論文の内容の要旨

論文題目　**Regulation of dendrite development and synaptogenesis in hippocampal neurons by Dlx transcription factors**

（Dlx 転写因子の神経細胞形態形成における役割）

氏名　戴　暁　婧（Dai Xiaojing)

The excitatory pyramidal neurons and inhibitory interneurons are two major building blocks of the forebrain neurons network, with their own characteristic dendritic arborization patterns. In general, pyramidal neurons have elaborate dendritic arborization decorated with numerous dendritic spines. In contrast, most interneurons have much lower density of spines with simpler dendritic branches. Although inhibitory interneurons only occupy small population, they are critical in regulation of synchronized firing and generation of epileptic. However, the basic mechanisms that regulate unique dendritic morphology of interneurons have not yet been clarified.

The transcription factor in charge is Dlx1, a member of Dlx homeobox domain gene family, the orthologs of Distal-less in Drosophila. Previous studies demonstrate that Dlx1 is preferentially expressed in distinct interneuron subtypes and is involved in their migration and differentiation. Recent finding have shown that, aside from role in migration and early differentiation of neuronal precursors, Dlx1 appears to participate in postnatal maturation of postmitotic interneurons.

To understand the roles of Dlx1 in dendritic and postsynaptic differentiation, we manipulated Dlx1 expression in excitatory pyramidal neurons because Dlx1 expression in excitatory pyramidal neurons is negligible and phenotypes obtained by ectopic expression of Dlx1 in pyramidal neurons may reflect its unique functions not present in the transcriptional machinery and signaling pathways of the excitatory neurons. We found that exogenous expression of Dlx1 from 5 days in vitro (DIV) to 14 DIV in pyramidal neurons, which lack endogenous Dlx1, resulted in reduced complexity of dendritic complexity by Sholl analyses. Similar extent of reduction in dendritic complexity was observed in neurons expressing Dlx2, whereas the effect of Dlx5α and Dlx6 was less prominent. Besides, we could not detect further enhancement in growth suppression by cotransfection of Dlx1 and Dlx2 in pyramidal neurons, suggesting that there might not exist cooperation of these two proteins. We also found that Dlx1 could suppress axonal growth when we exogenously overexpressed Dlx1 from 2 DIV to 7 DIV.

We next analyzed the effects of Dlx1 on postsynaptic differentiation by examining dendritic morphology and distribution of postsynaptic densities (PSD-95) in pyramidal neurons expressing ectopic Dlx1 from 9 DIV to 14 DIV. We detected that significant decrease of PSD-95 puncta density in Dlx1-expressing pyramidal neurons, but the area size of PSD-95 puncta did not change. Besides, there was a dramatic reduction in spine density in Dlx1-expressing neurons, while the average size of remaining protrusions was similar to that of control spine. The overall morphology of Dlx1-expressing dendrites of pyramidal neurons resembled that of inhibitory neuron dendrites with few spines, such as basket cell dendrites.

Transcriptional regulation by Dlx1 should be important in regulation of neuronal morphology. To confirm this point, we generated six different types of Dlx1 mutants. Two mutants were deletions of either the C-terminal domain (N+HD) or the N-terminal domain (HD+C) with the intact homeodomain. Other two mutants were also deletions of either the C-terminal or the N-terminal domain, but contained Q50E substitution in the homeodomain, which was reported to abolish DNA binding affinity of the homeodomain (N+HD(Q50E) and HD+C(Q50E)). The remaining two constructs (N-term and C-term) corresponded to the N-terminal domain and the C-terminal domain without the middle homeodomain. To analyze the functions of these mutant Dlx1 proteins in pyramidal neurons, we expressed Dlx1 mutants from 5 DIV to 14 DIV in pyramidal neurons and examined their dendritic morphology. Sholl analyses showed that prominent reduction of dendritic complexity by expression of N+HD and HD+C, which contained the intact homeodomain, whereas Dlx1 mutants containing Q50E substitution in the homeodomain (N+HD(Q50E) and HD+C(Q50E), together with mutants without the homeodomain (N-term and C-term), could not induce reduction of dendritic complexity. These results indicate the essential role of the DNA-binding motif in Dlx1-dependent suppression of dendritic growth.

Our transfection experiments of Dlx1 mutants indicate importance of Dlx1-dependent transcriptional regulation in dendritic morphogenesis. There are several candidates of downstream effectors of Dlx1 and Dlx2. Among these candidate effectors, we focused on two molecules, Npn-2 and PAK3, which had been shown to be involved in dendritic morphogenesis. Expression of these molecules were reported to be repressed by Dlx1 and Dlx2. Neuropilin-1 and 2 are receptors for the class 3 semaphorin family and involved in axon outgrowth and spine formation. PAK3 belongs to group I of the PAK family and regulate dendritic spine formation and maturation. We transfected Npn-2 or PAK3 expression plasmids or their shRNA constructs into hippocampal pyramidal neurons at 5 DIV and examined dendritic morphology at 14 DIV. Sholl analyses of transfected neurons revealed slight increase of dendritic branching by overexpression of Npn-2 and PAK3. Knockdown of Npn-2 and PAK3 induced more prominent changes in dendritic morphology. Besides, we found that modest

improvement of Npn-2 knockdown phenotype by overexpression of PAK3 suggests the existence of non-overlapping functions of Npn-2 and PAK3. Similar to the effects on dendritic morphology, the total axonal lengths did not show prominent increase by overexpression of Npn-2 and PAK3, but significantly reduced by knockdown of Npn-2 and PAK3.

We next tested if suppression of dendritic growth by ectopic expression of Dlx1 in pyramidal neurons can be rescued by overexpression of Npn-2 or PAK3. Neurons were transfected at 5 DIV and observed at 14 DIV. Sholl analyses indicated that overexpression of either Npn-2 or PAK3 could partially rescue the phenotype of Dlx1 expression. Importantly, co-expression of Npn-2 and PAK3 could completely rescue Dlx1-dependent suppression of dendritic growth. These data support the idea that the effects of Dlx1 on dendritic growth were mediated by distinct functions of Npn-2 and PAK3.

In addition, we found that severe suppression of spine density by Dlx1 expression could be rescued by overexpression of either Npn-2 or PAK3, indicating overlapping functions of Npn-2 and PAK3 in regulation of spine formation and its perturbation by Dlx1.

Although pyramidal neurons are a useful cell type for ectopic expression of Dlx1 and analysis of its effects on neuronal structure and function, Dlx1-dependent regulation of dendritic growth and postsynaptic differentiation should be critically evaluated in neurons containing endogenous Dlx1. We utilized electroporation-mediated gene transfer technique to introduce expression plasmids in dissociated hippocampal neurons with high efficiency. By using this transfection protocol, we introduced Dlx1 knockdown construct and expression plasmids of Npn-2 and PAK3 at 0 DIV and visualized the morphology of dendrites and their identity as interneurons by GAD67 immunoreactivity at 14 DIV. Knockdown of Dlx1 in interneurons enhanced dendritic growth, suggesting functions of endogenous Dlx1 in restriction of dendritic complexity. Overexpression of Npn-2 and PAK3 induced similar enhancement of dendritic growth. Moreover, knockdown of Dlx1 in interneurons also induced generation of immature spine-like structure on dendrites. These results indicate that reduction of Dlx1 can support enhancement of dendritic growth and the amounts of Npn-2 and PAK3 are not saturated in interneurons with respect to their functions in dendritic growth.

Enhancement of dendritic complexity by overexpression of Npn-2 suggests possible role of semaphorin 3F (Sema3F) in enhancement of dendritic growth in interneurons. To test whether cultured hippocampal interneurons show enhancement of dendritic growth in response to different concentration of recombinant Sema3F. We observed significant increase in the number of dendritic branch points when neurons were

incubated with Sema3F at the concentration of 1-10 nM. These results indicate the ability of hippocampal interneurons to increase their dendrite complexity in response to Sema3F-dependent signaling pathway.

In this study we provided the evidence that Dlx1, a homeobox transcriptional factor, is involved in both morphogenesis of dendrites and differentiation of postsynaptic structures. Negative regulation of dendritic growth by Dlx1 is likely to be mediated via two critical regulators of dendritic growth, Sema3F-Npn-2-Plexin A3 signaling pathway and PAK3-dependent cytoskeletal remodeling. We propose that Dlx1 functions as a critical switch of dendritic growth pattern through the transcriptional regulation of multiple effector molecules.