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발생중인 기니피그 소뇌 신경발생영역에서 PCNA 면역반응에 관한 연구

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Immunoreactivity of PCNA in the neurogenic zone of developing guinea pig cerebellum

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ABSTRACT

발생중인 기니피그 소뇌 신경발생영역에서 PCNA 면역반응에 관한 연구

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설치류 중 기니피그는 비교적 긴 임신 기간을 가지고 있으며 출생 전에 주요 뇌 발생이 진행된다는 점에서 신경발생 연구에 많이 이용되고 있다. 특히 신경발생 과정 중 하나인 세포 증식은 다양한 조건을 주어 정상적인 발생 과정에서의 세포 증식 양상과 비교 연구되고 있으나 기니피그 소뇌의 세포 증식의 연령별 변화에 대한 연구는 잘 알려져 있지 않다. 본 연구는 기니피그 소뇌에서 신경발생영역에 해당하는 바깥과립층(external granular layer)의 연령별 발달과 성숙 양상을 알아보고자 시행되었다. 태생 50일과 60일의 태자 및 생후 1주일의 신생 기니피그의 체중과 뇌의 무게를 측정하였다. Proliferating cell nuclear antigen (PCNA) 면역조직화학염색을 이용하여 세포 증식의 연령별 변화를 관찰하고 바깥과립층 두께의 변화도 측정하였다. 본 연구 결과, 소뇌 소엽 I 의 바깥과립층에서 전체 세포 수에 대한 PCNA 면역반응에 양성을 보인 증식세포 수의 비율의 차이는 태생 60일과 생후 1주일 사이에 통계학적으로 유의하게 증가하였으나 다른 연령 사이에는 통계학적 의의가 없었다. 또한 바깥과립층의 두께는 소뇌 소엽 I 과 Ⅷ 모두에서 태생 50일에서 60일 및 태생 60일에서 생후 1주일로 각각 연령이 증가함에 따라 유의하게 감소하였다. 이상의 연구 결과는 기니피그 소뇌의 바깥과립층은 생후 1주까지도 완전히 사라지지 않고 1~2층의 세포층을 이루고 있어 출생 후 바로 사라지지 않는다는 것을 나타내 주었다. 또한 발생 시기별로 신경세포 증식 양상의 변화를 알 수 있어, 추후 소뇌 발달에 영향을 미칠 수 있는 환경에서의 이상 발생과의 비교 연구에 정확한 기준을 제공해 줄 것으로 생각된다.

Ⅰ. INTRODUCTION

Neurogenesis is a very complicated process, which produces new neurons from neural precursor cells via the pathways including proliferation, migration, differentiation and survival of progenitor cells (Caviness et al., 1995). During development, neural stem cells in the ventricular zone proliferate and differentiate into neurons and macroglial cells. In addition, neurogenesis continues in the neurogenic zones in the forebrain which are the subventricular zone, subgranular zones of the dentate gyrus and olfactory bulb, and the external granular layer (EGL) in the cerebellum of the adult mammalian brain (Lichtenwalner and Parent, 2006). Besides rodents, neurogenesis is known to appear in the hippocampus of the adult mammalian brain, including the human (Eriksson et al., 1998) and non-human primate (Gould et al., 1999). Recently, postnatal neurogenesis in the dentate gyrus of the guinea pig has also been characterized (Guidi et al., 2005).

On the other hand, in the mammalian cerebellum, neurogenesis is thought to be limited to the early postnatal period, coinciding with the end of the granule cell development and disappearance of the EGL (Altman and Bayer, 1997). Previous reports on the neurogenesis in the mammalian cerebellum have shown that the EGL persists after birth on the surface of molecular layer until it provides the granule cell formation by cell migration during early postnatal periods. But the period of its disappearance is different depending on the species (Fujita et al., 1966, Altman, 1969, Rakic, 1971, Abrahám et al., 2001). More recently, neurogenesis in the cerebellar cortex is demonstrated in peripuberal and adult rabbits (Ponti et al., 2008).

Especially, the width of the EGL starts to decrease and completely disappears by postnatal month in the developing human cerebellum (Abrahám et al., 2001). This report has revealed that significant cell formation and migration of the newly formed neurons occurs in the human cerebellar cortex during the third trimester and the early postnatal period (Abrahám et al., 2001). Ponti et al (2006) has shown that in the rabbit cerebellum, between the fourth and the fifth postnatal week the EGL is replaced by a proliferative germinal layer called 'subpial layer' (SPL) which persists beyond puberty on the cerebellar surface. The rabbit SPL is transient because it is completely disappeared about sixth month of postnatal life (Ponti et al., 2006).

The persistence of neurogenesis after birth raises the possibility that newly born neural progenitor cells may be stimulated to proliferate via exogenous factors such as growth factors or exercise in order to replace dying neurons (Lichtenwalner and Parent 2006). In addition to this suggestion, recent studies are concentrated in structural and functional changes of the neurogenic zones in the central nervous system of fetuses, neonates and young adults using the guinea pig (Mallard et al., 2000, Dieni and Rees, 2005, Munro et al., 2009, Tolcos et al., 2011). Guinea pig is ideal for experiment model of brain development, because it has a long gestation and its dendritic and axonal proliferation occur *in utero* like in the human (Nitsos and Rees 1990, Mallard et al., 2000, Dieni and Rees 2002). In particular, many researchers have chosen to examine cerebellum for studying neurogenesis because this region is vulnerable to prenatal insults (De Haan et al. 1997, Keunen et al. 1999, Mallard et al. 1998, 2000). However, detailed information is lacking about proliferating EGL in the cerebellum of developing guinea pig. Also, in the EGL of developing guinea pig, the correlation of cell proliferation according to the age is not clear and needs to be clarified.

Cell proliferation is essential for the development of the nervous system (Lossi et al., 2002). The assessment of cell proliferation can be achieved by various methods: counting of mitotic cells, flow cytometry, incorporation ratio of actively labeled DNA precursors, incorporation of bromodeoxyuridine, or by immunohistochemical detection of the proliferating cell marker Ki-67 (Hall and Coates, 1995) and proliferating cell nuclear antigen (PCNA) (Wullimann and Knipp, 2000; Ekstrom et al., 2001). Recent studies using PCNA as marker of endogenous cell cycle and dividing cell were performed in the animal under abnormal condition (Gil et al., 2005, He et al., 2005). Therefore, the aim of the present study is to investigate the cell proliferation of the cerebellar cortex in guinea pig at different stages of gestation and postnatal life on the basis of PCNA expression patterns.

Ⅱ. MATERIALS and METHODS

1. Animals

All procedures were carried out under the approval of the Chosun University Animal Experimentation and Ethics Committee in accordance with international guidelines. The numbers of experimental animal involved in this study was the minimum required for statistical analysis.

Dunkin - Hartley guinea pigs were received from a certified breeding center (Damul Laboratory Animals, Korea). After mating, pregnant female guinea pigs were separated from the males. All animals were bred in the same environment.

2. Tissue preparation

Pregnant guinea pigs (n = 13) at 50 and 60 days of gestation (dg, term approximately 67 days) were deeply anesthetized with an intramuscular injection of xylazine (Rompun®, Bayer korea) and ethyl ether (Duksan pure chemical, Korea). Fetuses at 50 (n = 6) and 60 dg (n = 6) were removed from the uterine horns by cesarean section. 1 week-old guinea pigs (n = 6) were also anesthetized with ethyl ether. The animals were perfused through the heart with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the brains were rapidly removed from the skull and cerebellum postfixed overnight at 4℃ in the same fixative.

Fetal and neonatal cerebellar tissues were dehydrated through graded series of ethanol solutions, and embedded in paraffin. Sagittal sections of 12 ㎛ were cut, and placed on gelatin-coated slides (Fisher Scientific, USA).

3. Immunohistochemistry

Deparaffinized sections were rehydrated and washed with 0.1 M PB (pH 7.4). For the identification of PCNA immunoreactivity, the slides were put in plastic jars filled with 0.01 M sodium citrate buffer (pH 6.0) and applied heat to buffer using microwave. After this antigen retrieval step, the sections were pretreated 0.3% hydrogen peroxide to block endogenous peroxidase activity. After rinsing in PB, the sections were incubated with monoclonal mouse anti-PCNA (1:3000, Sigma, USA) overnight at 4℃. Controls for immunohistochemical label were performed by incubation of normal horse serum at the same time. All slides were then rinsed several times in PB. Binding was visualized with biotinylated anti-mouse IgG and the avidin-biotin-peroxidase (ABC) detection system (Vectastain ABC Elite Kit, Vector Laboratories, USA), and developed in 3,3′-diaminobenzidine (DAB). Slides were washed in tap water and dipped in 0.1% thionin. Following the counterstaining, the sections were dehydrated, cleared with xylene and mounted with polymount mounting medium (Polysciences, USA).

4. Quantitative Analysis

The sections were examined with the aid of a light microscope (BX41, Olympus) attached to a digital CCD camera. The numbers of PCNA-immunoreactive (IR) cells were counted in the EGL of the cerebellar lobule Ⅰ and Ⅷ. Three sections of cerebellum from each animal were chosen for measurement. The interval of section was 240 ㎛ apart from each other. The proportions of PCNA-IR cells in the EGL of lobule I and VIII were assessed using a counting frame of a defined size placed over the three areas randomly each section. Their results were expressed as percentage (%).

For measurement of the EGL thickness, three PCNA-IR sections of

cerebellum 240 ㎛ apart were chosen from lobule I and VIII in each animal. Sections were observed under a 40× objective lens on a light microscope (Olympus) and then the thickness (㎛) of the EGL was measured along the length of the EGL overlying the molecular layer.

5. Statistical analysis

Before postfixation, the weights of body, brain, liver, brain stem, olfactory bulb, and cerebellum were measured. Crown rump length (CRL), brain-to-liver weight ratio, and brain-to-body weight ratio were also determined. All data were analyzed using the Statistical Package for Social Sciences (Information Analysis Systems, SPSS, USA). The mean value of each parameter for fetal and neonatal age was determined by averaging the mean values from each animal. The thickness of the EGL and proportion of PCNA-IR cells were compared between 50 and 60 dg, as well as 60 dg and neonate 1 week using Student's t-test. The level of statistical significance was accepted if $p < 0.05$.

Ⅲ. RESULTS

1. Prenatal and postnatal development of guinea pig

The mean weights of the body, brain, liver and cerebellum and mean CRL were significantly increased ($p < 0.005$) when compared to previous age (Table 1, 2). The mean brain/liver weight ratio was not significant between the ages. But the mean brain/ body weight ratio was significantly reduced ($p < 0.0005$) when compared to previous age (Table 1, 2). These findings reflected normal growth of the organs during fetal and postnatal life.

2. The proportion of PCNA-IR cells to the total cell number in the EGL

There was no significant difference in the proportion of PCNA-IR cells to the total cell number in the EGL between fetal stages, and fetal and postnatal stages in lobule I and VIII ($p > 0.05$) except between at 60 dg and 1 week after birth in lobule I ($p < 0.05$) (Fig. 1). The proportion of PCNA-IR cells to the total cell number was around 50~60% in lobule I and below 60% in lobule VIII at all developmental stages (Fig. 1). In the lobule Ⅷ, the proportion of PCNA-IR cells to the total cell number in the EGL at 50 dg was similar to that of 60 dg and 1 week after birth (Fig 1).

3. Thickness of the cerebellar cortex

When compared to fetal stages, the thickness of the EGL at 1 week after birth ($p < 0.05$) was significantly decreased in lobule I and VIII (Fig. 2). The thickness of the molecular layer and internal granular layer

were increased in both lobules according to increasing age (Fig. 3). However, the thickness of the EGL and Purkinje cell layer were progressively reduced in both lobules according to increasing age (Figs. 4, 5)

Especially, the thickness of the EGL was gradually reduced between 50 dg and 60 dg in the lobules Ⅰ and Ⅷ. Moreover, it became one or two cell layers in both lobules at 1 week after birth (Fig. 5). Whereas the EGL was thick and comprised of several layers of tightly packed cells at fetal ages.

4. PCNA immunoreactivity

After immunostaining with the PCNA antibody, proliferating cells were observed at fetal and postnatal stages (Figs. 3-5). Strong PCNA immunoreactivity was detected in the EGL of lobule Ⅰ and VIII at all developmental stages (Figs. 3, 5). The other layers of the cerebellar cortex had some scattered PCNA-IR cells at fetal stages. A few PCNA-IR cells with large nuclei were also observed in the Purkinje cell layer in both lobules at all developmental stages (Fig. 4). In the EGL, most of PCNA-IR cells were detected in the outer half or two-thirds of whole layer, which contained the neural precursor cells in both lobules (Fig. 5).

Table 1. Fetal body, brain, cerebellum and liver weights, crown rump length, and brain/liver and brain/body weight ratios at 50 and 60 days of gestation

	50 dg (n = 6)	60 dg (n = 6)	*P*
Body weight	39.533 ± 1.074	96.000 ± 3.521	***
Brain weight	1.807 ± 0.088	2.628 ± 0.078	***
Cerebellum weight	0.154 ± 0.009	0.285 ± 0.020	***
Liver weight	2.784 ± 0.216	5.347 ± 0.422	***
CRL	10.983 ± 0.310	13.500 ± 0.190	***
Brain/Liver ratio	67.0 ± 0.068	50.6 ± 0.039	
Brain/Body ratio	4.6 ± 0.002	2.8 ± 0.001	***

Values are expressed as Mean ± SEM, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to previous fetal age (Student's *t* test). The weights of organs are expressed as grams, the uint of CRL is centimeter. Brain/Body and Brain/liver weight ratios expressed as percentages. CRL: crown rump length.

Table 2. Fetal and postnatal body, brain, cerebellum and liver weights, crown rump length, and brain/liver and brain/body weight ratios at 60 days of gestation and 1 week after birth

	60 dg (n = 6)	1wk (n = 6)	*P*
Birth weight		108.500 ± 2.884	
Body weight	96.000 ± 3.521	163.167 ± 3.919	***
Brain weight	2.628 ± 0.078	3.132 ± 0.078	***
Cerebellum weight	0.285 ± 0.020	0.343 ± 0.006	**
Liver weight	5.347 ± 0.422	6.740 ± 0.374	**
CRL	13.500 ± 0.190	15.800 ± 0.526	***
Brain/Liver ratio	50.6 ± 0.039	47.2 ± 0.025	
Brain/Body ratio	2.8 ± 0.001	1.9 ± 0.001	***

Values are expressed as Mean ± SEM, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to previous fetal age (Student's t test). The weights of organs are expressed as grams, the uint of CRL is centimeter. Brain/Body and Brain/liver weight ratios expressed as percentages. CRL: crown rump length.

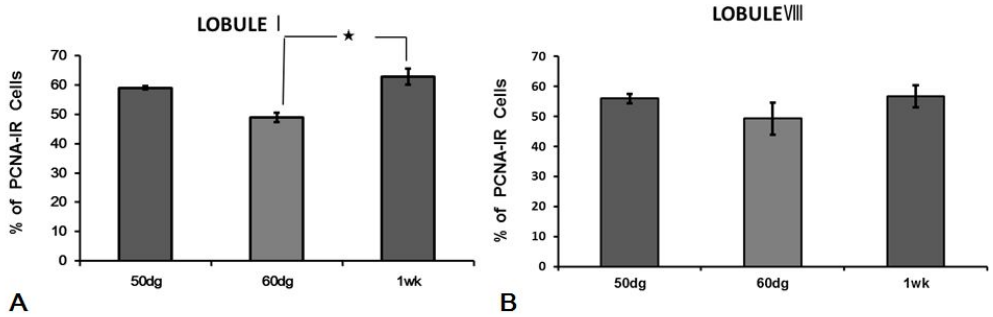

Fig. 1 The proportions of PCNA-IR cells to the total cell number in the EGL of cerebellar cortex in lobule Ⅰ (A) and lobule Ⅷ (B). In lobule Ⅰ, the proportion of proliferating cells to the total cell number was significantly greater at 1 week after birth than at 60 dg. Values are expressed as a Mean ± SEM. ★ $p < 0.05$ compared to previous age (Student's t-test).

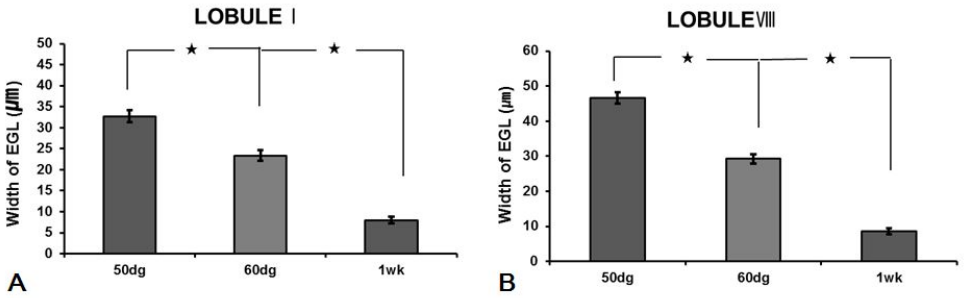

Fig. 2 The thickness of the EGL in lobule Ⅰ (A) and Ⅷ (B) in the cerebellar cortex. According to increasing age, the thickness of the EGL was significantly reduced in both lobules compared to previous age. Values are expressed as a Mean ± SEM. ★ $p < 0.05$ compared to previous age (Student's t-test).

Ⅳ. DIDCUSSION

This study demonstrates cell proliferation in the cerebellar cortex of guinea pig during fetal and perinatal period. The body and brain weights were increased by increasing age like previous study (Dobbing and Sands 1970). The investigation of the embryonic development of the cerebellum has a long history. In the first application of thymidine radiography which researched the embryonic development of the cerebellum, Altman and Bayer (1978) determined that the neurons of deep nuclei were produced between days E13 and E15, with a peak (about 60%) on day E14 in prenatal rats. Recently, one study reported that zone of cell proliferation was present in the cerebellum in which PCNA-labeled cells were located in the granular layer and the inner molecular layer (Raucci et al., 2006). Results of present study support to explain the cell proliferation in the cortical layers of the developing guinea pig cerebellum.

To examine the pattern of cell proliferation, lobules I and VIII of the cerebellum were selected because they were the first and the last lobule to develop in the cerebellum (Nathaniel et al., 1999). In the present study, proliferating cells were observed using PCNA immunohistochemistry. PCNA is a nuclear protein with a molecular weight of 36 (Ogata et al., 1985). It has been demonstrated to be identical both to cyclin (Bravo and Celis 1980), and to the auxiliary protein of the DNA polymerase-δ (Mathews et al., 1984). PCNA and the polymerase-δ auxiliary protein are functionally equivalent in chromosomal DNA replication (Prelich et al. 1987). A current model proposes that PCNA is essential for binding polymerase-δ during the leading-strand DNA synthesis (Fairman, 1990). PCNA expression levels vary with the cell cycle. It appears in late G1 phase and reaches the maximal level during the S phase. Then, it

decreases during other phases including G2 and mitotic phase (Candal et al., 2005). Based on these characteristics of PCNA, a strong nuclear expression indicates that cells are in the S phase, whereas a weak labeling represents that cells are in the G1 or G2 phases (Morris and Mathews, 1989). In this study, PCNA-unlabeled cells are thought to be undifferentiated precursor cells or postmiotic cells in the cortical layer of cerebellum as described by kee et al (2001).

In the present study, the proportion of PCNA-IR cells to the total cell number in the EGL was greater at 1 week after birth than at 60 dg in the lobule I. However, in the lobule VIII, there was no difference in the proportion of PCNA-IR cells to the total cell number in the EGL between 60 dg and 1 week after birth. Considering that the lobules I and VIII in the cerebellum are early-developing and late-developing lobules in the progress of cerebellar maturation, there is a regional difference between two lobules at the same stage of development.

In this study, PCNA-IR cells were mainly detected in the EGL. The thickness of the EGL continued to decline until 1 week after birth. Proliferative zone beneath the pia, the EGL emerges in the late stage of the embryonic development and exists for a period that varies according to the species, but eventually reduces in thickness and ultimately disappears (Ryder and Cepko 1994). The EGL disappears at about the time of birth or soon after birth in precocial animals, but it persists for longer periods in altricial animals such as the mouse, cat, and human (Lossi et al., 2002). In the mouse, EGL does not disappear until 21 days after birth and cell proliferation occurs at the high-rate until the second week (Fujita et al., 1966). In the rat, EGL decreases to postnatal day 20 and disappears at postnatal day 22 (Seress, 1978). The guinea pig is a precocial rodent, which means that most brain development happens before birth (Ryder and Cepko, 1994). A previous study showed that the EGL was

disappeared at birth (Lossi et al., 2002). However, the EGL remained with one or two cell layers until 1 week after birth in this study. As above mentioned, the proportion of PCNA-IR cells to the total cell number were increased between 60 dg and 1 week after birth in this study. It suggests that cell proliferation is more brisk between 60 dg and 1 week after birth than between 50 dg and 60 dg. It is related with the existence of EGL at 1 week after birth. The cells of EGL give rise to neurons of internal granular layer due to proliferation and inward migration through the molecular layer (Gao and Hatten, 1994). The PCNA-IR cells were found in the outer zone of the IGL in this study. A few PCNA-IR cells which have large nuclei are presumably Bergmann glia cells. These cells are related with migration of postmiotic granule cells in the growing cerebellar cortex (Rakic, 1971).

Briefly, the proportion of PCNA-IR cells to the total cell number in the EGL of lobule I was significantly greater at 1 week after birth than at 60 dg. After 50 dg, the thickness of the EGL continued to decline until 1 week after birth in both lobules, as maturation of EGL. These results demonstrate the pattern of PCNA immunoreactivity in the neurogenic zone of developing guinea pig cerebellum which provides a basis for studying neurogenesis under the abnormal condition.

V. CONCLUSION

Guinea pig is ideal for experiment model of brain development, because it has a long gestation among rodents and most brain development happens before birth. Cell proliferation is essential for the development of the nervous system. The assessment of cell proliferation can be achieved by various methods. However, detailed information is lacking about proliferating EGL in the cerebellum of developing guinea pig. Therefore, the aim of this study is to investigate the cell proliferation of the EGL, which is a neurogenic zone in the guinea pig cerebellum at different stages of gestation and postnatal life on the basis of PCNA expression patterns. Fetuses were obtained at 50 dg, 60 dg and 1 week after birth. The body and organ weights measured. The brain sections were incubated with monoclonal mouse anti-PCNA which used to determine cell proliferation. The proportion of PCNA-IR cells was significantly greater at 1 week after birth than at 60 dg in the lobule I. However, there was no difference in the proportion of PCNA-IR cells between at 50 dg and 60 dg. The thickness of the EGL continued to decline until 1 week after birth and remained until the same stage. These results demonstrate the pattern of PCNA immunoreactivity in the neurogenic zone of developing guinea pig cerebellum. This would provide a guideline to study neurogenesis under the abnormal condition.

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Legends for figures

Fig. 3 PCNA immunoreactivity in the cerebellar cortex of lobule Ⅰ and Ⅷ at 50 and 60 dg, and 1 week after birth. Strong-stained PCNA-IR cells were observed in the EGL of lobule Ⅰ and Ⅷ at all developmental stages. Some scattered PCNA-IR cells were observed in the molecular and internal granular layer at fetal stages. When compared to previous stage, the thickness of the molecular layer were increased in both lobules. The thickness of the Purkinje cell layer was progressively reduced in both lobules according to increasing age. EGL: external granular layer; IGL: internal granular layer; ML: molecular layer; PCL: Purkinje cell layer. Scale bars = 100 ㎛

Fig. 4 Representative photomicrographs of PCNA immunoreactivity in the cerebellar layers except EGL of lobule Ⅰ and Ⅷ at 50 and 60 dg, and 1 week after birth. A few PCNA-IR Bergmann glial cells with large nuclei were observed in the Purkinje cell layer in both lobules at all developmental stages. Scale bars = 100 ㎛

Fig. 5 Representative photomicrographs of PCNA immunoreactivity in the EGL of lobule Ⅰ and Ⅷ at 50 and 60 dg and 1 week after birth. According to increasing age, the thickness of the EGL was gradually reduced in both lobules. The EGL was consists of several layers of tightly packed cells at 50 and 60 dg. At 1 week after birth, only one or two cell layers PCNA-IR cells formed the EGL. Scale bars=100 ㎛

Fig. 3

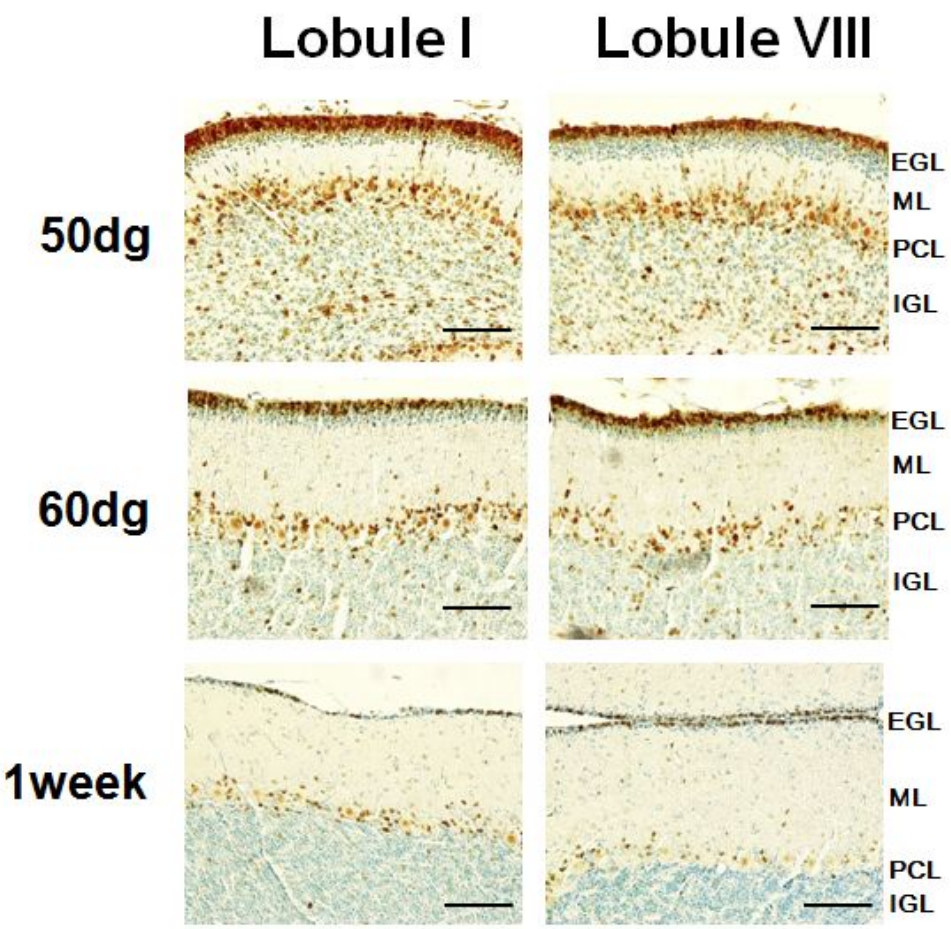

Fig. 4

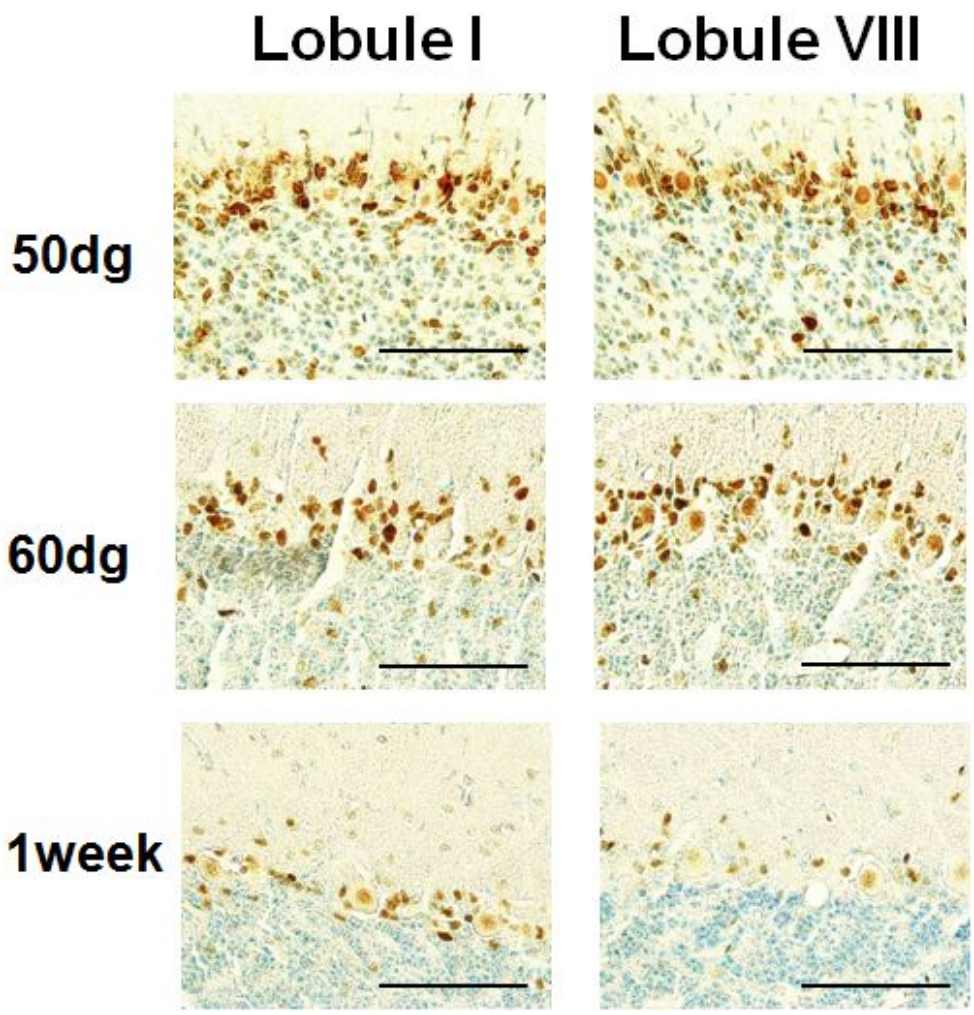

Fig. 5