Current Topics

Recent Advances in Research on Biological Membranes That Regulate the Central Nervous System

Review

Molecular Investigations of the Development and Diseases of Cerebral Cortex Folding using Gyrencephalic Mammal Ferrets

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Folds of the cerebral cortex (gyri and sulci) are among the most important properties of the mammalian brain. Uncovering the physiological roles, developmental mechanisms and evolution of the cortical folds would greatly facilitate our understanding of the human brain and its diseases. Although the anatomical features of the cortical folds have been intensively investigated, our knowledge about their molecular bases is still limited. To overcome this limitation, we recently established rapid and efficient genetic manipulation techniques for the brain of gyrencephalic mammal ferrets (*Mustela putorius furo*). Using these techniques, we successfully uncovered the molecular mechanisms of cortical folding. In this article, I will summarize our recent research on the molecular mechanisms of development and diseases of cortical folding.

Key words cerebral cortex; cortical folding; gyrus; ferret; *in utero* electroporation

1. INTRODUCTION

One of the most prominent features of the mammalian brain is the folds of the cerebral cortex (*i.e.*, cortical gyri and sulci). Humans, monkeys and ferrets have gyrencephalic brains (*i.e.*, brains with folded cerebral cortices), whereas the brains of rodents are often lissencephalic (*i.e.*, lacking cortical folds). Because it is believed that the creation of cortical folds during evolution was a major milestone, leading to an increase in the number of neurons in the cerebral cortex and to obtaining higher brain functions,[1–3] investigations of the physiological importance, developmental mechanisms and diseases related to cortical folds would greatly facilitate our understanding of the human brain and its diseases. Indeed, malformations of cortical folds in human patients are associated with severe intellectual disabilities, epilepsy and diseases such as lissencephaly, polymicrogyria, schizophrenia and autism.[4–6] Therefore, the molecular and cellular mechanisms underlying the evolution, formation and diseases of cortical folds are of great interest.

However, our knowledge about cortical folding is still limited, mainly because the mechanisms of cortical folding had been difficult to test experimentally *in vivo* due to the lack of rapid and efficient genetic manipulation techniques that can be applied to gyrencephalic mammalian brains. To overcome this difficulty, we recently developed genetic manipulation techniques for gyrencephalic carnivore ferrets (*Mustela putorius furo*) using *in utero* electroporation and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system.[7–10] By taking the advantage of our *in utero* electroporation technique for ferrets, we successfully uncovered the molecular mechanisms of cortical folding.[11,12] In this article, I will summarize our recent experimental results on the molecular mechanisms of cortical folding using ferrets.

2. GENETIC MANIPULATION OF FERRET BRAIN USING *IN UTERO* ELECTROPORATION

The ferret belongs to the Mustelidae, a family of carnivorous mammals; adult ferrets have an average length 50 cm and weight 1–2 kg (Fig. 1). Ferrets have a venerable history in research because they have well-developed brain structures, such as cortical folds and the visual system, that mice do not have.[12–15] Because *in utero* electroporation has been widely used for expressing transgenes in the brain of rodents,[16–18] we tried to apply *in utero* electroporation to the ferret brain. Fortunately, we succeeded in establishing a rapid and efficient procedure of *in utero* electroporation for the ferret brain[7–9] (Fig. 2). This technique takes only a few hours to perform electroporation using ferret embryos, and after a couple of days, newborn ferrets expressing transgenes are available. Expression of transgenes is detectable even in the embryo and persists at least several months after ferrets are born. Transgenes can be introduced in both superficial and deep neurons in the cerebral cortex, depending on the age at which *in utero* electroporation is performed during embryonic development. *In utero* electroporation performed at embryonic day 31 (E31) and at E37 results in transgene expression in deep and superficial cortical neurons, respectively. Using our electroporation procedure, transgenes can be expressed in not only post-mitotic neurons but also neural progenitors.[7–9] Furthermore, by combining *in utero* electroporation and the CRISPR/Cas9 system, we established gene knockout procedures for the cerebral cortex of mice and ferrets.[10,19,20] Our genetic manipulation techniques for ferrets enabled us to investigate the molecular mechanisms underlying the formation and malformation of

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Fig. 1. Appearance of Ferrets

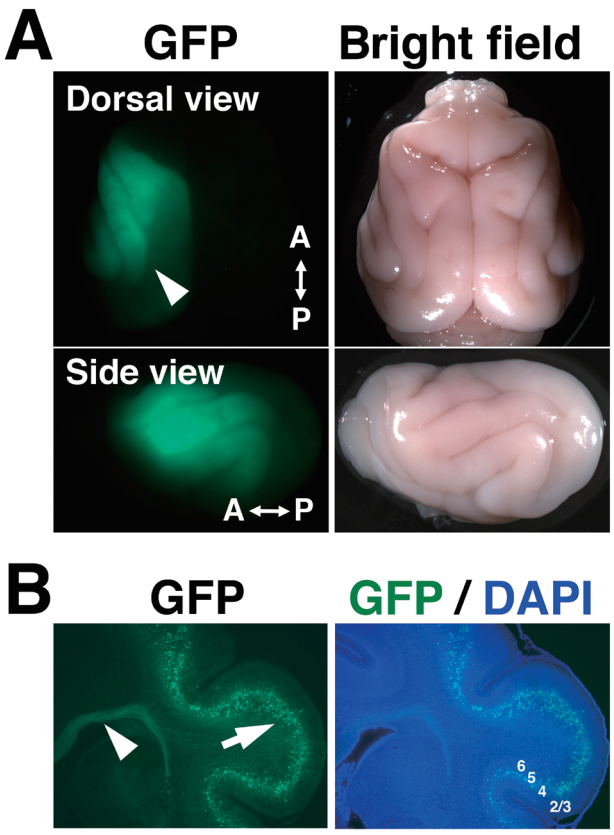

Fig. 2. GFP Expression in the Ferret Brain using *in utero* Electroporation

(A) Dorsal view and side view of the electroporated ferret brain. GFP fluorescence was observed (arrowhead). A, anterior; P, posterior. (B) GFP signals in a coronal section of the electroporated ferret brain. GFP-positive cells (arrow) and axons (arrowhead) can be observed. Numbers indicate corresponding layers in the cerebral cortex. (Adapted from Ref. 7.)

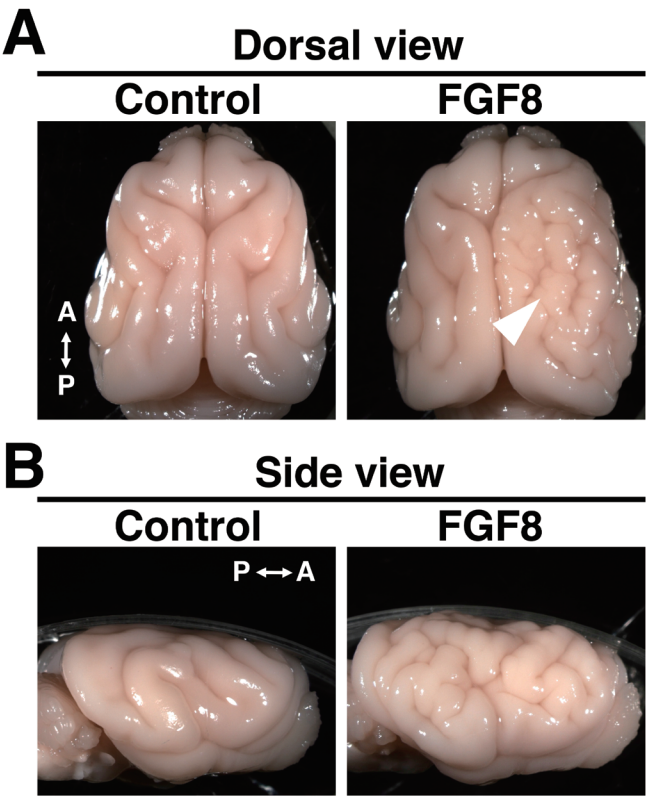

Fig. 3. Polymicrogyria of the Ferret Brain Induced by FGF8

(A) Dorsal view of the ferret brain electroporated with FGF8. Note that polymicrogyria was produced by FGF8 (arrowhead). (B) Side view of the FGF8-electroporated ferret brain. A, anterior; P, posterior. (Adapted from Ref. 31.)

cortical folds.

3. FERRET DISEASE MODELS OF POLYMICROGYRIA

Thanatophoric dysplasia (TD) is a relatively common skeletal dysplasia in which the cerebral cortex displays polymicrogyria (the brain with multiple small gyri creating excessive folding), megalencephaly and neuronal heterotopia.[21,22] TD is caused by activating mutations of the fibroblast growth factor receptor 3 (FGFR3) gene.[23–28] Because knockin mice that have the same activating mutation in the FGFR3 gene did not exhibit polymicrogyria,[29,30] it was controversial whether the activation of FGF signaling is indeed responsible for the

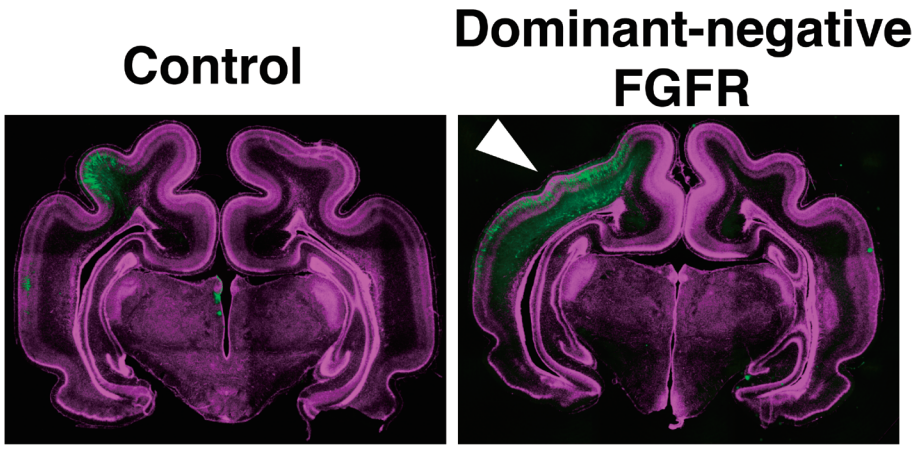

Fig. 4. FGF Signaling Is Required for Cortical Folding

Dominant-negative FGFR and GFP were co-electroporated into the ferret cortex. Coronal sections stained with Hoechst 33342 (purple) and anti-GFP antibody (green) are shown. Note that cortical folding was severely impaired with dominant-negative FGFR (arrowhead), whereas it was not affected in the control brain. (Adapted from Ref. 34.)

pathogenesis of polymicrogyria. To test this point, we expressed fibroblast growth factor 8 (FGF8), which has a high affinity to FGFR3, into the ferret cerebral cortex using our technique.[31] We successfully recapitulated the cortical phenotypes of TD by expressing FGF8 in the ferret cerebral cortex.[31] Strikingly, our TD ferret model showed not only megalencephaly but also polymicrogyria, periventricular nodular heterotopia and leptomeningeal heterotopia[31–33] (Fig. 3). We further uncovered that subventricular zone (SVZ) progenitors, including outer radial glial cells (oRG cells) and intermediate progenitor cells (IP cells), were markedly increased. It seemed likely that these increases underlie the pathogenesis of polymicrogyria.

4. MOLECULAR INVESTIGATIONS OF CORTICAL FOLDING USING FERRETS

As described above, introduction of FGF8 into the ferret cortex resulted in polymicrogyria, suggesting that activation of FGF signaling is sufficient for producing cortical folds.[31] Therefore, we next examined the role of endogenous FGF signaling in cortical folding.[34] We first examined the expression of *FGFRs* and found that *FGFR1*, *FGFR2*, and *FGFR3* are expressed in the developing ferret cortex. To suppress the functions of FGFRs all at once, we introduced a dominant-negative form of FGFR in the ferret cortex. We found that cortical folding was severely impaired by the dominant-negative FGFR (Fig. 4), suggesting that FGF signaling is required for cortical folding.[34] Taken together, our findings indicate that FGF signaling is essential and sufficient for cortical folding (Fig. 5). FGF signaling is the first signaling pathway shown to regulate cortical folding experimentally.

The next question was the mechanisms of cortical folding downstream of FGF signaling. We examined the distribution patterns of FGFRs and found that most FGFR-positive cells in the outer subventricular zone (OSVZ) are oRG cells.[34] The number of oRG cells was significantly reduced by the dominant-negative FGFR,[34] whereas it was markedly increased by FGF8.[31] These results indicate that FGF signaling plays a pivotal role in producing oRG cells in the OSVZ. Furthermore, our finding raised the possibility that oRG cells mediate cortical folding downstream of FGF signaling. Consistently,

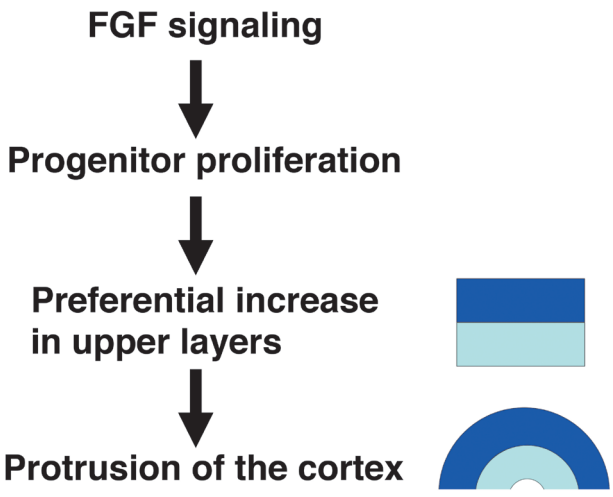

Fig. 5. Our Model of the Mechanisms Underlying Cortical Folding

Dark blue and light blue represent upper and lower layers in sections of the cerebral cortex, respectively.

it has been proposed that an increase in the numbers of SVZ progenitors is responsible for the acquisition of cortical folds during evolution.[35–38] To test this hypothesis, we introduced a dominant-negative form of Tbr2, which is a transcription factor expressed in IP cells. We found that cortical folding was significantly impaired by the dominant-negative Tbr2, suggesting an essential role of SVZ progenitors in cortical folding[39] (Fig. 5). Interestingly, there were regional differences in the abundance of SVZ progenitors in the developing cerebral cortex in ferrets and monkeys even before cortical folds were formed.[39–41] The regional differences in the abundance of SVZ progenitors may determine the positions of the gyri and the sulci. It should be noted that the numbers of both oRG cells and IP cells were markedly reduced by inhibiting Tbr2 transcription factor.[39] Therefore, it would be important to examine which SVZ progenitors, oRG cells or IP cells, are responsible for cortical folding.

Another intriguing question was the mechanisms leading to morphological formation of cortical gyri. As described above, when dominant-negative FGFR or dominant-negative Tbr2 was expressed, cortical folding was inhibited. We examined

the thickness of each cortical layer and found that the thickness of layer 2/3 was selectively reduced by dominant-negative FGFR or dominant-negative Tbr2.[34,39] Therefore, we hypothesized that FGF signaling and Tbr2 preferentially increase layer 2/3 neurons, and as a result, protrusion of cortical gyri is produced. To test this hypothesis, we focused on Cdk5, which is a serine/threonine kinase crucial for radial migration of post-mitotic neurons in the cerebral cortex. Because a mutation was reported in a human patient with lissencephaly,[42] it seemed likely that Cdk5 is involved in cortical folding. We found that cortical folding was severely impaired by knocking out *Cdk5* in the ferret cortex,[10] suggesting that Cdk5 is indeed involved in cortical folding. Next, we introduced dominant-negative Cdk5 selectively into layer 2/3, layer 4 or layer 5/6. Cortical folding was significantly impaired when dominant-negative Cdk5 was expressed in layer 2/3, whereas it was not affected if it was expressed in either layer 4 or layer 5/6.[10] Because Cdk5 is required for radial migration of post-mitotic neurons, our results suggest that increase in neurons in layer 2/3 is crucial for cortical folding[10] (Fig. 5). Consistently, it was proposed that the ratio of cortical neurons in upper layers compared with lower layers determines the curvature of the cortical surface.[43,44] In addition, it was reported that the thickness of upper layers is selectively larger in the gyrencephalic human brain compared with the lissencephalic rodent brains.[45] Thus, increase in layer 2/3 seems an important process for obtaining cortical folds.

5. FEATURES OF *IN UTERO* ELECTROPORATION

In utero electroporation has several important advantages compared with transgenic animals. First, the procedure of *in utero* electroporation is simple and rapid. It requires relatively little time and effort to express genes of interest. It takes only a few hours for performing electroporation, and transfected embryos can be collected within a few days. Second, various kinds of experimental conditions can be tested easily. Multiple genes can be expressed simultaneously by simply using a mixture of several kinds of plasmids. Electroporation using a mixture of GFP and mCherry expression plasmids resulted in most mCherry-positive neurons also being GFP-positive in the ferret cerebral cortex,[7] indicating efficient co-transfection. Different experimental conditions can be used for multiple embryos in one pregnant mother. Third, by adjusting the direction of the electrodes and by changing the age of the embryo at which *in utero* electroporation is performed, the identities of transfected neurons can be controlled. While we mainly transfected the cerebral cortex of ferrets, based on the results obtained using rodents,[46–51] it seems plausible that *in utero* electroporation is applicable to other brain regions, such as the hippocampus, the thalamus, the retina and the amygdala in ferrets. Finally, large plasmids can be introduced using *in utero* electroporation. Cell type-specific promoters, which are often quite large, seem useful for controlling expression patterns of transgenes. For example, we recently transfected only a small number of neurons in mice by combining *in utero* electroporation and the Thy1S promoter.[52] In addition, because *in utero* electroporation works well not only in rodents but also in carnivores, it could also be applicable to other higher mammals.

Besides *in utero* electroporation, postnatal electroporation can be used for expressing transgenes in the ferret cerebral cortex.[53,54] It should be noted that when postnatal electroporation was performed, transfected neurons were mostly distributed in layer 2/3 in the ferret cerebral cortex. This is presumably because most post-mitotic neurons had already migrated into the cortical plate when postnatal electroporation was performed. Almost all excitatory neurons in the cerebral cortex can be transfected using *in utero* electroporation and postnatal electroporation in ferrets.

6. FEATURES OF FERRETS COMPARED WITH MARMOSETS

Previous pioneering studies used virus vectors to create transgenic primates such as monkeys and marmosets.[55–57] For example, injection of a lentiviral vector into eggs resulted in marmosets expressing transgenes in several organs.[57] Although the establishment of transgenic marmosets would provide new opportunities to make animal models for human diseases,[58] making new transgenic marmosets requires time, effort and special animal facilities. In contrast, genetic manipulation of ferrets using *in utero* electroporation described herein is rather simple and rapid.[9] In addition, ferrets and marmosets differ in several important points. Cortical folds are well developed in ferrets, whereas they are rather obscure in marmosets, suggesting that ferrets are more suitable for research on cortical folds. The gestation period of ferrets is shorter than that of marmosets (42 *versus* 150 d). About 6–8 babies are born from one pregnant ferret mother, while 1–2 are born from a pregnant marmoset. Because of the larger number of ferret babies per pregnant mother, many experimental samples can be obtained using ferrets. Ferrets and marmosets become sexually mature at age 8 and 14 months, respectively, and the average life span is 5–10 years in ferrets and 10–15 years in marmosets. The ferret is therefore an attractive option for exploring the brain structures unique to higher mammals including cortical folds. Interestingly, our results demonstrated that genetic manipulation of Cdk5 and FGF signaling, which have been reported responsible for human patients with malformation of cortical folds,[23,42,59,60] also produced similar phenotypes in ferrets.[10,31,34] This suggests that similar mechanisms are used to make cortical folds in ferrets and humans. Because marmosets and ferrets have their own unique features, combining transgenic marmosets and electroporated ferrets would facilitate our understanding of the brain of higher mammals.

7. CONCLUSION

Herein I have described our recent experimental results of research on cortical folds using ferrets. One of the ultimate goals of neuroscience is to understand the human brain, and therefore molecular investigations of the brain of carnivores and primates are of great importance. Because a rapid and efficient genetic manipulation is now available for ferrets, the ferret should be an important option for neuroscience research involving higher mammals.

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Conflict of Interest The author declares no conflict of interest.

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