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# 출생전 저산소증에 의한 기니픽 태자 뇌의 신경발생 감소

조선대학교 대학원

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전 용 현

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Prenatal hypoxia reduces neurogenesis in the brain of fetal guinea pig

2014년 2월 25일

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지 도 교 수 정 윤 영

이 논문을 의학박사학위 신청 논문으로 제출함

2013년 10월

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의 학 과

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# 전용현의 박사학위논문을 인준함

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## 조선대학교 대학원

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# ABSTRACT

## 출생전 저산소증에 의한 기니픽 태자 뇌의 신경발생 감소

**전 용 현**
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부적절한 자궁내 발달은 비정상적인 뇌 발달을 초래하여 뇌성마비나 인지 및 행동 장애와 같은 신경학적 결함을 일으킬 수 있다. 이러한 질환들은 저산소증, 저혈당증 등의 증상을 보이는 만성태반부전과 관련이 있는데 특히 저산소증이 뇌 발생에 미치는 영향을 밝히고자 다양한 연구기법을 이용하여 신경발생 과정에 대한 연구가 이루어지고 있다. 신경발생 과정은 신경세포로 발생할 전구세포들의 증식, 이동, 분화 및 생존 등을 포함하는 매우 복잡한 과정으로 저산소증이 신경발생 과정에 어떠한 영향을 미치는지에 대해서는 일치된 견해가 없다. 따라서 본 연구는 기니픽을 이용하여 출생전 지속적인 저산소증이 신경발생 과정에 미치는 영향을 알아보고자 하였다. 임신 30일 된 기니픽의 한쪽 자궁동맥을 결찰하여 저산소증을 유발한 뒤 태생 50일과 60일에 증식세포 표지자인 PCNA, 성숙 신경세포 표지자인 NeuN 및 신경영양물질 표지자인 BDNF를 이용하여 면역조직화학염색을 시행하였다. 주요 신경발생 부위인 뇌실막밑층과 과립밑층에서 PCNA 양성반응 세포들이 태생 50일과 60일의 정상군과 성장지연군 사이에 큰 차이 없이 관찰되었다. 태생 60일의 대뇌겉질과 치아이랑에서 성장지연군의 NeuN 양성반응 세포 수가 정상군에 비해 통계학적으로 유의하게 감소하였으며, 동일 연령의 BDNF 양성반응 세포 수도 성장지연군에서 정상군보다 감소되었다. 이상의 연구 결과는 저산소증에

의한 자궁속성장지연이 뇌 발달에 미치는 영향은 신경발생 과정 중 세포 증식의 변화로 인한 것이라기보다는 세포 생존의 감소가 주요 원인이 될 수 있다는 것을 시사하였다.

# Ⅰ. Introduction

Intrauterine growth retardation (IUGR) causes abnormal prenatal development and neurological sequelae such as cerebral palsy, mental retardation, learning disability, and epilepsy (Towfighi et al. 1997; Tan et al. 1998; Berger and Garnier 1999). These neurological problems are related to chronic placental insufficiency (CPI), which leads to chronic hypoxemia and hypoglycemia (Jensen et al. 1996). Brain damage as a result of hypoxic injury during the fetal period is recognized as a major factor contributing to neonatal mortality (Towbin 1970).

There has been considerable disagreement as to the effect of hypoxia on neurogenesis, which is the process of generating new neurons from progenitor cells including the proliferation, migration, differentiation and survival of neural precursor cells (Caviness et al. 1995). For example, progenitors within the neonatal subventricular zone (SVZ) are vulnerable to hypoxic/ischemic insult (Tolsa et al. 2004). However, neurogenesis is found to occur in the SVZ after neonatal hypoxic injury (Kernie and Parent 2010). Neurogenesis is thought to be limited to the embryonic period, with the exceptions of the olfactory bulb, SVZ, and hippocampal dentate gyrus (Lichtenwalner and Parent 2006). One study shows that chronic perinatal hypoxia promotes cell proliferation in the SVZ (Fagel et al. 2006). However, it has not known whether these neurons survive in the brain.

Brain-derived neurotrophic factor (BDNF) promotes the growth and survival of dentate granule cells (Lowenstein and Arsenault 1996) and pyramidal neurons (Ip et al. 1993) in the brain. The effects of chronic damage on BDNF expression have not well known. It is shown that CPI reduces BDNF and tropomyosin receptor kinase B (Trk B) expression in the guinea pig hippocampus via reduced process

outgrowth, while not affecting the BDNF and Trk B expressions in the cerebellum (Dieni and Rees 2005). Furthermore, the hippocampal volume is found to be reduced at term (Mallard et al. 1999) and hippocampal cell dendrites are altered (Dieni and Rees 2003) in a guinea pig model of CPI, in which fetal hypoxia is induced via uterine artery ligation.

The guinea pig is a useful model for brain development as it has a long gestation period [term, ~67 days of gestation (dg)] with major developmental events occurring in utero (Spira 1975). The guinea pig CPI model mimics a reduction in the oxygen and nutrient supply to the fetus (Jones and Parer 1983). The present study used the guinea pig CPI model to investigate the effect of hypoxia on the development of mature neurons and to evaluate the relationship between BDNF and neurogenesis after chronic prenatal hypoxia.

# Ⅱ. Materials and methods

## A. Animal surgery

All animal experiments were approved by the Chosun University Institutional Animal Care and Use Committee (approval no. CIACUC2013-S0009). CPI was induced via unilateral uterine-artery ligation in pregnant Dunkin-Hartley guinea pigs, as described previously (Nitsos and Rees 1990; Mallard et al. 2000). Briefly, the animals were anesthetized by intramuscular injection of Zoletil (10 mg/kg; Virbac, France) and xylazine (0.15 mg/kg; Bayer, Germany) at 30~32 dg (term ~ 67 days). After shaving the surgical area, a midline incision was made below the umbilicus under aseptic conditions. The fat pad of the uterine horns, which contained the maternal blood vessels, was exposed and ligated with silk sutures (4/0) at the cervical end of the arterial cascade. After the procedure, the abdomen was disinfected using povidone-iodine solution. The animals were caged and raised in a common environment.

## B. Tissue preparation

The fetuses were delivered by cesarean section at 50 or 60 dg. Fetuses from the unoperated horn were assigned to the control group (50 dg, n=8; 60 dg, n=8), and those from the other, ligated horn were assigned to the growth-restricted (GR) group (50 dg, n=8; 60 dg, n=8). The fetuses were removed from the uterine horn and fixed in 4% paraformaldehyde (PFA) solution. The fetal brains were removed and transferred to fresh 4% PFA at 4℃ for 2 days. The forebrains were then washed with water, dehydrated through graded ethanol solutions, and finally embedded in paraffin. Series of 12 ㎛-thick sagittal sections were cut and mounted on gelatin-coated

slides (Fisher Scientific, USA).

### C. Immunohistochemistry

The deparaffinized sections were rinsed three times in 0.1 M phosphate-buffered saline (PBS; pH 7.4) and then incubated in 0.01 M sodium citrate buffer (pH 6.0) while being heated in a microwave oven for 10 minutes. After cooling, the slides were treated with 0.3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity, and rinsed in PBS. They were then incubated with one of the following primary antibodies overnight at 4℃: Rabbit anti-hypoxia induced factor 1α (HIF-1α; 1:500, Abcam, UK), monoclonal anti-proliferating cell nuclear antigen (PCNA; 1:3000, Sigma, USA), mouse anti-NeuN (1:100, Millipore, USA), and rabbit anti-BDNF (1:50, Santa Cruz Biotechnology, USA). On the following day the sections were washed several times in PBS and the immunoreactivity was visualized with biotinylated anti-mouse and anti- rabbit IgG and the avidin-biotin-peroxidase (ABC) detection system (Vectastain ABC Elite Kit, Vector Laboratories, USA), and chromogen 3, 3'-diamino-benzidine as the chromogen. The sections were counterstained with thionine and coverslipped using PolyMount mounting medium (Polysciences, USA).

### D. Quantification

The sections were observed and analyzed with the aid of a light microscope (BX41, Olympus) equipped with a digital CCD camera. The numbers of HIF1-α-, PCNA-, NeuN-, and BDNF-immunore- active (IR) cells were counted in the cerebral cortex, including the parietal cortex and hippocampus. Three sections of the forebrain 300 ㎛ apart were used from each animal. Each section was divided randomly into five areas, and the number of cells within a defined square

region was calculated in each area.

### E. Statistical analysis

All of the data were analyzed using the Statistical Package for Social Sciences (Information Analysis Systems, SPSS, USA). All measurements were compared between the control and GR groups using Student's t-test. The level of statistical significance was set at $p<0.05$.

# III. RESULTS

## A. Cerebral cortex

The density of HIF-1α-IR cells was higher in GR fetuses than in the controls at both 50 and 60 dg ($p<0.05$; Figs. 1 and 9). There was no significant difference between groups in the densities of PCNA-IR cells in SVZ at 50 and 60 dg (Figs. 2 and 10). The density of NeuN-IR cells in the cerebral cortex did not differ significantly between the control and GR fetuses at 50 dg (Figs. 3 and 11), but was lower in GR fetuses than in the controls at 60 dg ($p<0.05$; Figs. 3 and 11). Similarly, the density of BDNF-IR cells did not differ between the control and GR fetuses at 50 dg (Figs. 4 and 12), but was lower in GR fetuses than in the controls at 60 dg ($p<0.05$; Figs. 4 and 12).

## B. Dentate gyrus

The HIF-1α-, PCNA-, NeuN-, and BDNF-IR cells in the middle dentate gyrus were evaluated at 50 and 60 dg. Similar to the findings for the cerebral cortex, the density of HIF1α-IR cells was higher in GR fetuses than in the controls at both 50 and 60 dg (Figs. 5 and 13). Densities of PCNA-IR cells in the subgranular zone (SGZ) did not differ significantly ($p>0.05$) between the control and GR fetuses at 50 dg and 60 dg (Figs. 6 and 14). There was no significant difference in the density of NeuN-IR cells in the dentate gyrus between the control and GR fetuses at 50 dg (Figs. 7 and 15), but it was decreased in GR fetuses compared to the controls at 60 dg (Figs. 7 and 15). Similarly, there was no difference in the density of BDNF-IR cells between the control and GR groups at 50 dg (Figs. 8 and 16), but it was lower in GR fetuses than in the controls at 60 dg ($p<0.05$; Figs. 8 and 16).

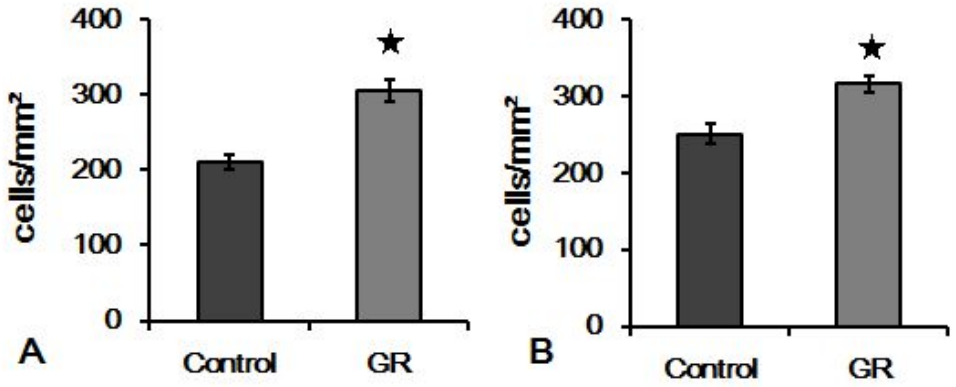


Fig. 1. Densities of HIF-1α-IR cells in the cerebral cortex in control and GR fetuses at 50 dg (A) and 60 dg (B). The cell density was significantly higher in GR fetuses than in the controls. The data are mean and SEM values. ★ $p<0.05$.

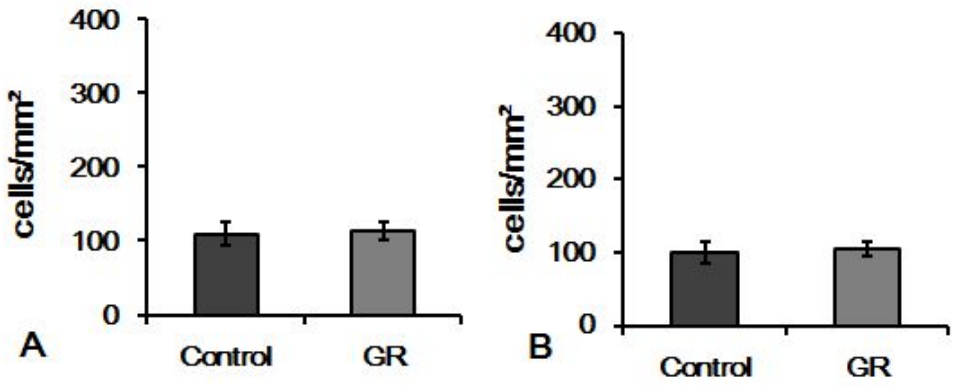


Fig. 2. Densities of PCNA-IR cells in the SVZ in control and GR fetuses at 50 dg (A) and 60 dg (B). The cell densities in the cerebral cortex did not differ significantly ($p>0.05$) between the control and GR fetuses at 50 dg and 60 dg. The data are mean and SEM values.

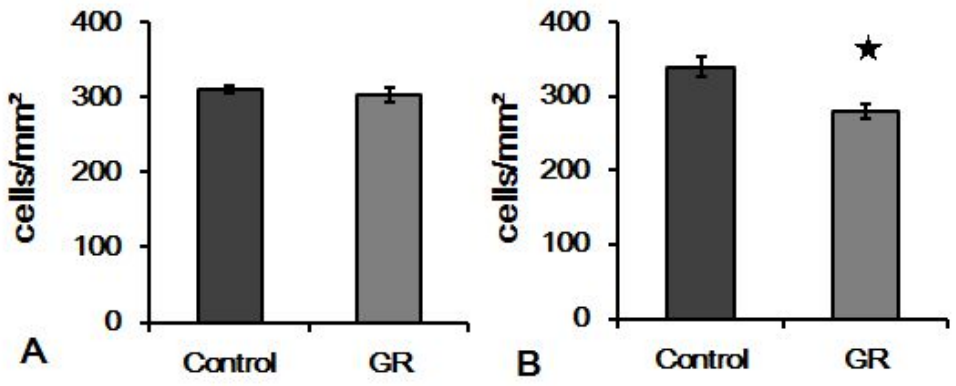


Fig. 3. Densities of NeuN-IR cells in the cerebral cortex in control and GR fetuses at 50 dg (A) and 60 dg (B). The cell density in the cerebral cortex did not differ significantly ($p>0.05$) between the control and GR fetuses at 50 dg. However, the cell density was lower in GR fetuses than in controls at 60 dg. The data are mean and SEM values. ★ $p<0.05$.

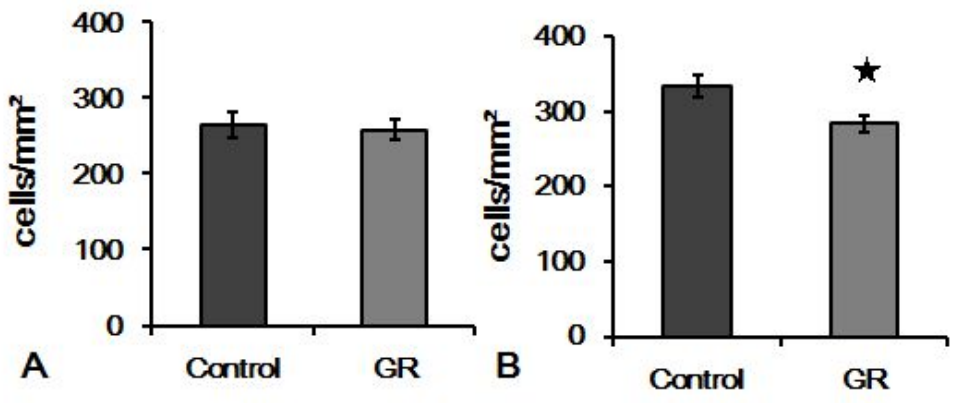


Fig. 4. Densities of BDNF-IR cells in the cerebral cortex in control and GR fetuses at 50 dg(A) and 60 dg (B). The cell density did not differ significantly between the control and GR groups but was lower in GR fetuses than in controls at 60 dg. The data are mean and SEM values. ★ $p<0.05$.

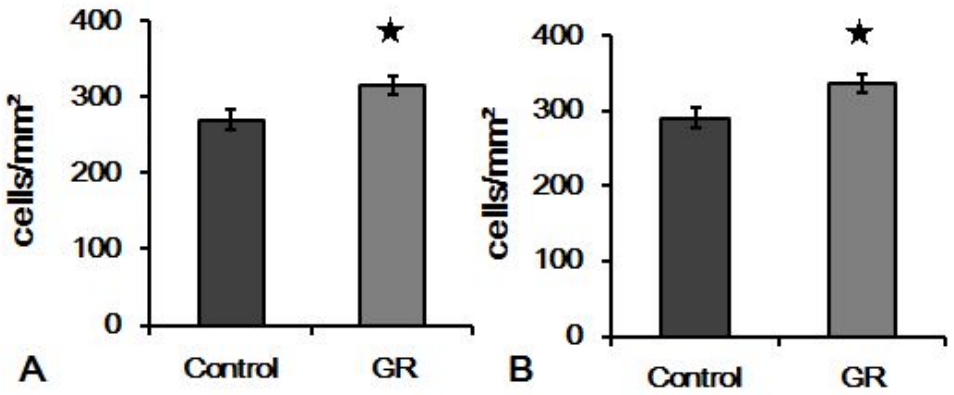


Fig. 5. Densities of HIF-1α-IR cells in the dentate gyrus in control and GR fetuses at 50 dg (A) and 60 dg (B). The cell density was higher in GR fetuses than in the controls at 50 and 60 dg. The data are mean and SEM values. ★ $p<0.05$.

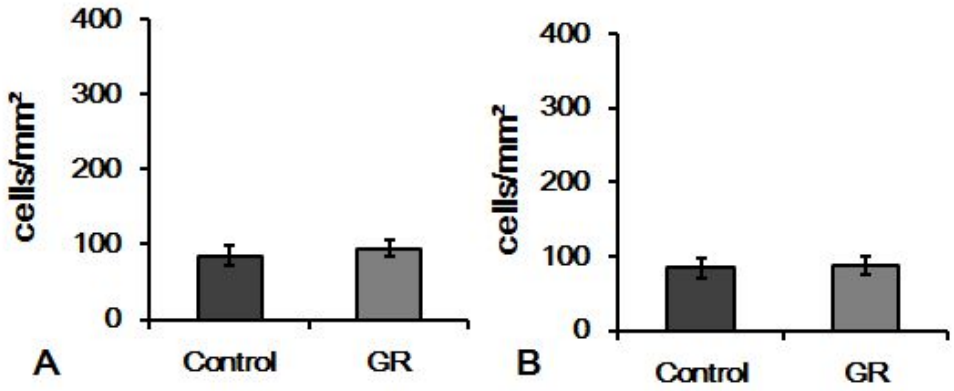


Fig. 6. Densities of PCNA-IR cells in the SGZ in control and GR fetuses at 50 dg (A) and 60 dg (B). The cell densities in the dentate gyrus did not differ significantly ($p>0.05$) between the control and GR fetuses at 50 and 60 dg. The data are mean and SEM values.

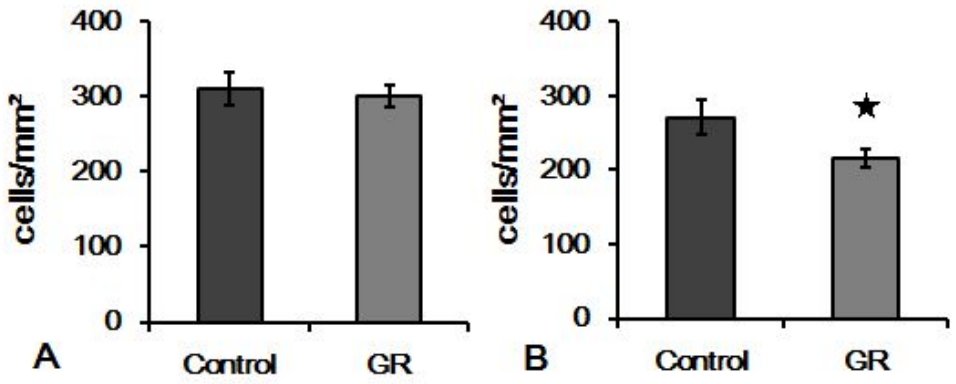


Fig. 7. Densities of NeuN-IR cells in the dentate gyrus in control and GR fetuses at 50 dg (A) and 60 dg (B). The cell density did not differ ($p>0.05$) between the control and GR fetuses at 50 dg, but was lower in GR fetuses than in controls at 60 dg. The data are mean and SEM values. ★ $p<0.05$

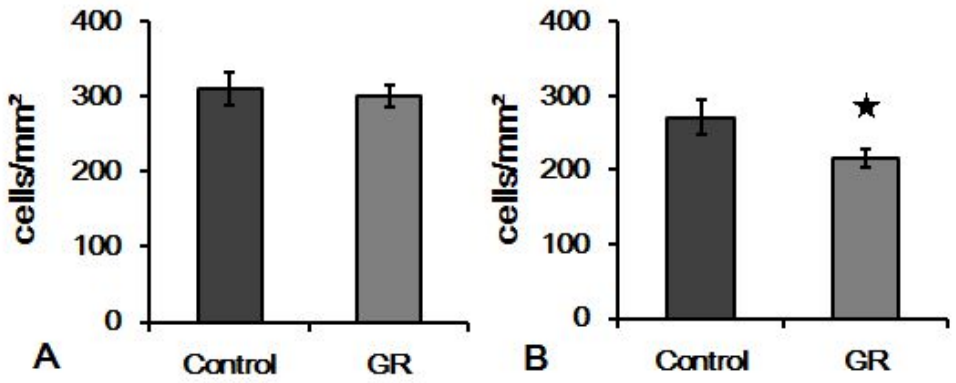


Fig. 8. Densities of BDNF-IR cells in the dentate gyrus in control and GR fetuses at 50 dg (A) and 60 dg (B). Density of BDNF-IR cells did not differ between the control and GR fetuses at 50 dg. However, the cell density was lower in GR fetuses than in the controls at 60 dg. The data are mean and SEM values. ★ $p<0.05$

# Ⅳ. Discussion

The findings of this study demonstrate that chronic hypoxia sustained for the later half of a pregnancy in the guinea pig CPI model can affect neuronal numbers in the cerebral cortex and dentate gyrus. The uterine artery was ligated unilaterally to induce a hypoxic status at 30 dg, reducing the placental blood flow by 25~45% (Jansson et al. 1986). A previous study found immature and undifferentiated neurons in the fetal guinea pig brain at 30 dg (Mallard et al. 2000). At 50 dg, many mature neurons were observed in the cerebral cortex in the present study, and their numbers did not differ between the GR and control animals. The situation in the dentate gyrus was similar to that in the cerebral cortex at 50 dg.

At 60 dg there were some differences between the control and GR groups. The mature neuron cell number was decreased in both the cerebral cortex and dentate gyrus of the GR group compared to the controls. Furthermore, the granular cell layer was thinner in the GR group than in the controls. The alteration in the number of BDNF cells resembled that in the number of mature neurons.

The density of HIF-1α-IR cells in the cerebral cortex and dentate gyrus was higher in the GR group than in the controls at 60 dg. HIF-1α is a nuclear protein complex that binds to a specific consensus sequence in hypoxia-responsive enhancers of many target genes (Semenza et al. 1994), the expression of which is induced by hypoxia-ischemia (Li et al. 2006). HIF-1α has a short half-life and is rapidly degraded during normoxia (Wang et al. 1995). The observed increase in the density of HIF-1α-IR cells in the GR group but not the control group confirms that the fetuses in the former group were in a hypoxic state.

The number of NeuN-IR cells in the cerebral cortex was lower in the GR group than in the controls at 60 dg. NeuN is a

neuronal-specific nuclear protein (Mullen et al. 1992), and NeuN immunoreactivity is used to determine estimates of total numbers of neuronal and nonneuronal cells independently (Herculano-Houzel and Lent 2005). A significant reduction in the number of NeuN-IR neuronal nuclei was shown in the developing chick brain (Rodricks et al. 2010). The present study found PCNA-IR cells in the SVZ. These findings suggest that the survival of neurons in the cerebral cortex is decreased by chronic prenatal hypoxia at 60 dg. In a separate investigation we found that the number of NeuN-IR cells was decreased in the cerebellar external granular layer (So et al. 2013). Bisignano and Rees (1998) reported that the number of cortical neurons was decreased in the cerebral cortex of fetal sheep. The number of NeuN-IR cells was also decreased in the dentate gyrus at 60 dg in the present study. This suggests that the survival of neurons in the dentate gyrus is affected by chronic prenatal hypoxia. However, neurogenesis was found to be enhanced after transient ischemia in the rat dentate gyrus (Liu et al. 1998; Kee et al. 2001). Comparison of the duration of hypoxia imposed using the CPI model and other model is required, since the hypoxic exposure period may be an important factor regarding the degree and type of hypoxic brain injury.

The number of BDNF-IR cells was also decreased in the cerebral cortex and dentate gyrus at 60 dg. BDNF is a member of the neurotrophin family, and promotes growth and reduces cell death in several different neuronal populations, such as cerebellar (Nonomura et al. 1996) and hippocampal cells (Labelle and Leclerc 2000). Dieni and Rees (2005) found that BDNF protein levels were reduced in GR fetuses. The growth of the developing brain is dependent upon the availability of growth factors such as BDNF (Bibel and Barde 2000). Reductions in BDNF levels may thus be associated with the growth retardation caused by chronic prenatal

hypoxia. The reduction in the number of neuronal cells observed in our GR group was associated with the observed reduction in BDNF protein found at 60 dg.

There was no significant difference between control and GR fetuses in the densities of PCNA-IR cells at 50 and 60 dg. The densities of mature neurons in the cerebral cortex and dentate gyrus were diminished in GR fetuses in the present study. Hippocampal progenitors proliferate and migrate into the granular layer of the SGZ, where they differentiate into hippocampal granule neurons (Stanfield and Trice 1988). Therefore, we suggest that prenatal hypoxia reduces neurogenesis in the brain. Since the present experiments were limited to observations at 50 and 60 dg, further studies are required with animals of different (both earlier and postnatal) ages to establish how chronic prenatal hypoxia affects neurogenesis.

# Ⅴ. Conclusion

Chronic prenatal hypoxia is considered to cause perinatal brain injury. It can result in neurological disorder such as cerebral palsy, learning disability, and epilepsy. These neurological problems are related to chronic placental insufficiency (CPI), which leads to chronic hypoxemia and hypoglycemia. How the prenatal hypoxia effect on the brain is not well-known. Chronic placental insufficiency was induced by unilateral uterine artery ligation at 30~32 days of gestation (dg: with term defined as ~67dg). At 50 and 60 dg, fetuses were sacrificed and assigned to either the growth-restricted (GR) or control (no ligation) group. After fixation, dissection, and sectioning of cerebral tissue from these animals immunohistochemistry was performed with HIF-1α, PCNA, NeuN and BDNF antibody in the cerebral cortex and dentate gyrus. The number of NeuN-immunoreactive (IR) cells in the cerebral cortex did not differ between the GR and control groups at 50dg. However, the number of NeuN-IR cells was lesser in GR fetuses than in controls at 60 dg ($p<0.05$). These findings show that chronic prenatal hypoxia affect the number of neuron in the cerebral cortex of guinea pig fetus at 60dg. The number of BDNF-IR cells was also decreased in the cerebral cortex and dentate gyrus at 60 dg. The growth of the developing brain is dependent upon the availability of growth factors such as BDNF (Bibel and Barde 2000). The reduction in the number of neuronal cells observed in our GR group was associated with the observed reduction in BDNF protein found at 60 dg. The approach used in this study is useful for extending our understanding of neurogenesis in the brain, and the findings may be useful for elucidating the brain injury caused by prenatal hypoxia.

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# Legends for Figures

Fig. 9. Representative photomicrographs of HIF-1α immunoreactivity in the cerebral parietal cortex from a control and a GR fetus at 50 and 60 dg. HIF-1α-IR cells in the GR groups were stained in sections strongly at 50 and 60 dg. Scale bars=100 ㎛

Fig. 10. Representative photomicrographs of PCNA immunoreactivity in the SVZ from a control and a GR fetus at 50 and 60 dg. PCNA-IR cells were seen somewhat in the SVZ in control and GR groups at 50 and 60 dg. Scale bars=100 ㎛

Fig. 11. Representative photomicrographs of NeuN immunoreactivity in the cerebral parietal cortex from a control and a GR fetus at 50 and 60 dg. At 60 dg, the NeuN-IR cells in the GR group were smaller and fewer in density than in the control group. Scale bars=100 ㎛

Fig. 12. Representative photomicrographs of BDNF immunoreactivity in the cerebral parietal cortex from a control and a GR fetus at 50 and 60 dg. The density of BDNF-IR cell was lower in GR fetuses than in controls at 60 dg. Scale bars=100 ㎛

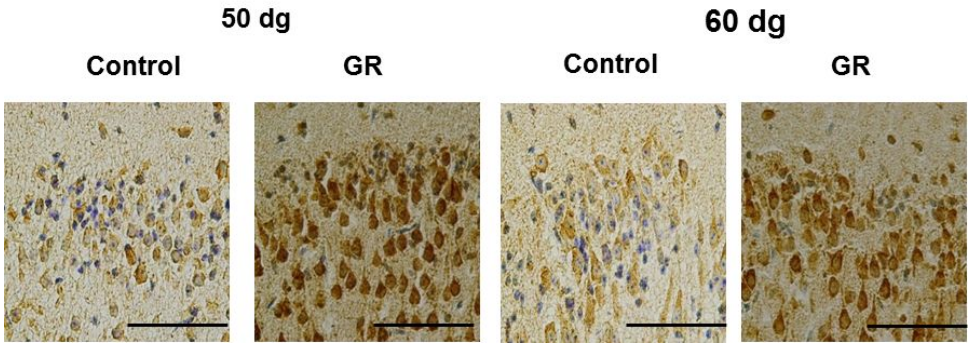
Fig. 13. Representative photomicrographs of HIF-1α immunoreactivity in the dentate gyrus from a control and a GR fetus at 50 and 60 dg. The granule cell layer (GCL) contained many HIF1α-IR cells in the GR group but not the control group at both 50 and 60 dg. Scale bars=100 ㎛

Fig. 14. Representative photomicrographs of PCNA immunoreactivity in the SGZ from a control and a GR fetus at 50 and 60 dg. The pattern of PCNA immunostaining in 60 dg fetuses was similar to that in 50 dg fetuses. Scale bars=100 ㎛

Fig. 15. Representative photomicrographs of NeuN immunoreactivity in the dentate gyrus from a control and a GR fetus at 50 and 60 dg. The NeuN-IR cells were smaller and the GCL was thinner in the GR group than in the control group at 60 dg. Scale bars=100 ㎛

Fig. 16. Representative photomicrographs of BDNF immunoreactivity in the dentate gyrus from a control and a GR fetus at 50 and 60 dg. The density of BDNF-IR cells was lower in GR fetuses than in the controls at 60 dg. Scale bars=100 ㎛

Fig. 9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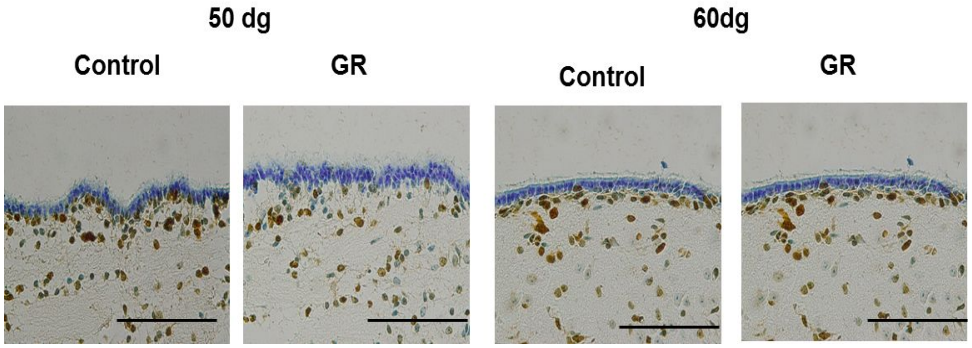
60 dg
Control
GR
Control
GR

Fig. 10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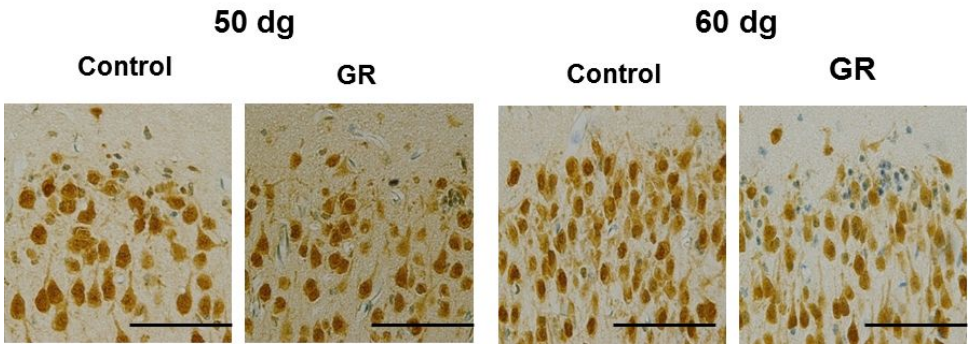
60dg
Control
GR
Control
GR

Fig. 11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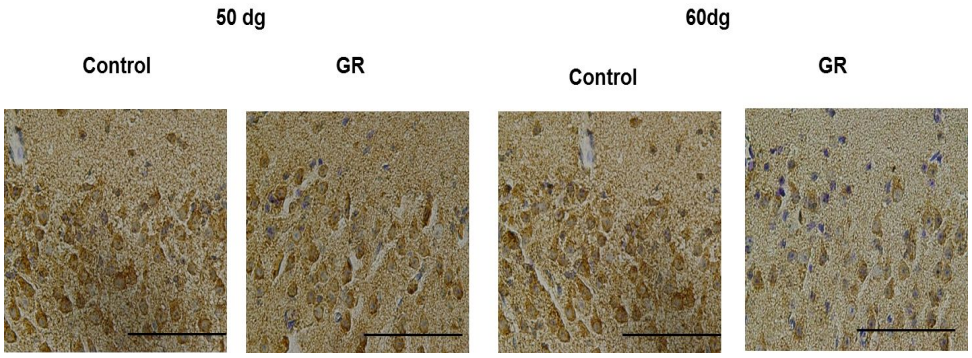
60 dg
Control
GR
Control
GR

Fig. 12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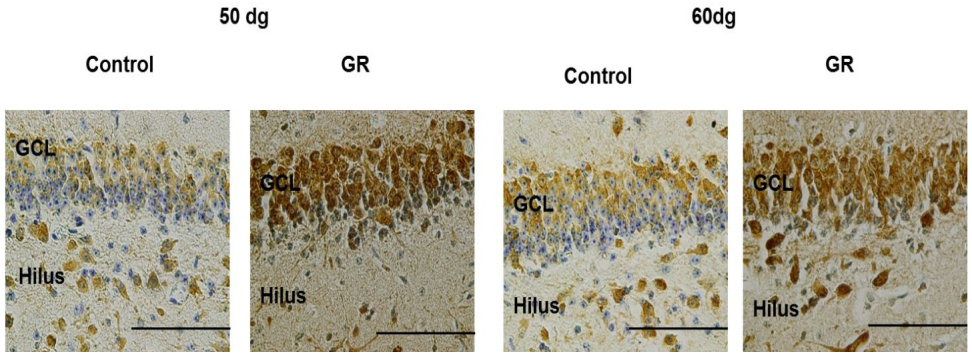
60dg
Control
GR
Control
GR

# Fig. 13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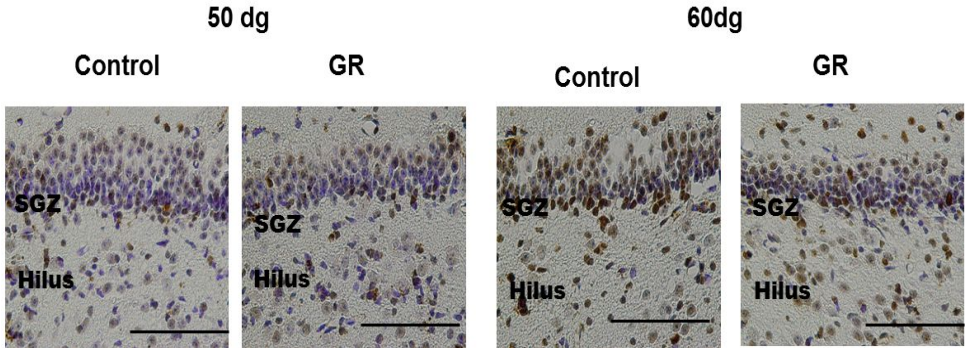
60dg
Control
GR
Control
GR
GCL
Hilus
GCL
Hilus
GCL
Hilus
GCL
Hilus

# Fig. 14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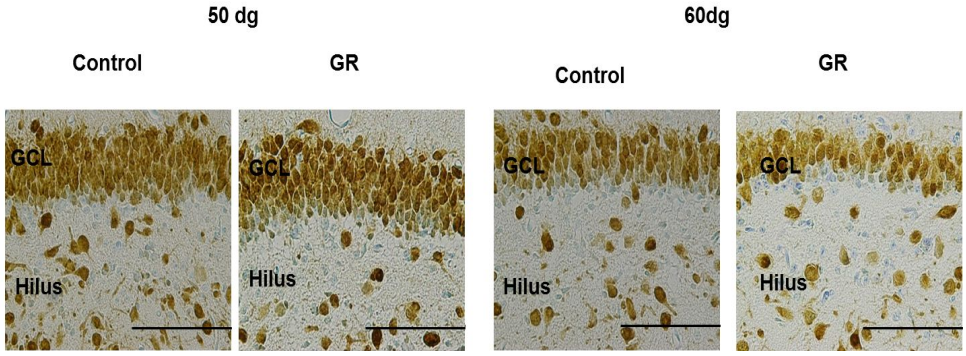
60dg
Control
GR
Control
GR
SGZ
Hilus
SGZ
Hilus
SGZ
Hilus
SGZ
Hilus

## Fig. 15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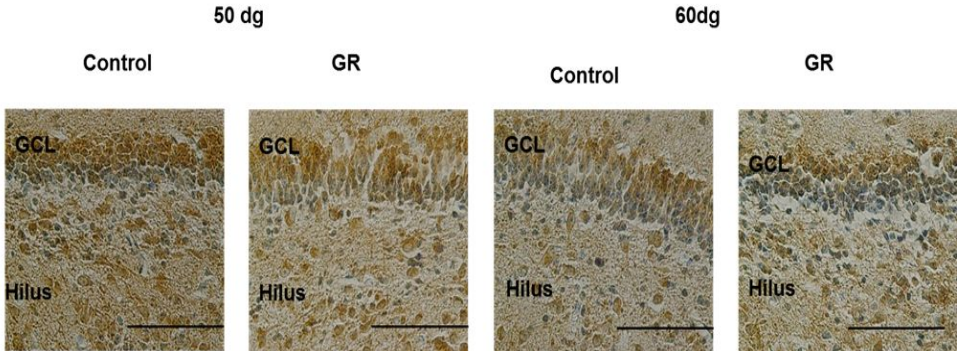
60dg
Control
GR
Control
GR
GCL
Hilus
GCL
Hilus
GCL
Hilus
GCL
Hilus

## Fig. 16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60dg
Control
GR
Control
GR
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Hilus
GCL
Hilus
GCL
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GCL
Hilus