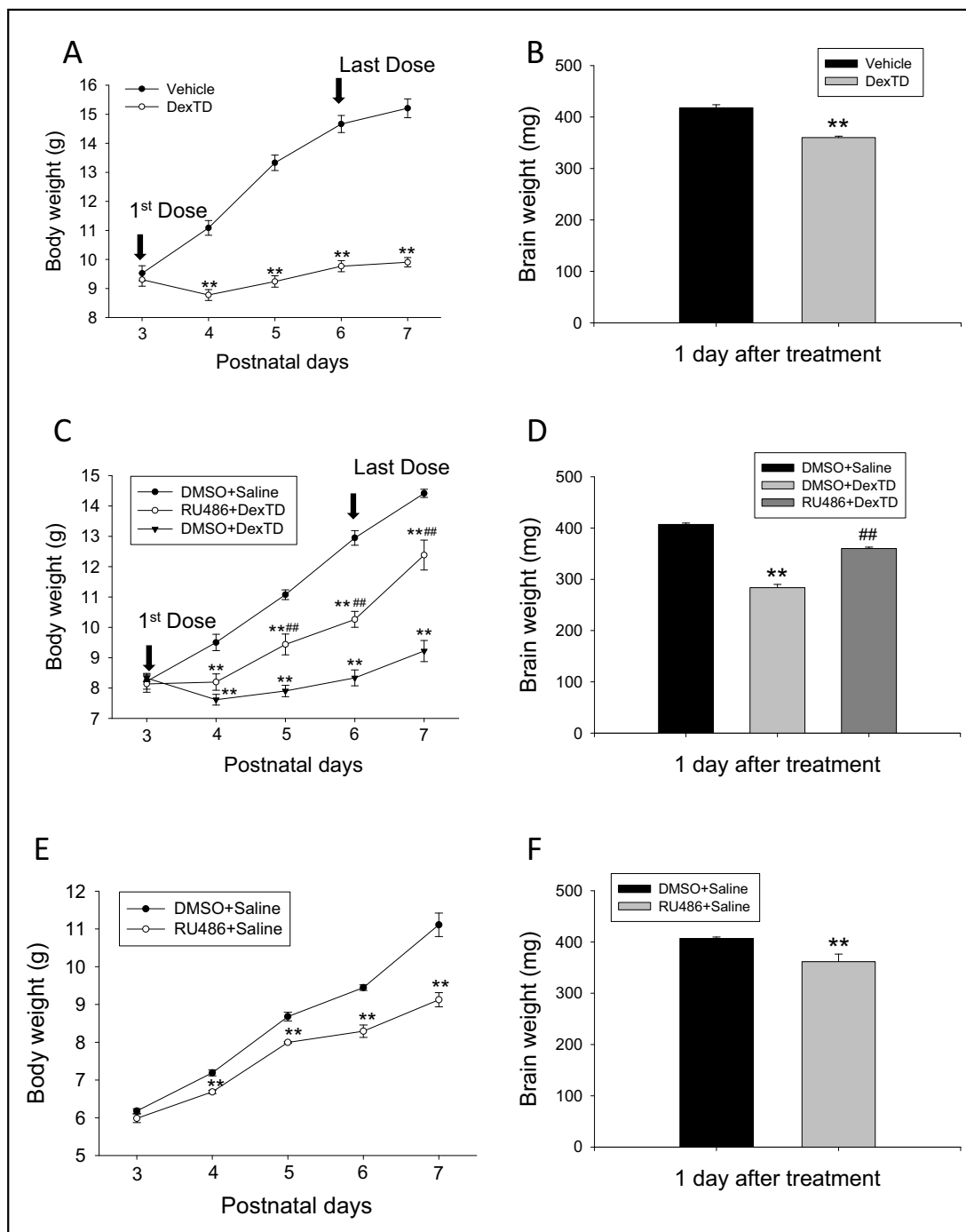
Fig. 1. Neonatal Dex exposure significantly decreased gain of body weight (*A*) and brain weight (*B*) (**$p<0.01$ vs. vehicle, n=10–12 in each group). Treatment with GR blocker RU486 significantly inhibited these decreases of gain of body weight (*C*) and brain weight (*D*) (**$p<0.01$ vs. vehicle, ##$p<0.01$ vs. DMSO + DexTD, n=5–6 per group). Treatment with RU486 alone caused less but also significant decrease in gain of body weight (*E*) and brain weight (*F*) compared to DMSO + saline group (**$p<0.01$, n=4 in each group). Data are presented as mean ± S.E.M.

RU486 significantly decreased the gain of body and brain weight compared to DMSO+saline (Figure 1*E, F*, $p<0.01$ vs. vehicle).

DexTD increased caspase-3 activity in pup brain while RU486 inhibited these effects

Eighty-four rat pups were evaluated for caspase-3 activity. As previously reported (Feng *et al.* 2009), there

was a significant increase in caspase-3 activity in the brain cortices at 6, 12 h and 1 day after DexTD treatment compared with the corresponding vehicle group (Figure 2*A*, $p<0.05$) but there was no change at 3 and 7 days after Dex treatment (Figure 2*A*). In a separate experiment, treatment with RU486 significantly inhibited the increase of caspase-3 activity induced by DexTD at 24 h after treatment (Figure 2*B*, $p<0.01$ vs.

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vehicle, $p<0.01$ vs. DMSO + DexTD). No change in caspase-3 activity was noted between RU486 + saline and DMSO + saline groups ($p>0.05$).

GR blocker prevented the DexTD induced reduction in IGF-I and -II protein in pup brain

We first tested the time-course of DexTD on IGF-I and -II protein. DexTD significantly decreased IGF-I and -II protein compared with the vehicles at 6, 12 and 24 h after treatment (Figure 3A, B, $p<0.01$). We further investigated the effect of GR blocker RU486 on the DexTD-induced decrease in IGF-I and -II protein at 24 h after treatment. RU486 significantly prevented the DexTD-induced reduction in IGF-I {3596.3±194.3 pg/mg in the vehicle, 2598.8±124.9 pg/mg in the DexTD and 3646.2±273.0 pg/mg in the RU486+Dex treated groups ($p<0.01$)} and IGF-II proteins {956.9±66.2 pg/mg in the vehicle, 362.9±30.0 pg/mg in the DexTD and 1018.8±77.9 pg/mg in the RU486+DexTD treated group ($p<0.01$)} in the neonatal rat brain (Fig.3C,D). Treatment with RU486 + saline alone tended to down-regulate protein of IGF-I and -II compared with DMSO + saline but there are no significant differences ($p>0.05$, data not shown).

DexTD decreased the expression of mRNA of IGF-I and -II in pup brain

DexTD decreased the expression of mRNA of IGF-I at 24 h but not at 6 and 12 h after treatment (Figure 4A, $p<0.01$ vs. vehicle). DexTD decreased the expression of mRNA of IGF-II at 6, 12 and 24 h after treatment (Figure 4A, $p<0.01$ vs. vehicle). In a separate experiment, the expression of mRNA of IGF-I decreased by 49% ($p<0.01$, vs. vehicle), and IGF-II decreased by 31% ($p<0.01$, vs. vehicle) at 24 h after DexTD treatment, while treatment with RU486 prevented this decrease in IGF-II (decreased by 81%, $p<0.01$) but not IGF-I (decreased by 55%, $p>0.05$) compared with the DMSO + DexTD group at 24 after treatment in the neonatal rat brain (Figure 4B).

Effect of DexSD and DexRD on IGF-I and IGF-II in pup brain

IGF-I and -II protein were measured by ELISA and the results are expressed relative to total protein for each sample. DexRD (0.5 and 1 mg/kg) significantly decreased levels of IGF-I and -II protein compared with the vehicles at 1 day after treatment (Figure 5A, B). Single dose 0.125 mg/kg to 0.5 mg/kg but not 0.06 mg/kg of Dex also significantly decreased IGF-I protein compared with the vehicles at 1 day after treatment (Figure 5C). Single dose 0.125 mg/kg to 0.5 mg/kg of Dex also significantly decreased IGF-II protein (data not shown). For the time course experiment, the single dose 0.5 mg/kg of Dex significantly decreased IGF-I and -II protein at 6 h, 12 h and 1 day after treatment but there was no change at 3 and 5 days after treatment (Figure 5D, E).

DexSD of 0.5 mg/kg significantly decreased IGF-II mRNA, but did not decrease IGF-I mRNA compared to the vehicle at 1 day after treatment (Figure 5F). There were no changes in mRNA of IGF-I and -II in DexSD 0.25 mg/kg group, but treatment with the DexSD 0.125 mg/kg significantly increased IGF-I and -II mRNA compared with the vehicle at 1 day after treatment (Figure 5F).
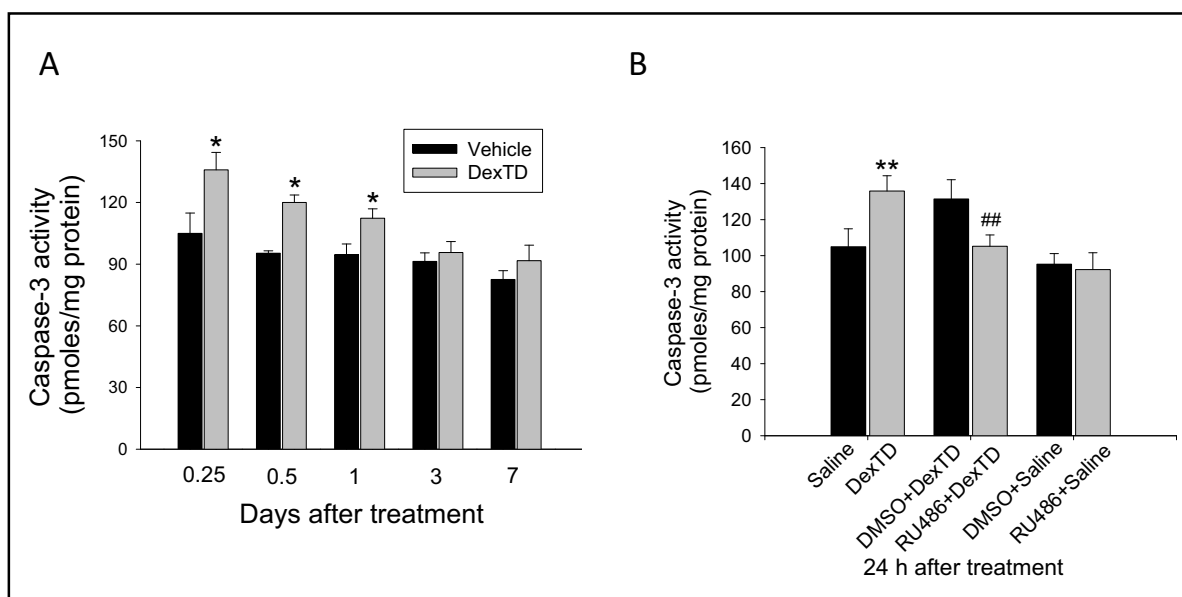

Fig. 2. DexTD increased the caspase-3 activity in the pup brain while RU486 inhibited these effects. DexTD increased in caspase-3 activity in the brain cortices at 6, 12 h and 1 day after treatment compared with the corresponding vehicle group (*A*). Treatment with glucocorticoid receptor blocker RU486 significantly inhibited the increase of caspase-3 activity induced by DexTD at 24 h after treatment (*B*). **$p<0.01$ vs. vehicle; ##$p<0.01$ vs. DMSO + DexTD. Data are presented as mean ± S.E.M. n=5–6 per group.
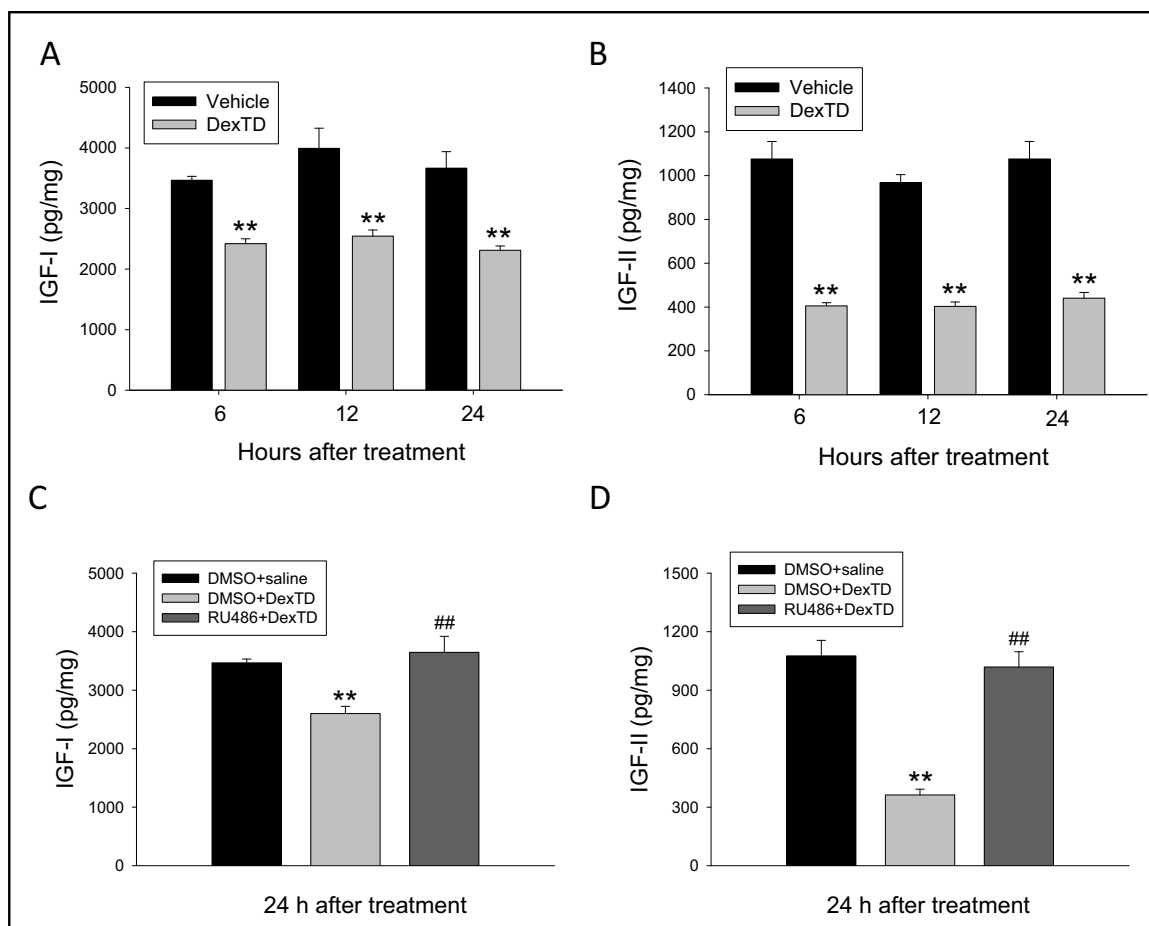
Fig. 3. Glucocorticoid receptor blocker prevented the DexTD induced down-regulation of IGF-I and -II protein in pup brain. DexTD significantly decreased IGF-I and -II protein in brain cortices compared with the vehicles at 6, 12 and 24 h after treatment (*A,B*) (**$p<0.01$, n=6 per group). In a separate experiment, GR blocker RU486 significantly prevented the DexTD-induced reduction in IGF-I and -II proteins in the neonatal rat brain at 24 h after treatment (*C,D*) (**$p<0.01$ vs. DMSO+Saline; ##$p<0.01$ vs. DMSO+DexTD, n=6 per group).

Dex increased BDNF and NGF mRNA expression, but did not change NGF or BDNF protein in pup brain

At 24 h after treatment, DexSD of 0.25 mg/kg significantly increased both BDNF and NGF mRNA while DexSD of 0.125 mg/kg increased NGF mRNA but did not affect BDNF mRNA (Figure 6*A*). For the time course experiment, the DexSD of 0.5 mg/kg of Dex significantly increased mRNA of BDNF at 3h after treatment but there was no change at 6 h and 1 to 3 days after treatment (Figure 6*A&B*). The DexSD of 0.5 mg/kg of Dex significantly increased mRNA of NGF at 3 and 6 h after treatment, but there was no change at 1 to 3 days after treatment (Figure 6*A&B*).

Compared to the vehicle, DexTD significantly increased the expression of mRNA of NGF (1.3 fold, $p<0.01$) in rat pup brain at 24 after treatment but there was no significant change in BDNF mRNA ($p>0.05$, Figure 6*C*). NGF and BDNF proteins were measured by ELISA at 24 after treatment and the results are expressed relative to total protein for each sample. Brain NGF level was 60.6±3.1 pg/mg in the vehicle and 58.0±3.9 pg/mg in the DexTD group ($p>0.05$, n=8/group). Brain

BDNF level was 675.6±89.5 pg/mg in the vehicle and 680.2±90.3 pg/mg in the DexTD group ($p>0.05$, n=8/group). Thus DexTD did not affect NGF and BDNF protein.

DISCUSSION

In this study, we found that Dex caused a marked decrease in IGF-I and -II mRNA expression and protein levels in the developing rat brain. GR inhibition prevented these effects on IGF-I and II protein and IGF-II mRNA. Additionally, DexTD caused minimal increase in NGF mRNA but no change in NGF protein in the brain. No change in BDNF mRNA or protein was noted following DexTD treatment.

Similar to our previous study (Feng *et al.* 2009), Dex decreased body and brain weight. Decrease in brain weight by Dex is crucial considering that many clinical studies in premature infants have shown deleterious effects of Dex treatment on the brain. In a clinical study by Murphy *et al.* (2001) there was a 30% reduction in cerebral tissue volume in premature infants treated with
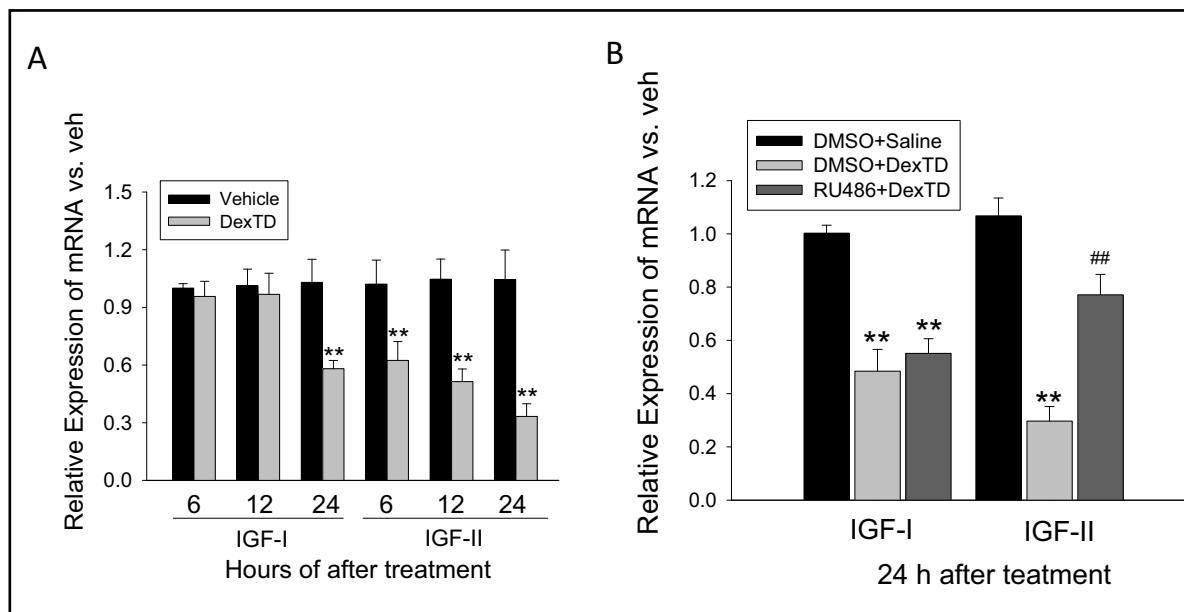
Fig. 4. DexTD decreased the expression of mRNA of IGF-I and -II in the pup brain. The summary of results derived from real-time RT-PCR at 24 h after treatment in the brain cortices of rat pups following DexTD or vehicles for IGF-I and -II mRNA. Each mRNA level was first normalized to that of a housekeeping gene GAPDH and further calculated by the $2^{-\Delta\Delta Ct}$ method and plotted as fold change vs. vehicle. DexTD decreased the expression of mRNA of IGF-I at 24 h but not at 6 and 12 h after treatment (A, **$p<0.01$ vs. vehicle, n=5–6 per group). DexTD decreased the expression of mRNA of IGF-II at 6, 12 and 24 h after treatment (A, **$p<0.01$ vs. vehicle, n=5–6 per group). Treatment with GR blocker RU486 prevented the decrease in IGF-II (B) (##$p<0.01$ vs. DexTD, n=5–6) but did not affect IGF-I (B) ($p>0.05$ vs. DexTD, n=5–6) measured at 24 after treatment in the neonatal rat brain.

Dex compared with untreated age-matched controls. Other studies have shown that Dex increases the incidence of cerebral palsy in premature infants (Shinwell *et al.* 2000; O'Shea *et al.* 2007). Thus, Dex causes permanent damage to the developing brain. This is in contrast to the effects on the adult brain in which recovery of both the morphological (hippocampal atrophy) and behavioral changes (mood disorders) caused by elevated corticosteroid secretion are possible after normo-cortisolemia has been re-established (Crochemore *et al.* 2005; Starkman *et al.* 1999). In adult animals, Dex treatment caused apoptosis in striatum, granular and hilar cells in the dentate gyrus, as well as pyramidal cells of the CA3 subfield of the hippocampus (Haynes *et al.* 2001; Hassan *et al.* 1996). In a neonatal rat model, Dex caused apoptosis in CA1 and CA3 subfield of the hippocampus (Duksal *et al.* 2009). In immature mice, a single GC injection produced rapid and selective apoptotic cell death of the proliferating neural progenitor cells in the cerebellar external granule layer and permanent reductions in neuronal cell counts of their progeny, the cerebellar internal granule layer neurons (Noguchi *et al.* 2008). Several putative mechanisms have been implicated in Dex-induced neuronal damage, but the role of GR is strongly implicated. In our study, Dex increased caspase-3 activity as demonstrated previously. More importantly, treatment with GR blocker inhibited the decrease of brain and body weight and reduced the increase of caspase-3 activity induced by Dex. These findings indicate that Dex induces neurodegenerative

effects by activating GR. One possible scenario might be that GCs inhibit glucose utilization, thereby compromising the activity of energy dependent excitatory amino acids transporters, thus causing an increase in extracellular glutamate and increase in intracellular calcium levels (Reagan & McEwen 1997). Interestingly, GCs were also reported to increase peak voltage-gated calcium channel (VGCC) currents (Fuller *et al.* 1997), basal cytosolic free calcium concentrations (Elliott & Sapolsky 1993) and mRNA levels of VGCC in cultured hippocampal neurons (Fomina *et al.* 1996). Additionally, loss or impairment of trophic growth factors in the brain following Dex exposure might be involved.

Our results showed that DexTD caused minimal increase in NGF mRNA, but no change in NGF protein in the brain. No change in BDNF mRNA or protein was noted following DexTD exposure. These results imply that NGF or BDNF are not involved in the Dex-induced neurotoxicity. Interestingly, DexTD caused significant reduction in IGF-I and -II mRNA and protein in the brain. Other studies have shown similar effects of Dex on IGF-1. Adamo *et al.* (1988) showed that the Dex reduced IGF-I mRNA levels by 60% and 40% in neuronal cell and glial cell cultures, respectively. The authors conclude that their results suggest that GCs-induced reductions in IGF-I production could occur at the level of transcription and may underlie some of the actions of GCs in causing growth retardation and inhibition of cell proliferation (Adamo *et al.* 1988). Another study showed that high levels of corti-
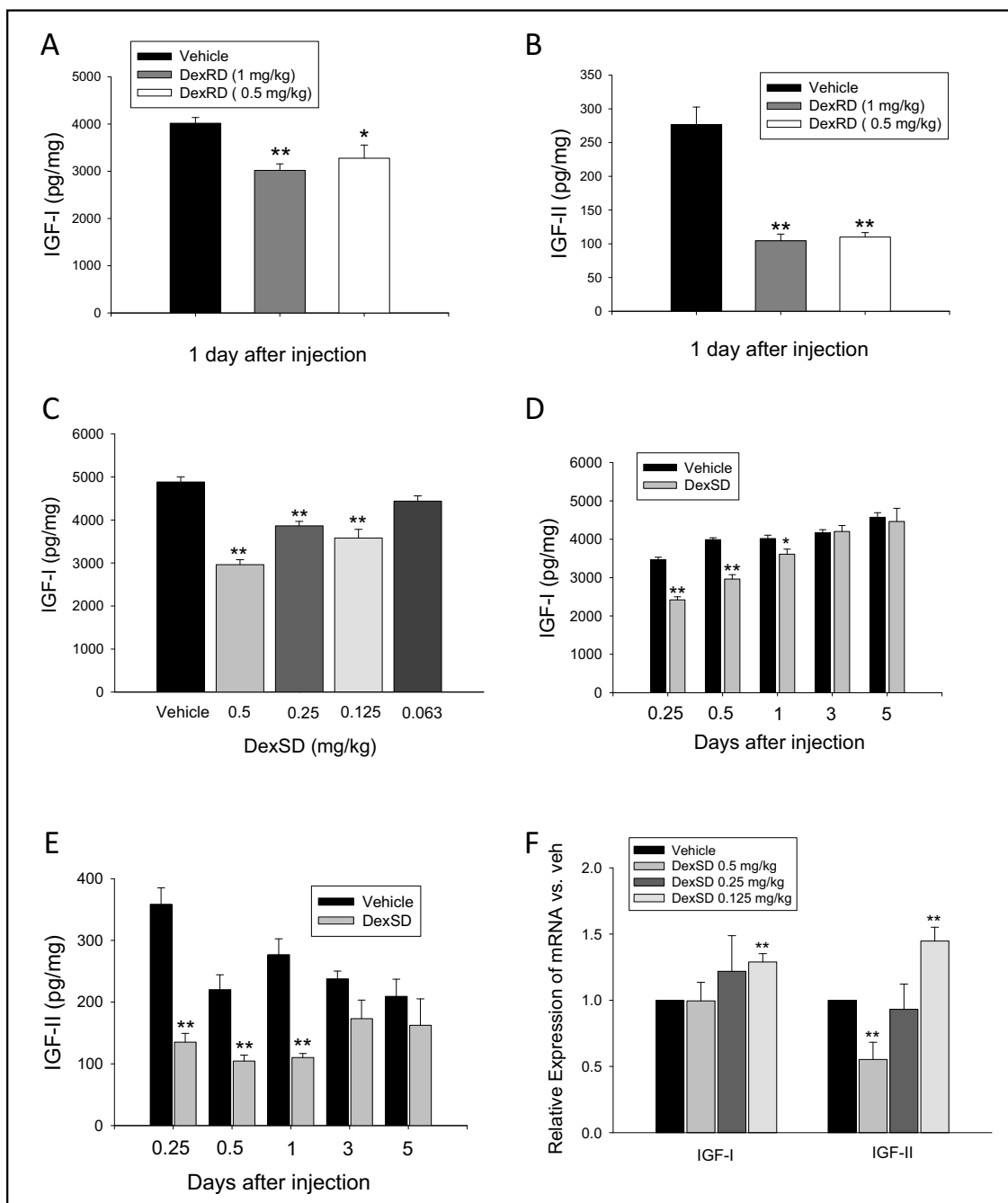
Fig. 5. Effect of DexSD and DexRD on IGF-I and IGF-II in the pup brain. IGF-I and -II protein in brain cortices were measured by ELISA and the results are expressed relative to total protein for each sample. DexRD (0.5 and 1 mg/kg) significantly decreased levels of IGF-I and -II protein compared with the vehicles at 1 day after treatment (*A,B*) (*$p<0.05$, **$p<0.01$, n=5–6 per group). The single dose of 0.125 mg/kg to 0.5 mg/kg but not 0.06 mg/kg of Dex also significantly decreased IGF-I protein compared with the vehicles at 1 day after treatment (*C*) (**$p<0.01$, n=5–6 per group). For the time course experiment, the single dose 0.5 mg/kg of Dex significantly decreased IGF-I and -II protein at 6 h, 12 h and 1 day after treatment compared with the vehicles (*D,E*) (*$p<0.05$, **$p<0.01$, n=5–6 per group) but there was no change at 3 and 5 days after treatment (*D,E*) ($p>0.05$, n=5–6 per group). DexSD 0.5 mg/kg significantly decreased IGF-II mRNA but did not decrease IGF-I mRNA compared with the vehicle at 1 day after treatment (*F*) (**$p<0.01$, n=5–6 per group). There were no changes in mRNA of IGF-I and IGF-II in DexSD 0.25 mg/kg group but treatment with DexSD 0.125 mg/kg significantly increased IGF-I and IGF-II mRNA compared with the vehicle at 1 day after treatment (*F*) (**$p<0.01$, n=5–6 per group).

sol decreased skeletal IGF-I synthesis by reducing IGF-I transcript levels and suggested that this effect probably contributed to the inhibitory influence of cortisol on bone formation (McCarthy *et al.* 1990). Dex inhibited

growth hormone induction of IGF-I mRNA in different tissues examined (brain was not examined) in hypophysectomized rats and reduced IGF-I mRNA abundance in the intact rat (Luo & Murphy 1989). Additionally,
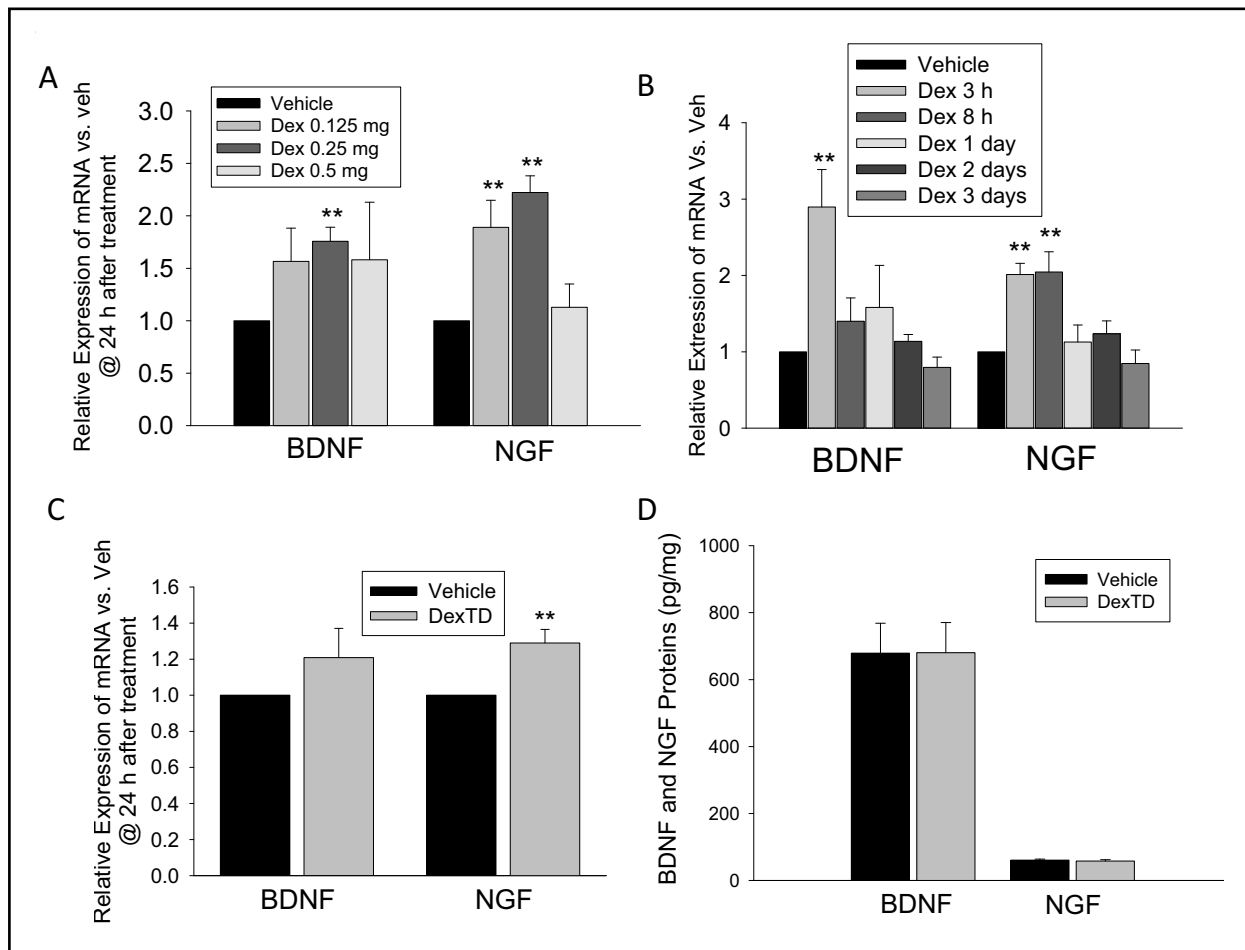
Fig. 6. Effect of Dex on NGF and BDNF in the pup brain. The summary of results derived from real-time RT-PCR at 24 h after treatment in brain cortices of rat pups following DexSD, DexTD or vehicles for BDNF and NGF mRNA. Each mRNA level was first normalized to that of a housekeeping gene GAPDH and further calculated by the $2^{-\Delta\Delta Ct}$ method and plotted as fold change vs. vehicle. At 24 h after treatment, DexSD 0.125 mg/kg increased NGF mRNA (*A*) (**$p<0.01$ vs. vehicle, n=5–6 per group) but did not affect BDNF mRNA (*A*) ($p>0.05$); DexSD 0.25 mg/kg significantly increased both NGF and BDNF mRNA (*A*) (**$p<0.01$ vs. vehicle, n=5–6 per group) but DexSD 0.5 mg/kg did not (*A*) ($p>0.05$, n=5–6 per group). For the time course experiment, the single dose 0.5 mg/kg of Dex significantly increased mRNA of NGF at 3 and 6 h after treatment (*B*) (**$p<0.01$ vs. vehicle, n=5–6 per group) but there was no change at 1 to 3 days after treatment (*B*) ($p>0.05$); single dose 0.5 mg/kg of Dex also significantly increased mRNA of BDNF at 3h after treatment (*B*) (**$p<0.01$ vs. vehicle, n=5–6 per group) but there was no change at 6 h and 1 to 3 days after treatment (*B*) ($p>0.05$). DexTD significantly increased the expression of mRNA of NGF (*C*) (1.3 fold, **$p<0.01$, n=6–8 per group) at 24h after treatment but no significant change in BDNF mRNA was noted (*C*) ($p>0.05$, n=6–8 per group). There are no differences in BDNF and NGF protein between vehicle and DexTD groups (*D*) ($p>0.05$, n=4 per group). Data are presented as mean ± SEM.

Dex attenuated reactive axonal sprouting by inhibiting the microglial production of IGF-I (Woods *et al.* 1999). A study involving transgenic mice expressing an IGF-I-Luciferase fusion gene showed that Dex significantly reduced transgene expression in the developing cerebral cortex, hippocampus, brain stem, and cerebellum indicating that GCs might be involved in brain IGF-I gene expression during development (Ye *et al.* 1997). Little is known about the effects of Dex on IGF-II or regulation of IGF biosynthesis in the CNS. Ours is the first to examine and show the significant effects of Dex on IGF-I and -II in rat brain but the mechanism of regulation IGF-I and -II by Dex is not known. GR inhibition prevented Dex-induced decrease in IGF-I mRNA and IGF-II mRNA and protein, while IGF-I mRNA was not affected. We speculate that Dex via GR regulates

IGF-I at the translation level and also regulates IGF-II at both the translational and transcription levels. The mechanism of Dex-induced increase in IGF-I mRNA is not clear and probably involves non-genomic pathways. Further studies will be required to address these important questions.

IGFs exert potent neurotrophic and neuroprotective/antiapoptotic activities (Russo *et al.* 2005; Beilharz *et al.* 1995; Knusel *et al.* 1990). Thus, Dex-induced reduction in IGFs can explain its apoptotic neurodegenerative effects. Additionally, reduction in brain IGF-I adversely affects neurogenesis, oligodendrocyte proliferation and maturation, axonal growth and myelination (Ye *et al.* 2002; Beck *et al.* 1995; Russo *et al.* 2005). Interestingly, adverse effects of Dex on the developing brain include its deleterious effects on neurogenesis, axonal degen-

eration and myelination (Kanagawa *et al.* 2006; Tan *et al.* 2002; Antonow-Schlorke *et al.* 2009). Thus, pleiotropic functions of the IGF system may play an important role in the Dex-induced neurotoxicity by a variety of mechanisms and merit further research.

A variety of molecular mechanisms of Dex-induced neuronal apoptosis have been suggested. In the rat hippocampus, activation of GR increases levels of the tumor suppressor protein p53 and induces cell death by increasing the ratio of the proapoptotic molecule Bax relative to the antiapoptotic molecules Bcl-2 or Bcl-xL (Almeida *et al.* 2000). Alternatively, regulation of the death pathway by proapoptotic BcL-2 family member p53 upregulated mediator apoptosis independent of p53 expression has been suggested (Noguchi *et al.* 2008). Previous *in vitro* and *in vivo* studies have shown that elevated levels of IGF-I act to reduce neuronal apoptosis by increasing expression of BcL-2 and Bcl-xL (Chrysis *et al.* 2001) and decreasing expression of Bad, Bax and Bim (Gleichmann *et al.* 2000; Chrysis *et al.* 2001; Linseman *et al.* 2002), leading to decreased activation of caspase-9 and caspase-3 (Chrysis *et al.* 2001; Linseman *et al.* 2002). Thus, it is possible that Dex-induced reduction of IGF-I can change the ratio of proapoptotic and antiapoptotic molecules of Bcl-2 family proteins and cause neuronal apoptosis.

It is interesting to note that the effect of Dex on IGF-I and IGF-II differed at the different doses. Overall, all three different regimens of Dex had inhibitory effects on IGF-I and II protein and mRNA. The lower single dose (0.25 mg/kg) of Dex increased IGF-I and II mRNA; but decreased protein at 24 h after treatment. It is possible that a fall in protein concentration results in a rebound increase in respective mRNA at the lower dose but not at the higher doses of Dex. Alternatively, at lower doses Dex may regulate IGF levels at translational levels and/or by non-genomic mechanisms.

CONCLUSION

Our findings indicate that Dex decreases IGF-I and -II levels and causes neurodegeneration via GR-dependent mechanisms in the developing rat brain. Further research to confirm a causative role of changes in the IGF signaling pathways in the Dex-induced neurotoxicity is needed. If confirmed, it can help to establish potentially new therapeutic targets and their exploitation may lead to new treatment to prevent or treat brain damage in premature infants following Dex exposure.

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