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論文内容の要旨
Summary of thesis contents

Glycine is a major inhibitory neurotransmitter in the spinal cord and the brainstem. A glycine-mediated inhibitory neurotransmission (glycinergic transmission) is responsible for physiological functions that include motor control, afferent sensory transduction, visual and auditory transductions. Disruptions of glycinergic transmission caused by mutations to glycine receptors (GlyRs) are associated with hyperekplexia (so-called "startle disease"). Despite the physiological importance of glycinergic transmission, the primary mechanism of glycinergic synapse formation is still not fully elucidated.

The clustering of postsynaptic glycine receptors is considered to be established at immature stages by depolarization mediated increase in Ca^{2+}, and is disturbed by the chronic presence of strychnine, a selective glycine receptor antagonist. In mature neurons, however, the neuronal response to glycine receptor activation is the opposite, a hyperpolarization. Thus, glycinergic inputs evoke depolarizing responses at immature stage, but hyperpolarizing responses at mature stage due to the developmental upregulation of KCC2, an intracellular Cl^- extruder. The timing of this glycine-induced depolarizing to hyperpolarizing switch occurs at around the 1st postnatal week in mice and rats. Nevertheless, remodeling of inhibitory glycinergic synapses continues in the mature neuronal system. Therefore, the basic hypothesis that GlyR activation regulates its localization at synapses in mature neuronal system needs investigation.

In this study, I have analyzed whether GlyR activation is responsible for synaptic localization and neurotransmission in mature cultured spinal cord neurons. To elucidate this question, I applied various experimental techniques to quantify GlyRs in neurons and synapses: conventional immunostaining, dynamic imaging techniques on living cells including fluorescence recovery after photobleaching (FRAP), combinations of fluorescence loss in photobleaching (FLIP), and a single particle tracking. Finally, I combined these with the voltage-clamp configurations of electrophysiological patch clamp recordings to measure functional synaptic transmission.

Primary cultures of spinal cord neurons were prepared from wildtype Wistar rats at embryonic day 14 (E14) for immunocytochemistry; from VGAT-Venus transgenic C57BL/6 mice E13-15 for single particle tracking; and from wildtype C57BL/6J mice E13-15 for FRAP, FRAP-FLIP and electrophysiological experiments. All procedures carried out in this study were approved by the Okazaki Institutional Animal Care and Use Committee, and conducted in accorded with guidelines defined by the National Institute of Natural Sciences. All the efforts were made to minimize suffering and the number of animals used in this study. Procedures of primary cultured neurons were mostly similar to standard protocols for primary hippocampal neurons.

In most of the present studies, three set of groups were compared. One was chronically treated with strychnine (1 μM final concentration, cSTR) to block GlyR

activation from the first day of culture. The second group (WASH) had under one hour GlyR activation by removing chronically applied strychnine only one hour prior to each experiment. The third group (control) was incubated without strychnine from the first day of culture plating. All the neurons were incubated on polyethylenimine-coated cover glasses with Neurobasal medium at 37 °C and 5% CO_2 for 14-42 days in vitro (div).

To examine whether chronic blockade of GlyR affects its synaptic localization, immunostaining was performed in control, cSTR and WASH groups. The results showed that chronic strychnine caused a significant decrease in the intensity of GlyR staining at synapses. The result of surface cell assay showed intracellular GlyR expression was increased in cSTR compared with control group, but the total surface expression that includes synaptic and extrasynaptic GlyRs was similar in each group. These results indicated that GlyRs on the neuronal membrane are diffused away from synapses in cSTR. Thus, I next examined GlyR dynamics in living neuronal culture by using FRAP and FRAP-FLIP experiments, using a pH-sensitive GFP (superecliptic pHluorin: SEP)-tagged GlyR to specifically visualize cell surface GlyRs.

This GFP-tagged GlyRα1 was firstly constructed, then transfected to cultured spinal cord neurons by Lipofectamine 2000 at 13-15 days in vitro, with experiments conducted within 48 hours after transfection. The results from the FRAP experiments suggested that GlyR activation is responsible for its surface mobility. To distinguish contributions of exocytosis, insertions from intracellular stores, and lateral diffusion on the surface mobility, I performed a combination of FRAP-FLIP experiments. The result demonstrated that chronic blockade of GlyR induces an increase in the lateral diffusion of cell surface GlyRs without effects on GlyR exocytosis. In contrast, one hour GlyR activation (WASH group) was sufficient to reduce this change in diffusion properties in mature neurons.

For distinguishing GlyR lateral diffusion at synaptic vs. extrasynaptic location, mCherry-gephyrin (the postsynaptic scaffolding protein of GlyR) was cotransfected into neuronal cultures and focal FRAP (1 μm diameter) was performed at mCherry-expressed synaptic regions. The results showed that synaptic GlyRs had a significantly lower lateral diffusion following the activation of GlyRs. To quantify activation-dependent GlyR diffusion at synaptic and extrasynaptic locations, single particle tracking quantum dot-labeled GlyRs was employed, with FM 4-64 used to identify active presynaptic sites. The results indicated that laterally diffusing GlyRs stay for longer dwell times at synapse following GlyR activation.

Finally, I examined whether functional glycinergic transmission was modulated by chronic GlyR block using electrophysiological patch-clamp techniques. After activation of chronically blocked GlyRs, the amplitude of spontaneous miniature glycinergic currents was gradually increased after strychnine removal in cSTR group, but not in the control group. This supports the observations in live cell imaging that diffusion rate of synaptic GlyRs were decreased under the absence of strychnine. The recording by

gramicidin-perforated patch-clamp verified that the neurons used in the present study were mature, in terms of glycine-induced hyperpolarizations.

Overall, these results suggested that the activation of glycine receptor regulates the diffusion properties of the receptor and thereby functional glycine neurotransmission in mature neurons. Because glycine induces a hyperpolarization rather than depolarization in this matured stage, the previously proposed mechanism of glycinergic synapse formation at immature stage is insufficient to explain the results. Although the signaling mechanism is not fully elucidated, this is a novel report of activation-dependent glycinergic synapse formation in mature neurons.

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博士論文の審査結果の要旨
Summary of the results of the doctoral thesis screening

グリシンは脊髄や脳幹における主要な抑制性神経伝達物質であり、グリシン作動性シナプス伝達は運動制御や、感覚情報の伝導に重要な役割を果たしている。また、ヒトのグリシン受容体遺伝子の変異はてんかんやびっくり病とも関連している。これまでに、ラットの発達初期段階(生後 1 週以前)ではグリシン受容体の活性化(脱分極)がグリシン受容体のシナプス部位への集積に必要であることが報告されている。しかし、生後 1 週頃に生じる脱分極−過分極スイッチ後の成熟期においてグリシン受容体の活性化(過分極)がグリシン受容体の局在、動態、機能にどのような影響を与えるかは不明であった。本研究では「成熟期(生後 2-6 週)におけるグリシン受容体の動態および機能が神経活動によりどのように制御されているか」をマウスあるいはラットの脊髄初代培養神経細胞を用いて明らかにした。

本研究では以下の３群の比較により実験、解析を進めた。すなわち、グリシン受容体阻害剤（ストリキニーネ, STR）を培養直後から長期投与し続けた群(cSTR 群)、阻害剤長期投与後実験開始 1 時間前に阻害剤を除去した群(WASH 群)、阻害剤を投与しない群(control 群)を比較した。まず、固定標本での免疫染色により内在性のグリシン受容体の局在を検討したところ、cSTR 群においてのみ有意にグリシン受容体のシナプス局在が減少していることを見出した。すなわち、グリシン受容体の活性化がグリシン受容体のシナプス局在に必要であることが示唆された。次に、pH 感受性 GFP(SEP)グリシン受容体と光褪色後蛍光回復法(FRAP)、および 光褪色による蛍光消失法(FLIP)を組み合わせることにより、cSTR 群では細胞表面におけるグリシン受容体の移動（入れ替わり）が促進していることを見出した。また、このグリシン受容体の細胞表面における速い入れ替わりは、エクソサイトーシス(exocytosis)によるものではなく側方拡散(lateral diffusion)の促進によるものであることも明らかとなった。興味深いことに、control 群では gephyrin 陽性のシナプス部位においてグリシン受容体は遅い動態（入れ替わり）を示すのに対し、cSTR 群ではグリシン受容体はシナプス領域でもシナプス外領域においても速い動態を示した。すなわち、グリシン受容体はグリシン受容体の活性化により、シナプス部位に集積することが明らかになった。加えて、薬理学的手法によりこの過程に蛋白質リン酸化酵素が関与していることも突き止めた。また、出願者は量子ドットを用いてグリシン受容体の 1 分子イメージングを行い、グリシン受容体が活性化される条件下でグリシン受容体はより長くシナプス部位に局在することを示した。さらに、出願者はグリシン受容体を介したシナプス伝達機能がグリシン受容体の活性化により制御されるか否かを電気生理学的パッチクランプ法を用いて検討した。培養初期から長期間グリシン受容体の活性化を阻害してきた cSTR 群から STR を除去すると、グリシン作動性微小シナプス電流の振幅が除去直後から 50 分間の間、徐々に持続的に増強することが明らかとなった。以上の結果から、成熟期におけるグリシン受容体は動的に細胞膜上を拡散移動する性質を有する一方で、シナプス前終末から放出されるグリシンによって活性化されると、より長時間シナプス部位に停留し、結果としてシナプス部位にグリシン受容体が集積することが明らかになった。

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本研究は、「成熟期におけるグリシン受容体の神経活動依存的な動態制御機構の解明」を研究テーマに掲げ、分子細胞生物学（生細胞イメージング、1 分子イメージング）や電気生理学等の手法を駆使して行われたものである。本研究内容は国際誌へ投稿準備中である。また、すでに関連した内容で 2 報の筆頭論文、および 2 報の共著論文を発表している。以上のことも鑑みて、本研究が学位論文としてふさわしいものであることに、審査委員全員の意見が一致した。