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学 位 論 文 題 目	M6 proteins regulate axon outgrowth in mouse callosal neurons
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Corpus callosum (CC) is the largest fiber tract in the human brain that connects the left and right cerebral hemispheres. The absence of the CC results in impairment of higher-order cognitive functions because interhemispheric transfer of information is disrupted. The formation of the CC is intricately regulated by a large number of molecules, which have not been fully characterized, while actively searched. In this study, I show that additional molecules, M6 proteins, are also involved in the CC development.

M6 proteins consist of M6a and M6b, highly homologous four-transmembrane proteins. M6a was originally found by the antibody screening for candidate molecules that are involved in guidance of axons. Previously, we found that when anti-M6a antibody binds to M6a protein in cultured neurons, the axons stall the elongation. Therefore, it seemed that M6a is involved in axon elongation of developing neurons. However, no study has so far examined the function of M6 proteins in vivo. To address this issue, I analyzed brain morphology and axonal projections in knockout mice for the M6a and M6b genes.

First, to assess whether the two M6 proteins have redundant functions, I investigated expression patterns of M6a and M6b proteins in the wildtype mouse brains during developmental stages, using a newly prepared anti-M6b specific antibody. M6a was expressed mainly in growing axons as previously reported. The new anti-M6b antibody revealed that M6b was well co-localized with M6a in these axons. The expression level of M6b on the axons appeared lower at E14.5 compared with M6a, and subsequently intensified at E16.5 and P0, displaying comparable overlapping patterns with M6a. I also examined the subcellular distribution of M6 proteins in neurons in dissociated culture. As described previously, M6a protein was enriched in the edge of growth cones and the shaft filopodia of axons in cultured neurons. M6b protein was also expressed in the growth cones and the shaft filopodia, and co-localized with M6a. Previous studies have shown that binding of anti-M6a antibodies to M6a induces aggregation of the protein over the growth cone membrane. I found that, during this reaction, M6b proteins also changed the distribution and were aggregated together with M6a proteins. Because the anti-M6a antibody does not bind to M6b protein, the redistribution of M6b proteins can not be due to the direct action of the antibody binding. Instead, when M6a proteins change the location upon binding of the anti-M6a antibody, they might be accompanied by physically interacting M6b proteins. These observation suggest that M6a and M6b are co-localized and behave together in the subcellular level, supporting the idea that these proteins function redundantly.

Because redundant functions for M6a and M6b were postulated, I examined the brain phenotypes in the M6a-/-; M6b-/- double knockout mice. Histological analyses of the brain sections revealed that the major axon tracts basically existed in the double

knockout mice. However, the size of the CC bundle was abnormally small in the M6a-/-; M6b-/- knockout mice. This hypoplasia in the CC was not clear at early developmental stages, but became obvious by P7 and throughout life. I quantified the size of the CC in wildtype, single and double mutant mice at P7, and found that the defect was most pronounced in the double knockout mice, indicating the redundant function of the two M6 proteins.

I considered the following three possibilities to explain the smaller CC bundles in the double knockout mice. 1) The number of the callosal neurons is reduced. 2) The axons of the callosal neurons are misdirected from callosal pathway. 3) The axon outgrowth is impaired in the callosal neurons. First, I measured the density of cortical neurons, and found that the density in the mutant cortex was comparable to that of the wild-type cortex in the motor, somatosensory, and visual areas. This result suggests that the number of callosal neurons was not reduced in the M6a-/-; M6b-/- mice. Second, I labeled the callosal projection in the cortex of M6a-/-; M6b-/- double knockout mice by selectively electroporating GFP-expressing vector into cortical neurons. This labeling visualized a significantly smaller number of axons reached the contralateral cortex in the double knockout mice. Also, there were ectopic bundles of axons coursing ventrally toward the septum region in the double knockout mice. More detailed observations by single cell labeling confirmed that these ectopic projections to the septum indeed originated from the cell bodies in the position specified for the callosal neurons. The results suggest that at least some callosal axons were misdirected from callosal pathway in the double knockout mice.

Lastly, I examined the third possibility that the axon outgrowth was impaired in the callosal neurons. Cortical neurons were isolated from the mutants and cultured to evaluate the intrinsic capability of neurite length. The quantification indicated that the axonal length of any single and double mutant neurons was significantly smaller than that of wild-type neurons. The impairment in neurite outgrowth was similarly observed in cortical neurons, regardless of the layer position or the birth time, suggesting that the defect of M6a-/-; M6b-/- was not limited in the callosal neurons, but rather common to all cortical neurons. I next examined whether the expression of M6 proteins rescued the impairment of axon growth in M6a-/-; M6b-/- neurons. When M6a or M6b expression plasmid was transfected into the cortical neurons of the double knockout mice, either of the proteins restored the axonal length to the normal level. These results confirm that M6a or M6b proteins have redundant functions in the axon outgrowth. Finally, using culture system, I mapped the functional domain of M6a that was required for the axonal growth. The expression plasmids for M6a deletion constructs were transfected into cortical neurons of M6a-/-; M6b-/-. Through assays of various deletion constructs, I found that the second intracellular loop (amino acid 105-128) was required to restore the axonal outgrowth in the double knockout neurons.

In this study, I showed that M6 proteins have an essential role in the formation

of the CC by regulating the axonal growth of callosal neurons. M6a and M6b are functionally redundant in this process. Based on the results from the primary cultured neurons, the defect of the CC in the M6a-/-; M6b-/- double knockout mice is at least partly due to the reduced length of axons, which can be attributed to the impairment in the intrinsic ability of individual callosal neurons to extend the sufficient length of axons. Furthermore, the study revealed that some callosal axons are misguided ventrally in the double knockout mice, which should also contribute to the hypoplasia in the CC observed in this study.

博士論文の審査結果の要旨

神経回路形成過程での軸索伸長において，神経細胞の細胞膜は，細胞外環境の検出器であるとともに，「細い突起」という特殊な構造を形作る．このような細胞膜の「高次機能」を担う分子については未知の点が多い．三田さくらさんは，4回膜貫通型の親脂質性タンパク質であるM6ファミリーに着目し，その軸索伸長と神経回路形成における役割を解析した．

これまでの研究から，M6ファミリーメンバーの一つであるM6aは，「抗体処理をすると神経軸索伸長が停止する」，「過剰発現すると細胞骨格を含まない細い突起を生じる」等の興味深い活性を持つことが知られていたので，三田さんはこのファミリーの生体内機能の解明を目指した．まずM6ファミリーメンバーのうちM6aとM6bがマウス脳の多くの神経細胞で共発現しており，軸索や成長円錐に共局在していることを示した．培養下の神経細胞においてM6a抗体処理をおこなうと，M6AとM6bが共に成長円錐から消失し，軸索シャフトから伸びる細い突起と成長円錐の付け根に集積することを見いだし，M6AとM6bが協調的に作用する可能性を示した．

M6aとM6bの生理機能を解明するために，これらの遺伝子のノックアウト系統を用いた解析を行った．どちらの遺伝子のノックアウト系統でも，培養した大脳皮質の各層の神経細胞で突起の長さが短いことが分かり，ノックアウト系統では正常型より軸索伸長速度が遅いことが示唆された．この軸索伸長に関する機能について，M6ファミリーの3つのメンバーが同様の活性を持つことや，第2細胞内ループが必要であることを示した．

神経回路形成における役割を解析する系として，三田さんは左右の大脳半球を繋ぐ太い軸索束である脳梁を選んだ．脳梁は主に大脳皮質のII, III, V層の神経細胞の軸索から構成され，M6aとM6bが共発現している．M6a，M6bの単独ノックアウト系統では脳梁に異常は認められなかったが，2重変異では脳梁の太さが正常型よりも有意に細くなっていた．

大脳皮質のII, III層の細胞を選択的にラベルしてその軸索走行をしらべると，殆どの細胞で正常型より軸索の長さが短く，異所的な場所に投射している軸索も多数存在した．この結果はM6a･M6bの軸索伸長に関わる機能が正常な神経回路形成に必須であることを強く示唆している．異所的な投射が引き起こされる機構として，三田さんは軸索伸長速度が低下しているために，軸索経路の選択点で一過的に提供されるガイダンスシグナルを受け取ることができなかったと推測している．

従来の神経回路形成機構の解析では軸索ガイダンス分子やその受容体が注目され，軸索伸長という細胞の基本的性質の役割に目が向けられることは少なかった．三田さんの仕事はM6ファミリーの軸索伸長における生理機能を明らかにしただけでなく，伸長速度が神経回路形成過程にどういう意味を持つかを認識させる重要な手がかりを提供した．以上のことから，博士号授与の要件を満たすと審査員全員一致で判断した．