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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研 究 報 告 書**

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1．助 成 金 額：600,000　円

2．研 究 テ ー マ

皮質凍結損傷で誘発したマウス微小脳回の形成期におけるGABAとglutamate作動性ニューロンの特異な集積

3．成 果 の 概 要（100字程度）

大脳皮質凍結損傷による層構造形成異常動物を作製し、異常皮質の形成過程を調査した。その結果、表層部にGABA細胞が先に集積し、続いて胎生17.5日に発生した本来浅層を形成する錐体細胞がその回りに移動してくることを見出した。さらに、異常皮質形成には、GABAとCl-ホメオスタシスが関与する可能性を示した。

4．研 究 業 績

(1) 学会における発表　　無　・　(有)（学会名・演題）

第３１回日本神経科学学会

Aberrant cortical organization of freeze lesion-induced microgyrus in mouse model of polymicrogyria.

第５５回中部日本生理学会

Accumulation of GABAergic interneurons during organization of freeze lesion-induced microgyrus in mouse.

研究領域「脳の機能発達と学習メカニズムの解明」第６回領域内研究報告会

皮質凍結損傷で誘発したマウス微小脳回の形成期におけるGABAとglutamate作動性ニューロンの特異な集積

(2) 発表した論文　　(無)　・　有（雑誌名・題名）

―日中医学協会助成事業―

皮質凍結損傷で誘発したマウス微小脳回の形成期における GABA と glutamate 作動性ニューロンの特異な集積

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Abstract

Freeze lesion-induced microgyrus resembling human polymicrogyria are associated with seizures. In order to investigate cortical organization with respect to GABAergic and glutamatergic neurons in microgyrus, we made a focal freeze lesion on the GAD67-EGFP knock-in mouse cerebral cortex at P0. The microgyrus was identified with a small sulcus and 3- or 4-layered dysplastic cortex at P7. We found GABAergic interneurons accumulated in superficial layer as well as in the boundary of necrosis at P4. Birthdate analysis showed that the microgyrus contained BrdU-labeled glutamatergic neurons generated on E17. They accumulated around necrotic center at P4 and then formed the cell dense portions in layer ii of microgyrus. The chemokine Cxcl12, a chemoattractant for cortical interneurons, was expressed in the meninges overlying the dysplastic cortex at P4 after freeze lesion. However, significant induction of Cxcl12/Cxcr4 mRNA expression in the dysplastic cortex could not be detected, suggesting Cxcl12/Cxcr4 signaling was not be responsible for accumulation of GABAergic interneurons at P4. Imaging studies on endogenous GABA, another molecule for modulating the migration of immature cortical neurons, revealed GABA release was aberrant in the lesioned area at P4. Thus, neonatal freeze lesion can cause assembly of certain populations of neurons associated with characteristic release of GABA at early stage, which may underlie the formation of microgyrus.

Keyword: freeze-lesion, microgyrus, GABAergic interneuron, glutamatergic pyramidal neuron, GABA

Introduction

Structural abnormalities of the human cortex including polymicrogyria are frequently associated with epilepsy. Cortical malformation can be induced by freeze lesion on the cortex of neonatal rat (Luhmann & Raabe, 1996; Luhmann et al., 1998a, 2000). The abnormal structure of cortex can be characterized by a small sulcus and three- or four-layered dysplastic cortex in the freeze-lesioned rat (Luhmann et al., 1998b). In vitro electrophysiological studies showed lesion-induced microgyrus can cause focal hyperexcitability (Jacobs et al., 1996).

Microgyrus with epileptic activities can be induced by freeze-lesion only on the day of birth (P0) or P1. So far most of studies involved in molecular and electro-physiological changes in the function of GABAergic inhibition and glutamatergic excitation in the microgyrus (Shimizu-Okabe et al., 2007; Zilles et al., 1998) Although laminar abnormalities in freeze lesion-induced microgyrus have been thought to be a migratory defect, the mechanism of the development of microgyrus is not completely understood yet.

Newly born cortical neurons undergo extensive migration before reaching their final destination in the brain. GABAergic interneurons derive from the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE) of the subpallium and reach the developing cortex through a long tangential migration. These neurons migrate in two main streams, one

migrating through the marginal zone (MZ) and another one in the lower intermediate zone (IZ)/ subventricular zone (SVZ). Glutamatergic neurons originate throughout the ventricular zone (VZ) of the pallium and migrate radially to establish the different layers of the cortex in an inside-out progression. Interactions between GABAergic interneurons and glutamatergic pyramidal neurons have been thought to be involved in cortical organization (Lujan et al., 2005). For example, GABAA receptor activation can modulate movement and migration of immature cortical neurons and also provide stop signal once the cells reached their destination (Heck et al., 2007). Therefore, we focused on cortical organization with respect to GABAergic and glutamatergic neurons in microgyrus.

Materials and methods

Animal: We used glutamic acid decarboxylase (GAD)67-enhanced green fluorescent protein (GFP) knock-in (KI) mice in which GFP is expressed under the regulation of the endogenous GAD67 promoter (Tamamaki et al.,2003). GABAergic interneurons can be easily detected by expression of GFP in GAD67-GFP KI mice.

1. Induce a cortical freeze-lesion on the newborn GAD67-GFP KI mice: A liquid nitrogen-cooled copper rod with a tip diameter of 0.5 mm was positioned for 5 sec on the surface of the exposed calvaria of P0 mice. In order to produce a longitudinal microgyrus, three focal freeze-lesions were made in a line near to the midline in a rostro-caudal direction, with a distance of 0.5 mm between the lesions.

2. Birthdates analysis: Pregnant mice were injected intraperitoneally with a single dose of an S-phase marker BrdU (50 mg/kg) on either E14.5 or E17.5. Then the location of BrdU positive neurons was determined at different stages by mean of immunohistochemistry..

3. Immunohistochemistry: Coronal sections were cut at a thickness of 25-μm with a cryostat. Free-floating method was used to perform immunostaining with specific antibodies (anti-GFP, BrdU, Cux1 and Tbr1 antibodies).

4. In situ hybridization: Coronal brain sections of 16-μm thickness were prepared from freeze lesioned-mice at P4. Digoxigenin-labeled cRNA probes for Cxcl12 and Cxcr4 were used. The hybridization signals of Cxcl12 and Cxcr4 mRNAs were enhanced by Tyramide Signal Amplification (TSA) system.

5. GABA imaging: Living coronal brain slices with a thickness of 400-μm were prepared for GABA imaging. During the degradation of GABA by GABase, a fluorescent concomitant NADPH was produced. NADPH is excited by 340nm UV and can emit the fluorescence of 480nm. The fluorescence of NADPH was detected with CCD camera (Keyence, BZ-9000, JP). Endogenous GABA release can be quantified as it is released from cells in the brain slice by NADPH fluorescence intensity.

Result

Figure 1. Formation of a microgyrus after freeze lesion

A. A dorsal view of a mouse brain after freeze lesion. A longitudinal microgyrus can be observed at P7 in the right hemisphere (marked with arrowheads). B. Thionin-stained coronal sections at different stages. At P2, the position of the lesion was identified by necrotic tissue in the lesioned area. At P4, a cell dense layer was observed in the superficial part (arrow) as well as in the boundary (arrowheads) of lesioned area. At P7 and P10, a microgyric architecture could be detected with 3- or 4- layered dysplastic cortex.

Figure 2. GABAergic interneurons accumulated in the superficial part of lesioned area at P4.

Distribution of E14.5-/E17.5-born cells and GAD67-GFP positive cells in freeze-lesioned mouse cortex at P4. Coronal sections of neocortex stained with anti-BrdU and anti-GFP antibodies which injected with BrdU on either E14.5 or E17.5. Note GAD67-GFP positive cells accumulated in the superficial layer and boundary (arrows) of necrosis. BrdU positive cells generated on E14.5 were absent in the lesioned cortex, but BrdU positive cells generated on E17.5 were already accumulated around lesioned area at P4 (arrowheads).

Figure 3. High density of E17.5-generated cells in layer ii of dysplastic cortex at P7

Distribution of E14.5-/E17.5-born cells and GAD67-GFP positive cells in freeze-lesioned mouse cortex at P7. Coronal sections of neocortex stained with anti-BrdU and anti-GFP antibodies which injected with BrdU on either E14.5 or E17.5. Paucity of BrdU positive cells generated on E14.5 was observed in the dysplastic cortex. Note a high density of BrdU-labeled cells generated on E17.5 dispersed throughout the dysplastic cortex (marked with asterisks) at P7.

Figure 4. Cxcl12/Cxcr4 signaling was not involved in the accumulation of GABAergic interneuron at P4

In situ hybridization of Cxcl12 and Cxcr4 mRNA at P4. By using the TSA in situ hybridization, we found some positive signals of Cxcl12 in the meaninges overlying on the cerebral cortex at P4 as previously reported. Apparent expression of Cxcl12 could not be detected in/surrounding the boundary of necrotic tissue. At P4, significant induction of Cxcr4 mRNA expression in the dysplastic cortex could not be detected although GABAergic interneuron accumulated at this stage.

Figure 5. Endogenous GABA release at different stages after freeze-lesion at P0

At P2, lesioned area was identified with necrosis, the signal of GABA imaging in lesioned area was much lower than that in peri-lesioned area. At P4, GABA release was evident in the superficial layer as well as the boundary of lesioned area compared with peri-lesioned area (arrows). The position was correlated with accumulation of GABAergic interneurons at P4. These signals were disappeared in the absence of GABase (showed in negative control).

Discussion

To our Knowledge, this is the first study to demonstrate that there is a peculiar accumulation of GABAergic and glutamatergic neurons at the early stage of freeze lesion-induced microgyrus in mice. In this study, we developed a mouse model of microgyrus by focal freeze-lesion on the cerebral cortex of newborn GAD67-GFP KI mice. This model reproduces anatomical characters of the cortical malformation present in human polymicrogyia. By using the GAD67-GFP KI mice, we found GABAergic interneurons gathered in the superficial layer as well as boundary of lesioned area at P4. E17.5-born but not E14.5-born glutamatergic pyramidal neurons accumulated around GABA-rich necrotic center at P4 and then formed the cell dense portions in layer ii of microgyrus.

Functioning of the cerebral cortex requires the coordinated assembly of circuits involving glutamatergic projection neurons and GABAergic interneurons. Although much is known about the migration of interneurons from the subpallium to the cortex, our understanding of the mechanisms controlling their migration within the cortex is still limited. In freeze lesioned-mice model, we surprisingly found GABAergic interneurons gathered in the superficial layer as well as boundary of lesioned area at P4. Cxcl12 is a chemoattractant for migration of interneurons through its receptor Cxcr4 during the developmental stage (Stumm et al., 2003, López-Bendito G et al., 2008). Thus we tried to investigate the distribution pattern of Cxcl12 and Cxcr4 mRNAs by in situ hybridization. However, significant induction of Cxcl12/Cxcr4 mRNA expression in the dysplastic cortex could not be detected, suggesting Cxcl12/Cxcr4 signaling was not be responsible for accumulation of GABAergic interneurons at P4.

The major excitatory and inhibitory neurotransmitters in the brain, glutamate and GABA, activate both ionotropic and metabotropic receptors, and are generally associated with neuronal communication in the mature brain. Interestingly, besides their function in synaptic transmission, neurotransmitters have been shown to promote several developmental processes that contribute to the establishment and maintenance of the CNS. Therefore we performed GABA imaging by using the freeze lesioned-mice model. The results showed GABA was almost absent in the lesioned area at P2. However, the endogenous GABA concentration in the lesioned area was high compared with peri-lesioned area at P4. The position was correlated with accumulation of GABAergic interneurons at P4. GABA itself release from accumulated GABAergic interneurons at early stage might play a role in controlling the migration of both glutamatergic pyramidal neurons and GABAergic interneurons. Both GABAergic and later-born glutamatergic pyramidal neurons accumulated around

GABA-rich necrotic center at P4, as a result of the redifferentiation and its differential physiological responses to ambient GABA.

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Figure 1.Formation of a microgyrus after freeze lesion.

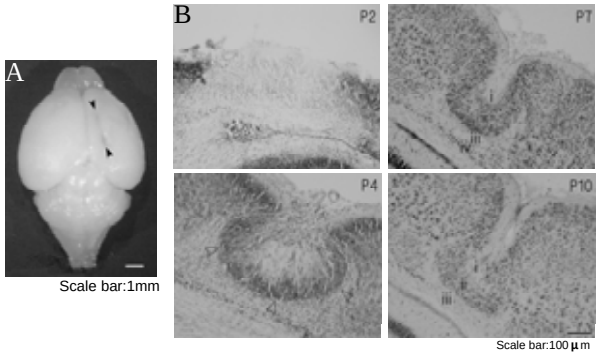


Figure 2. GABAergic interneurons accumulated in the superficial part of lesioned area at P4.

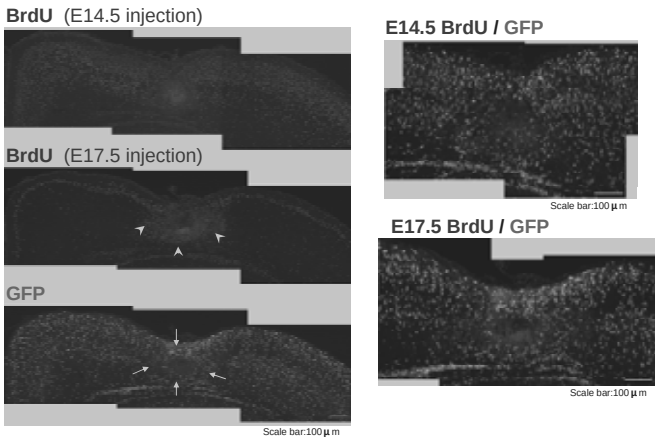


Figure 3. High density of E17.5-generated cells in layer ii of dysplastic cortex at P7

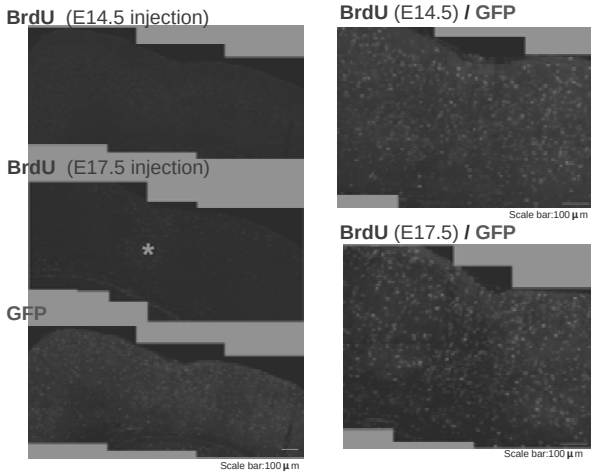


Figure 4. In situ hybridization of Cxcl12 and Cxcr4 mRNA at P4.

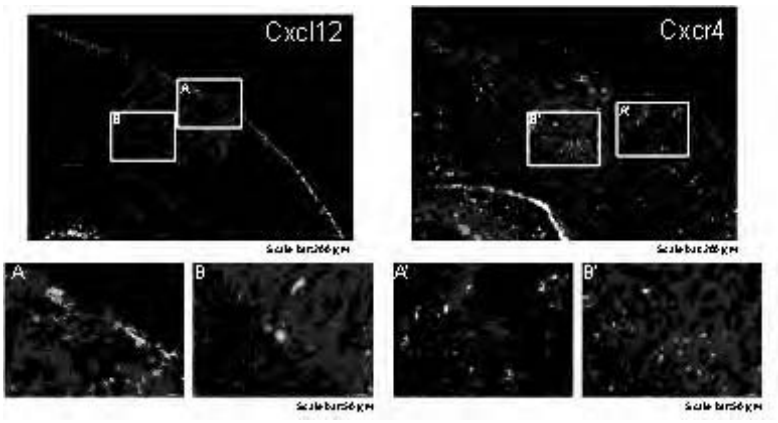
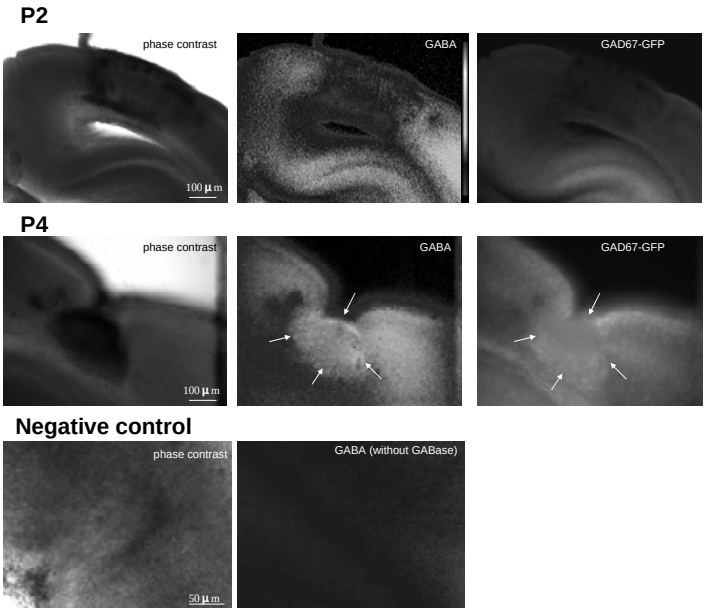


Figure 5. Endogenous GABA release at different stages after freeze-lesion at P0



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