氏　　名	斉藤　憲史
学位（専攻分野）	博士（学術）
学位記番号	総研大甲第1445号
学位授与の日付	平成２３年３月２４日
学位授与の要件	先導科学研究科　生命体科学専攻 学位規則第６条第１項該当
学位論文題目	The roles of vesicular GABA transporter during embryonic development
論文審査委員	主　査　教授　颯田　葉子 教授　堀内　嵩 教授　深川　竜郎 教授　柳川　右千夫　群馬大学 准教授　荒田　晶子　兵庫医科大学

論文内容の要旨

This thesis describes the generation and analysis of vesicular GABA transporter (VGAT) KO mice to elucidate the functional role of VGAT during embryonic development. I have four main points of discussion: (1) generation of VGAT KO mice, (2) morphological defects in the VGAT KO mice, (3) VGAT-independent GABA release, and (4) spinal circuit formation in the absence of VGAT.

In the mammalian central nervous system, inhibitory neurons release GABA and glycine as neurotransmitters. In GABAergic neurons, GABA is synthesized from glutamate by two glutamic acid decarboxylases (GADs), GAD65 and GAD67. GABA is transported into synaptic vesicles (SVs) by a VGAT and is released from axon terminals by Ca^{2+}-dependent exocytosis. GABA activates either ionotropic $GABA_A$ or metabotropic $GABA_B$ receptors, which localize to either pre- or post-synaptic membranes. The activation of the receptors is terminated by the reuptake of GABA into axon terminals and/or glial cells by plasma membrane GABA transporters (GATs). As GABA and glycine share the same vesicular transporter and VGAT is thought to be the only vesicular transporter for inhibitory amino acids, VGAT is essential for inhibitory neurotransmission via SVs.

Recent gene KO studies on inhibitory neurotransmission have elucidated not only essential roles in neural functions but also an unexpected contribution to development of non-neural tissue. For example, deletion of the GAD67 gene leads to a non-neural developmental defect, cleft palate, and loss of VGAT results in cleft palate as well as omphalocele. Omphalocele is a herniation of the gut and liver through the umbilical ring. These

studies have offered a conditional gene KO technique for further understanding the role of inhibitory neurotransmission.

(1) Conditional VGAT mice based on the Cre/loxP system were generated. The conditional VGAT mice were crossed with mice expressing Cre recombinase in germ cells to obtain VGAT KO mice. Western blotting revealed the VGAT protein to be successfully eliminated from the VGAT KO brain. In addition, VGAT KO mice exhibited substantial increases in overall GABA and glycine, but not glutamate, levels in the forebrain, while the expression of GABA-synthesizing enzymes did not differ between controls and KOs.

(2) Although previous studies elucidated that the deletion of genes related to inhibitory neurotransmission leads to unexpected developmental defects such as cleft palate and omphalocele, rather less attention has been paid to other developmental abnormalities. To further explore the role of inhibitory neurotransmission in proper embryonic development, a comprehensive histological analysis was performed in controls and KOs.

VGAT KO mice were dead at birth, and had a cleft palate and omphalocele, confirming previously reported phenotypes. Their body weight at embryonic day (E) 18.5 was significantly lower than that of wild-type littermates. Histological examination revealed a decrease in trapezius muscle mass, hepatic congestion, and little alveolar space in the VGAT KO mice.

(3) In the last two decades, there is increasing evidence of neurotransmission outside synapses. Non-vesicular release is thought to be involved in this process. However, since it remains unclear to what extent the vesicular release contributes to the amount of GABA released, it is important to determine whether GABA is present in the extracellular space in the

VGAT KO brain. To this end, whether or not GABA release could be confirmed in VGAT KO brain was investigated.

At first, $GABA_AR$-mediated synaptic currents were recorded using a whole-cell patch-clamp method. Electrophysiological recordings from E17.5 striatal neurons showed that the VGAT KO mice exhibited no spontaneous miniature inhibitory postsynaptic currents (IPSCs), suggesting the absence of vesicular release in the striatum. To investigate the presence of non-vesicular GABA release and the reversal of GAT-1, the amounts of GABA released from the forebrain slices were quantified. The slices were incubated in a small chamber containing artificial cerebrospinal fluid (ACSF) with or without a GAT-1 inhibitor, nipecotic acid, and the amount of GABA released into the ACSF was measured by HPLC. Without nipecotic acid, the amount of GABA in ACSF did not differ between controls and KOs. Blocking GAT-1 by nipecotic acid increased the amount of GABA in the ACSF. These results indicate that GABA can be released by VGAT-independent non-vesicular mechanisms in the embryonic mouse forebrain and that the plasma membrane GAT does not release, but rather than recovers the extracellular GABA.

(4) There is increasing evidence that GABA and glycine have neurotrophic effects in the developing nervous system, in which synapse formation are still immature. In such a situation, inhibitory neurotransmission must act non-synaptically. However, it is debatable whether or not vesicular GABA release is required for the neural circuit formation. To explore this issue, the responses to dorsal-root stimulation were examined in the control and VGAT KO mice.

Spontaneous IPSCs were absent in spinal cord motoneurons of VGAT KO mice.

However, electrical stimulation of the dorsal root evoked excitatory, but not inhibitory, responses in the motoneurons. The latency of this excitatory response was similar to that of the control preparations. These results indicate that the sensory pathway to motoneurons is formed in the absence of GABA- and glycine-mediated synaptic responses. VGAT KO mice at E17.5-18.5 were completely immobile and stiff, and none of them responded to mechanical stimuli by pinching of the tail. Therefore, the lack of inhibitory transmission to motoneurons in VGAT KO mice likely resulted in defects in the spontaneous and stimulus-induced movements *in vivo*.

This study provides evidence that VGAT has an essential role not only in GABA- and/or glycine-mediated neurotransmission but also in embryonic development. Another significant achievement of this study is the generation of conditional VGAT mice. This provides an opportunity to further understand roles of GABAergic neurotransmission. For example, distinct groups of inhibitory neurons contain different peptides and different calcium-binding proteins. Different classes of inhibitory neurons have precise patterns of axon targeting. These distinct subtypes of inhibitory neurons appear to contribute to specific functions in the brain. The conditional VGAT mouse provides a new tool to study the subtype-specific and circuit-specific role of inhibitory neurons in brain functions.

博士論文の審査結果の要旨

GABA とグリシンは中枢神経系における代表的な抑制性の神経伝達物質であり、神経の電位活動の制御に加えて、運動、感覚および呼吸など脳の機能を構築する上で中心的な役割を担っている。小胞型 GABA トランスポーター （VGAT）は GABA とグリシンをシナプス小胞へ輸送・蓄積することから、GABA とグリシンのシナプス伝達に重要な働きをする。従って、VGAT ノックアウトマウスを作製・解析することは、VGAT の生理学的役割や GABA とグリシンのシナプス伝達の役割について理解するための強力なアプローチになる。本論文で斎藤君は、VGAT ノックアウトマウスを作製し（下記 1)、胎生期に焦点を絞り細胞レベルから個体レベルまでの解析を行なった（下記 2-4)。

１：ES 細胞における相同組換えを利用した遺伝子標的法で VGAT 遺伝子のエクソン 2 と 3 の両側に loxP 配列を挿入した VGAT-flox マウスを作製した。VGAT-flox マウスと生殖細胞に Cre recombinase が発現する CAG-Cre マウスとを交配するなどして、最終的に VGAT ノックアウトマウスを作製した。VGAT ノックアウトマウスは出生日致死であった。胎生 18.5 日の VGAT ノックアウトマウス脳における VGAT 蛋白の欠損をウエスタンブロット法で確認した。VGAT ノックアウトマウス前脳では野生型マウスと比較して、GABA 含量とグリシン含量が 25%程度増加していたが、グルタミン酸含量に変化はなかった。

２：VGAT ノックアウトマウスの組織学的解析を行った結果、僧帽筋の減少、肝うっ血、肺胞腔の狭小が観察され、VGAT 欠損が筋肉、肝、肺の発達へ寄与することを明らかにした。また、VGAT ノックアウトマウスと GAD67（GABA 合成酵素）ノックアウトマウスで観察される口蓋裂、臍帯ヘルニアについて比較した結果、口蓋突起の所見や出現頻度からいずれも VGAT ノックアウトマウスの方が重症であることを明らかにした。

３：前脳スライス標本を用いて電気生理学的解析を行った結果、微小抑制性シナプス後電流（mIPSC）は野生型マウスで検出されたが、VGAT ノックアウトマウスでは検出されなかった。前脳スライス標本で細胞外に放出される GABA 濃度を測定した結果、VGAT ノックアウトマウスと野生型マウスで違いがなかった。また、前脳スライス標本外液に細胞膜 GABA トランスポーター（GAT）阻害剤を投与した結果、いずれのマウスでも細胞外の GABA 濃度の増加を観察した。これらの結果は、前脳において GAT が関与しない非小胞性 GABA 放出が存在することを示唆した。

４：VGAT ノックアウトマウス脊髄標本の運動ニューロンの電気生理学的解析を行った結果、自発性 IPSC が検出されなかった。一方、後根を刺激した場合には、運動ニュ

ーロンの興奮性応答が検出された。この結果は、GABA とグリシンの神経伝達が遮断されていても脊髄神経回路が形成されていることを示した。

今回の VGAT ノックアウトマウスの作製・解析に関する研究は、胎生期の VGAT の生理学的役割の理解に端緒をつけるもので、VGAT ノックアウトマウスがそのための強力なツールになることを示したものであり、先導科学研究科における学位論文の水準に十分に達していると判断した。今後は、作製した VGAT-flox マウスを用いて特定のニューロンあるいは特定の時期に限定した VGAT ノックアウトマウスを作製・解析することで、更なる神経研究に貢献できると期待される。