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論文内容の要旨

Neurons originate from neuroepithelial cells residing in the ventricular zone near the ventricle, migrate to their final positions, and mature to generate action potential, which is the most important characteristic of the neuron. However, molecular mechanism that regulate the maturation of action potential is still unknown. He has been trying to understand the maturation process of action potential by studying the developmental profile of expression of voltage-dependent ion channel genes.

In amphibian spinal cord neuron, it is well known that the properties of action potential, such as ionic dependency, amplitude and duration, change during the course of development, and it is reported that the maturation of action potential is controlled by developmental changes of voltage-dependent K^+ currents as follows. Barish(1986) observed that voltage-dependent K^+ currents changed from slowly activating current, in which the amplitude was very small, to large fast-activating current, and this change occurred in parallel with the developmental changes in the action potential. Based on this finding, he concluded that the expression of voltage-dependent K^+ current at early stage induces the alteration of ionic dependency of the action potential from Ca^{2+} to Na^+ by shifting membrane potential to hyperpolarized state. He also concluded that the expression of the voltage-dependent K^+ current shortens the duration of the action potential. This suggests that the expression of voltage-dependent K^+ current triggers the development of action potential. Moreover, Ribera and Spitzer(1990) found that fast-activating and fast-inactivating voltage-dependent K^+ current, known as A-type current, appeared at the final stage of neuronal maturation. This current is believed to control the frequency of repetitive firing. Thus, the expression of voltage-dependent K^+ current play a key role in the maturation of the action potential in amphibian neurons. It is reasonable to assume that K^+ currents play a similar role in the development of mammmalian neuron, however, few studies have been reported, partly due to the lack of convenient model system like amphibian motoneuron.

To clarify the molecular mechanism that regulate the maturation of action potential, he started to examine voltage-dependent K^+ currents of developing granule cells, by using mouse cerebellar microexplant culture.

This microexplant culture system was developed to investigate the process of neuronal differentiation in vitro by Nagata and Nakatsuji(1990). Unlike widely used primary culture methods of cerebellar granule cells, it is not necessary to culture cerebellar granule cells either in high-potassium medium or together with glia cells in this culture system. Based on their size, morphology,and inability to take up GABA, it was reported that more than 90% of

cells which radially migrated out from the explant were granule cells, and that granule cells in this culture formed small clusters. However, specific identification of granule cells in this culture system is not still completed.

In the present study, first, he identified and characterized granule cells in this microexplant culture system by immunocytochemical and electrophysiological analysis. The immunostaining with an anti-Zic antibody, which is a specific marker for cerebellar granule cells, revealed that about 50% of migrating cells in this culture was Zic-immunopositive, and that most of the Zic-immunopositive cells localized in small clusters. These results show that granule cells in this culture system migrated radially out from explants and then formed small clusters. The immunostaining with an anti-glutamate antibody, which recognize parallel fibers, revealed that most of the fine processes extending out from the explant were parallel fibers, and that the morphology of the granule cells with parallel fibers changed dramatically from bipolar to T-sharped cell during their migration. This unique morphological change is very similar to the process observed in vivo. The labeling with BrdU, which is a thymidine analogue, demonstrated that granule cells in the early stage of this culture were mitotically active, and that the proliferation of granule cells was gradually lost along with the culture period. In addition, to know whether properties of granule cells observed in vivo were maintained in this culture, he characterized the GABA response of granule cells electrophysiologically and pharmacologically, by using whole-cell patch-clamp technique. It showed that 86% of the cells(n=28) in the small clusters evoked marked inward currents after application of 10mM GABA, and that the GABA-induced currents were mediated through bicuculine-sensitive Cl^--channel-coupled $GABA_A$ receptors, which was consistent with that described in previous reports. This result suggested that the electrophysiological properties of granule cells in this culture are maintained normally; at least against their response toward GABA. From the present immunocytochemical and electrophysiological analysis for the microexplant culture, he concluded that this microexplant culture system was a powerful tool for investigating the differentiation of cerebellar granule cells.

Having established the cerebellar granule cell culture condition, he recorded voltage-dependent K^+ currents of developing granule cells in this culture. Immunostaining for glutamate indicated that migrating granule cells in the microexplant culture changed their morphology from bipolar to T-shaped during their migration. This unique morphological differentiation of granule cells mimics the in vivo developmental procedure. Based on this finding, voltage-gated K^+ currents of developing granule cells in the microexplant culture were recorded, under the observation of the morphology, using whole-cell patch-clamp technique. Simultaneous recording of the current and the morphology

were performed by using recording pipette including a fluorescent dye, Lucifer yellow.

Bipolar and T-shaped cells in this culture were observed by labeling with Lucifer yellow, and he considered that bipolar and T-shaped cells were corresponding to immature and mature granule cells respectively. Both bipolar and T-shaped cells exhibited fast activating K^+ current, however, the inactivation kinetics of their currents were remarkably different. T-shaped cells showed fast inactivating component in addition to slow inactivating component, in contrast, bipolar cells exhibited very small or no fast inactivating component. Activation and inactivation of the fast inactivating current component were voltage dependent, and this current component was 4-AP-sensitivite. These strongly indicates that this current is a typical A current. Therefore, these findings demonstrated that voltage-dependent K^+ currents of developing granule cells changed from delayed rectifier to A-type current in parallel with their morphological change from bipolar to T-shaped cell.

In the present study, developmental changes of voltage-dependent K^+ currents were similar to that found in amphibian spinal cord neurons. It was reported that the appearance of A current in amphibian spinal cord neurons was important to control the rate of repetitive firing of the action potential. Therefore, it is suggested that A current which appeared in the maturation process of cerebellar granule cells also plays a similar role in maturation of action potential. Interestingly, this A current appeared in parallel with the morphological change of developing granule cells. Therefore, he also speculate that this A current plays an important role not only in the maturation of the action potential but also in neuronal differentiation. Further examination using this culture system will provide the basis for understanding the role of voltage-dependent K^+ current in neuronal development.

論文の審査結果の要旨

脊椎動物中枢神経系の発生期において神経細胞とグリア細胞が分化する過程では、新たな mRNA の合成やそれに関わる調節因子など多様な変化が認められ、神経細胞の特徴の一つである活動電位もまたこの過程で発生する。しかしながら、その分子メカニズムは未解明のままである。本論文の目的は、この問題について電位依存性カリウムチャネルの発現という観点から明らかにすることである。

実験材料としてマウスの小脳顆粒細胞の microexplant culture 系を用い、免疫細胞化学的及び電気生理学的解析を行った。

顆粒細胞の同定は、小脳顆粒細胞に特異的に発現している転写因子である Zic に対する抗体を用いて行った。その結果、 explant から移動した細胞の５０％以上が Zic 陽性を示し、その多くは explant 周辺の cluster 内に認められることから、この培養系における顆粒細胞は移動後 cluster を形成する傾向があることが明らかとなった。

次に、 parallel fiber の同定を抗グルタミン酸抗体を用いて行った。グルタミン酸陽性線維を有する細胞の形態が bipolar や T-shape を示し、それらの割合は、培養経過に伴い後者が増えることから、 parallel fiber を有する顆粒細胞は bipolar から T-shape へと形態変化していることが示唆された。これは、小脳発生期に観察される顆粒細胞の形態変化と極めて良く一致するものであった。

これまで、この培養系を用いた電気生理学な解析は全く為されていないので、今回、ホールセル-パッチクランプ法を用いて、顆粒細胞の膜興奮性を検討した。cluster を形成している細胞の８０％以上が GABA 応答性を示し、この培養系における顆粒細胞は、機能的な $GABA_A$ 受容体を維持していることが示された。

以上の結果から、この microexplant culture は小脳顆粒細胞の分化の過程を追う上で有効なモデルであると考え、この培養系を用いて顆粒細胞の分化に伴う電位依存性カリウム電流の変化を検討することとした。

ルシファーイエローの注入により bipolar 型と T-shape 型の顆粒細胞を同定し、その電位依存性カリウム電流をホールセル-パッチクランプ法を用いて記録した。

すべての顆粒細胞は速い活性化を示したが、時定数が約１３ミリ秒の速い不活性化の成分が T-shape 型細胞に観察されるのに対し、 bipolar 型細胞にはほとんど認められなかった。この速い不活性化成分のみを分離し詳細に検討したところ、その活性化と不活性化は電位依存性であり、さらに 4-aminopyridime(4-AP) によって濃度依存的に阻害された。

以上の電位依存性及び薬理学性質は、これまでＡ電流について報告されたものと同様であることから、今回観察された速い不活性化成分はＡ電流であり、 microexplant culture における小脳顆粒細胞は、分化の過程において形態変化に伴いＡ電流が発現することが明らかとなった。Ａ電流により活動電位の反復発火が抑制されると考えられ、活動電位の反復発火が制御されると、それに伴う電位依存性カリウムチャネルを介する細胞内へのカルシウム流入量もまた調節されることから、Ａ電流の出現は、その後の神経細胞の分化に関与すると考えられる。

本研究は、神経細胞の形態学的分化と分子的、機能的分化が平行して進行することを明解に示しており、発生神経生物学に新たな知見を加えるものと学位に値する。

提出された論文の内容と背景、関連領域の理解度について試問したところ、満足すべき解答が得られた。すなわち、発生神経生物学、神経生物学、分子生物学の学識を十分有し、本研究が確かな基盤に立って自主的に遂行されたものであると判定された。

本論分は英語で書かれ、論旨が明解である。また本研究の内容は原著論文として国際誌に投稿され、改訂を終わって受理される見込みであることから、論文作成能力、語学力とも十分であると判定された。