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The Role of Reelin and Psoralidin in GABAergic Neurons

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GABA성 신경세포에서의 Reelin과 Psoralidin의 역할

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ABBREVIATIONS

DMSO	Dimethylsulfoxide
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
GABA	G-aminobutyric acid
ApoER2	Apolipoprotein E receptor 2
VLDLR	Very low-density lipoprotein receptor
MAPK	Mitogen-activated protein kinase
ERK1/2	Extracellular signal-related kinase1/2
NC	Nitrocellulose
RIPA	Radioimmunoprecipitation assay
PFA	Paraformaldehyde
NGS	Normal goat serum
VGAT	Vesicular GABA transporter
GAD	Glutamate decarboxylase
MAP 2	Microtubule-associated protein 2
PI3K	Phosphatidylinositol 3-kinase
SST	Somatostatin
PV	Parvalbumin
VIP	Vasoactive intestinal peptide

국 문 초 록

GABA성 신경세포에서의 Reelin과 Psoralidin의 역할

이 성 은

지 도 교 수: 이 금 화

약학과

조선대학교 대학원

중추신경계는 신속하고 정확한 정보 처리를 위해 다양한 신경 회로를 생성하고 회로 활성을 조절한다. 신경망의 흥분과 억제는 흥분성 신경세포와 억제성 신경세포의 균형적인 활동으로 조절된다. 대뇌피질에서 흥분성 신경세포는 총 신경세포의 대략 80%를 구성하며 주로 신경전달물질인 글루탐산을 분비하는데, 글루탐산은 시냅스후 막의 글루탐산염 수용체와 결합하여 탈분극을 유도하여 해당 신경세포에서 활동전위 생성을 촉진한다. 반면, 20%의 신경세포를 구성하는 억제성 신경세포는 주로 Gamma-aminobutyric acid (GABA)를 분비하고 이것이 시냅스후 막의 GABA 수용체에 결합하게 함으로써 표적 신경세포 막전위의 과분극화를 유발시켜 활동전위를 제어한다. 이러한 흥분성 혹은 억제성 신경세포의 기능적 장애는

신경망의 흥분과 억제의 불균형을 유발시켜 정보처리 과정에서 신호전달문제를 일으키고 자폐증과 조현병, 우울증 등 뇌신경질환을 초래한다.

Reelin은 뇌 발달과 신경 기능에 필요한 세포 외 분비 당단백질로 조현병, 우울증, 자폐를 비롯한 여러 정신질환과 유전적으로 연관된다. Reelin 은 뇌발달 단계에서 주로 대뇌피질의 표면층에 존재하는 Cajal-Retzius 세포에 의해 생성되어 피질 형성에 중요한 신경세포의 이동을 조절하고, 출생 후에는 주로 해마와 피질의 GABA성 신경세포에서 합성되어, 수상돌기의 성장과 spine 형성 그리고 학습과 기억능력에 요구되는 시냅스 가소성을 포함한 다양한 과정에 관여한다. 이러한 Reelin 은 신경세포의 막에 발현된 아포지단백 E 수용체 2 (ApoER2) 및 초 저밀도 지단백 수용체 (VLDLR)에 결합하고 세포내 어댑터 단백질 Dab1 의 티로신 인산화를 촉진한다. 인산화된 Dab1 은 세포 부착과 이동에 영향을 미치는 Crk/Rap1 신호 전달, 수상 돌기 성장 및 spine 형성을 촉진하는 포스파티딜이노시톨-3 키나제(PI3K)/Akt 및 mTOR 신호 전달을 포함한 여러 하위 신호 전달 경로를 활성화한다. 그러나 현재 Reelin의 역할에 대한 대부분 연구가 흥분성 신경세포 중심적으로 진행되어 있어 억제성 신경세포와 억제성 시냅스 발달에 미치는 Reelin의 영향에 대해서 알려진 바가 부족하다.

본 연구에서는 태아 생쥐의 대뇌와 해마로부터 분리한 신경세포에서 신경세포 유형-선택적인 Reelin의 신호전달 경로의 활성화와 Reelin의 억제성 시냅스 발달에 대한 영향을 연구하였다. 흥분성 신경세포와 억제성

신경세포에서의 신호전달경로 활성화 차이를 연구하기 위해 개개의 신경세포를 가시화할 수 있는 면역형광법과 공초점현미경을 이용하여 실험·분석하였다. 결과적으로 흥분성 신경세포에서 Reelin은 Akt와 리보솜S6단백질의 인산화를 통한 PI3K 신호전달 경로를 활성화시켰지만, 대부분의 억제성 신경세포에서는 인산화를 유도하지 않는 것을 최초로 밝혔다. 다만, 억제성 신경세포의 아유형 중 하나인 소마토스타틴-발현 억제성 신경세포에서는 놀랍게도 리보솜S6단백질의 인산화가 유도됨으로써 Reelin이 억제성 신경세포 내에서도 선별적인 반응을 유도할 수 있음을 알게 되었다. 그럼에도 불구하고 Reelin은 억제성 신경세포의 수상돌기의 총길이를 변화시키지 않는 것이 확인되었다. 다음으로, 해마 신경세포에서 Reelin을 통한 억제성 시냅스에 관련하는 단백질 또는 mRNA 의 발현량을 비교 분석하였고 억제성 시냅스의 발달에 미치는 영향을 연구하였다. Reelin 처리에 의해 시냅스관련 단백질의 발현은 변화되지 않았으나 억제성 시냅스의 밀도는 감소된다는 것을 발견하였다. 이러한 Reelin에 대한 선택적인 신경세포 반응의 기전을 살펴보기 위해 Reelin의 세포내 신호전달의 주요단백질인 Dab1의 발현을 연구하였다. 하지만 상업적으로 판매되는 Dab1의 항체는 개개의 신경세포에서 Dab1 발현을 가시화할 수 있는 면역형광법에서는 유효하지 않아 직접 맞춤형 항체를 주문하기로 하였고, Dab1 유전자의 일정부분을 GST 융합 벡터를 이용하여 클로닝하여 단백질 단편을 발현시켜 정제하고 이를 토끼에 주입하여 다클론 항체를 얻어 면역형광법 실험을 계속하고자 하였다.

다음 실험으로 억제성 신경세포의 기능에 미치는 인자를 연구하기 위해 Psoralidin을 이용하여 GABA 의 합성과 분비에 필수적 단백질인 글루탐산 탈탄산효소 (Glutamate decarboxylases, GADs)의 발현 기전을 살펴보았다. Psoralidin은 *Psoralea corylifolia*의 씨앗으로부터 분리된 천연 화합물로서 항산화, 항염증 효과를 포함한 여러가지 특성에 대해 보고가 되어있다. 이 연구에서는 우선 피질 신경세포와 해마 신경세포에서 억제성 신경전달 및 시냅스 관련 유전자 발현에 미치는 Psoralidin의 영향에 대해 실험하였고 그 결과, 초대 신경세포에서 Psoralidin은 GAD67과 GAD65 단백질의 발현을 감소시켰다. GADs 발현의 감소는 주로 NMDA 수용체를 통해 이루어지므로 NMDA 수용체 길항제를 사용하여 신호전달 관여 여부를 연구하였다. 놀랍게도 NMDA 수용체 길항제에 의한 GAD 발현 감소의 완화가 전혀 이루어지지 않아 Psoralidin은 기존의 조절체계와 다른 방법으로 GAD 의 발현을 조절한다는 것을 발견하였다. 또한 Psoralidin은 억제성 시냅스의 발달을 지연시키는 것으로 면역형광법과 공초점현미경을 통하여 밝혔다.

종합적으로, 본 연구에서는 초대 신경세포에서 생체 내 단백질인 Reelin의 신경세포 선택적인 신호전달과정과 억제성 시냅스의 발달에 미치는 영향을 분석하였다. 또한, 신경세포-선택적인 Reelin의 신호전달의 기전을 알아보기 위해 새로운 Dab1 항체를 생성하여 그 기전을 밝히고자 하였다. 덧붙여, 억제성 신경세포에서 GABA 의 합성과 분비에 필수적 단백질인 글루탐산 탈탄산효소의 발현 기전을 Psoralidin을 이용하여 연구하였다. 이 연구 결과들은

억제성 신경세포의 반응과 기능에 관련된 인자를 밝히고 신경망의 흥분/억제의 불균형으로 비롯된 신경학적 뇌질환의 분자적 기전을 이해하는데 기여할 것이다.

Ⅰ. INTRODUCTION

1. Inhibitory neuron in the forebrain

1-1. Cell in the central nervous system

Nervous system is an organ system responsible for monitoring body movements, sending and receiving signals to and from other parts of the body, and controlling body functions [1, 2]. Nervous system consists of the central nervous system (CNS), which constitutes the brain and spinal cord, and the peripheral nervous system (PNS), which connects the extremities of the body with the CNS. Brain performs movement, sense, language, memory and thought processing, and maintains the environment necessary for survival, such as maintaining homeostasis and regulating body metabolism.

At 3 weeks of fetal development, vertebrates begin to develop the neural plate from the thickened ectoderm. The neutral position is folded inward to create a neural groove and develops into the neural tube. Neural tube is divided into an anterior end and a posterior end. The anterior end forms forebrain, midbrain, and hindbrain, and the posterior end becomes the spinal cord. The forebrain is one of the brain regions in the early embryonic stage of vertebrates and consists of the telencephalon including the cerebral cortex and subcortical structures, and the diencephalon including the thalamus and hypothalamus. It is a basic structure that plays a central role in information processing related to cognitive activity, sensory and associative functions, and voluntary motor activity.

The forebrain forms the adult brain structure through the migration mechanism of neurons [3]. Neurons migrate from the ventricular zone to the pial surface via glial cells in

the cerebral cortex. Specifically, neocortex forms an intricate structure divided into six layers (I to VI) [4]. The cortical layer created by the migration of the first generated neurons becomes layer VI. Newly generated neurons reach the surface layer passing through pre-born neurons and organize the upper cortical layers. Consequently, neurons are distributed by type in a specific layer to develop the forebrain [5, 6].

Neurons and glial cells are the functional and structural basic elements of the nervous system. The structure of a neuron is basically composed of three parts: Cell body, Dendrite, and Axon (Figure 1A). Cell body has a nucleus as the center of the nerve cell. Dendrites that spread around the cell body play a role in receiving signals. And Axon is responsible for transmitting the signal received from the dendrite to other nerve cells through chemical and electrical synapses [7]. Glial cells are responsible for helping nerve cells transmit nerve impulses and supplying nutrients to nerve cells. The glial cell includes astrocyte, oligodendrocyte, microglia, and ependymal cell in CNS [8]. The glial cell exists in a larger proportion than neurons and has the ability to divide and regenerates damaged parts under certain conditions or induces a process of division. Oligodendrocytes present in CNS consisting of the brain and spinal cord form a myelin sheath surrounding the axon of the neurons in the CNS. The myelin sheath formed by oligodendrocytes functions as an insulator surrounding the axon, allowing the action potential generated in the axon hillock of the neurons to be transmitted to the nerve terminal at a high speed [9]. Astrocytes help blood brain barrier (BBB) stay intact by tightening nerve cells and capillary vessels using long projections to form a framework that supports the structure of CNS and promotes junctions that strongly block material passage between CNS capillary endothelial cells [10].

Two main types of neurons make up the cerebral cortex. About 80% of all neurons are

excitatory neurons, and the remaining 20% are GABAergic inhibitory neurons [11, 12]. Two types of neurons are generated from neural precursor cells that are insufficient to accommodate adult cortical cells [13, 14]. The population of neural progenitors divides into two identical neural progenitors by symmetrical mitosis. Symmetrical division expands the size of the pool of neural progenitors. At the end of cortical neurogenesis, mitosis changes from symmetric to asymmetric, and asymmetric division produces neural precursor cells and neurons. Neural progenitor cells generated after asymmetric division continue to divide in the proliferative region, but newborn neurons migrate to the developing neocortex [15-17]. Both types of neurons originate in specific regions of the developing forebrain [18, 19]. Excitatory neurons differentiate in the ventricular zone of the dorsal forebrain and migrate radially to reach the cortical plate [20]. This radial migration is a major process in the developing forebrain. The characteristic of radial migration is that neurons generated in the ventricular zone migrate perpendicularly to the cortical surface along the radial glial cells. The radial glial cells are precursor cells that exist in the ventricular zone, providing a scaffold for newborn neurons to follow. In contrast, GABAergic inhibitory neurons differentiate in the proliferative zone of the basal forebrain and migrate tangentially to reach the cortical plate of the dorsal neocortex. Tangential migration is the movement of neurons along an axon or other neuron parallel to the cerebral mantle [18, 21, 22].

Neurons located at their final destinations transmit signals and process information through various neurotransmitters. Representatively, excitatory neurons induce the excitability of neural networks through glutamate neurotransmitters, whereas inhibitory neurons produce and secrete γ-aminobutyric acid (GABA) neurotransmitters to offset the effects of excitatory neurons.

In the adult cerebral cortex, excitatory neurons are classified into pyramidal neurons and spiny stellate neurons according to their dendritic morphology [23-25]. Pyramidal neurons are multipolar neurons characterized by long apical dendrites found in all cortical layers except layer I. Although pyramidal neurons also exist in subcortical structures such as the hippocampus and amygdala, they make up about two-thirds of cortical neurons and are the major neocortical output neurons involved in many important cognitive processes. Several dendrites of spiny stellate neurons are similar in length and exist exclusively in layer IV of the V1 region of the visual cortex.

Excitatory neurons in other areas, including pyramidal neurons, are commonly involved in the transmission of nerve impulses through excitatory neurotransmitters [26, 27]. Among the excitatory neurotransmitters known so far, glutamate is representative and accounts for about 15-20% of the central nervous system. Glutamate plays an important role in opening sodium channels in post-synaptic neurons, which causes the influx of sodium ions to increase the potential inside the cell membrane. This promotes depolarization of post-synaptic cells, which is also known as excitatory post-synaptic potential (EPSP) [28]. Gamma-aminobutyric acid (GABA), a major neurotransmitter in inhibitory neurons, also binds to synaptic receptors and attenuates neuronal activity. In the cerebral cortex, inhibitory neurons are less distributed than excitatory neurons, but play a critical role in neurotransmission. GABA produced and released by inhibitory neurons opens chloride ion channels in the neuronal cell membrane, thereby lowering the potential by introducing negative charges in the post-synaptic neuron. The increase in negative charge leads to hyperpolarization of the post-synaptic neuron and reduces the frequency of action potential generation. The hyperpolarization potential of an inhibitory neuron is also called an inhibitory post-synaptic

potential (IPSP) [28, 29]. The inhibitory response of the central nervous system is mainly mediated by GABA and glycine, and the structural and physiological mechanisms of neurotransmitter receptors are the fundamental basis for drugs such as analgesics and antiepileptic drugs. For example, Benzodiazepin used for sedation enhance the binding of GABA neurotransmitter receptors, thereby enhancing the inhibitory action of GABA [30]. In addition to its role as a neurotransmitter during early development, GABA plays an important role as a trophic factor for neuroblast migration, regulation of dendrite growth and synapse formation [31-33].

1-2. GABAergic neuron

The generation and diversity of inhibitory neurons is a general strategy of the nervous system to ensure the functional complexity and flexibility of neural networks [34]. During embryonic development of mammals, GABAergic neuron are generated in the ganglionic eminence (GE) and travel tangentially to their final location in the brain [35]. The GE is a transient structure of nervous system development located in the abdomen of the telencephalon, which guides the movement of neurons and axons [36]. The ganglionic eminence is divided into medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE), and lateral ganglionic eminence (LGE) according to anatomical basis. In the case of primates, GABAergic neurons of the telencephalon arise from one of the MGE or CGE regions in the embryonic subcortical striatum. Thus, in the developing nervous system, MGE and CGE are the main sources of GABAergic neurons [37].

The subtypes of GABAergic neuron are strongly associated with specific progenitors, which gives rise to a diversity of inhibitory neuronal subtypes [11, 37, 38]. Within the cortex, MGE generates parvalbumin (PV)-expressing fast-spiking interneurons and somatostatin (SST)-expressing groups, of which Martinotti cells form the largest subset. CGE generates a relatively rare subtype that includes Neurogliaform cells (NGCs), bipolar and Vasoactive intestinal peptide (VIP)-expressing multipolar interneurons [39]. Active formation of cortical neural networks is facilitated by the genetic heterogeneity of inhibitory neurons. Classification of GABAergic interneuron subtypes based on the expression of combinations of genes represents cell types with distinct electrophysiology, morphology, connectivity, and molecular properties. [40-42].

The subtypes of GABAergic interneurons expressing calcium binding proteins or

neuropeptides and the microcellular types representing these subtypes have been investigated [43]. First, PV is a calcium-binding protein and is expressed in about 40% of all GABAergic interneurons in somatosensory cortex [44-46]. Most PV expression interneurons are basket cells, basket cells can be further subdivided into the size of the cell body and dendrites and axon projections [47-49]. Physiologically, PV-expressing basket cells are often fast-spiking, specified by high-frequency action potentials in which adaptation rarely occurs [50]. PV basket neurons innervate to the soma of excitatory pyramidal neurons and proximal parts of dendrites. Therefore, PV expression interneurons target the periphery and distal dendritic regions of postsynaptic excitatory neurons, and intracellular targets are the basis for the inherent inhibitory effects of excitatory neurons.

Somatostatin is a neuropeptide expressed in 30% of the interneuron of the cortex. SST expression interneurons rarely overlap PV expression interneurons [44-46]. Based on output connectivity, SST neurons are classified as cells targeting dendrites. A representative cell type belonging to SST expression neurons is Martinotti cells [51, 52]. This projects an ascending axon that splits horizontally from the first layer of the neocortex. Martinotti cells can be further divided into two subclasses depending on the presence or absence of calretinin (CR), a calcium-binding protein. SST+/CR+ or SST+/CR- Martinotti cells differ not only in dendritic tissues but also in input connectivity. In Layer II/III, SST+/CR+ cells are innervated into Layer II/III pyramidal neurons [53, 54]. On the other hand, SST+/CR- cells receive excitatory synaptic inputs from Layer II/III and Layer IV pyramidal neurons. In contrast to PV-expressing neurons, Martinotti cells are connected to highly accelerating excitatory synapses and induce feedback or feedforward inhibition to pyramidal neurons in an activity-dependent manner.

Neuropeptide VIP is expressed in a subset of interneurons that do not overlap with SST and PV expression neurons [45, 55]. A significant number of VIP neurons express CR and display a bitufted or bipolar form [56, 57]. As physiological characteristic, these neurons are generally irregularly-spiking cells. Other major subtypes of VIP neurons display the bitufted or multipolar form, but do not express CR. This subtype of neuron is a cell that adapts to rapid spikes. VIP neurons form synapses in the dendritic spines and shafts of the pyramidal neurons, and some of the VIP neurons are initially innervated into other interneurons. However, VIP neurons rarely inhibit pyramidal neurons and selectively inhibit SST neurons. This suggests that VIP neurons do not inhibit pyramid-shaped neurons [58-60].

The 5HT3a receptor (5HT3aR) is an ionic serotonin receptor that is expressed in GABAergic interneurons, which does not express PV and SST and constitutes approximately 30% of the total cortical interneuron [45]. Although 5HT3aR neurons are physiologically, anatomically, or biochemically very heterogeneous, they are largely divided into two subclasses: VIP neurons and non-VIP neurons. In somatosensory cortex, VIP neurons account for about 40% of the total 5HT3aR, while non-VIP 5HT3aR neurons, which account for 60% of 5HT3aR neurons, include Reelin expressive neurons. Reelin-positive neurons are the primary population of non-VIP 5HT3aR cells and are more than 80% [61].

Cholecystokinin (CCK) expression neurons consist of a type of basket interneuron that distributes nerves to soma and proximal dendrites of pyramidal neurons, and control the phasing and synchronization of neural ensembles like PV expression neurons [62]. However, neuropeptide CCK expression neurons make up different classes of basket cells and have unique molecular and physiological features different from PV expression basket cells. Another notable property of CCK basket cells is the plasticity change of the transmitter

release in response to a retrograde signal. Pre-synaptic terminals of CCK neurons express type 1 cannabinoid (CB1) receptors, which are activated by endogenous cannabinoid emitted from post-synaptic pyramidal neurons. Activation of CB1 receptors reduces GABA release [63-66]. GABAergic neurotransmission by CCK expression basket cells is mediated through α2-containing $GABA_A$ receptors that exhibit slow kinetics, while PV expression basket cells use α1-containing $GABA_A$ receptors that mediate rapid GABAergic reactions at postsynaptic sites [62, 67].

The GABAergic inhibitory neurons of cerebral cortex are a distinct group of neurons with various subtypes and functions. Each subtype of GABAergic neuron also has unique characteristics. However, due to the overlapping function and morphology among the subtypes, there are limitations to the clear definition of the GABAergic interneuron subtype. Therefore, it is necessary to identify the molecular factors that determine the physiological and anatomical characteristics of specific GABAergic interneuron subtypes.

1-3. Inhibitory synapses

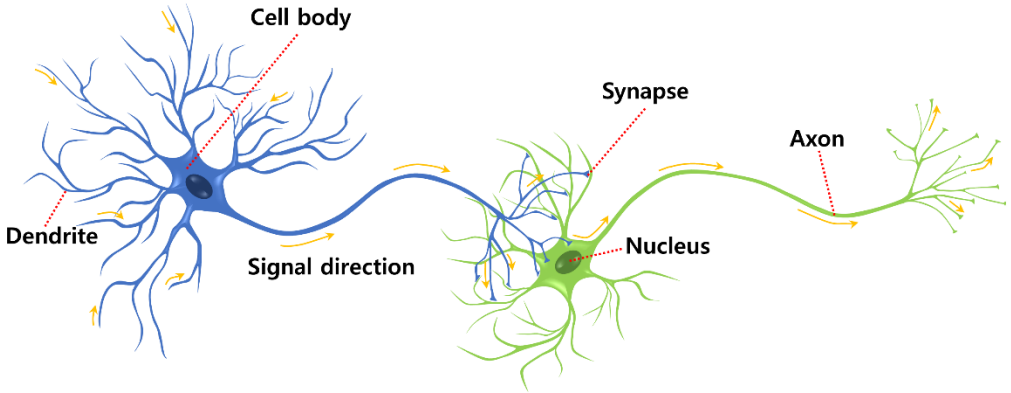
In the mammalian brain, neurons connect through synapses with other neurons to develop specific neural circuits that are central to overall brain function (Figure 1A). Morphologically, synapses are divided into two types. Type I is an asymmetric structure and is formed mainly in dendritic spines, whereas type II is a symmetric structure and develops mainly in somatic cells and dendritic shafts [68]. Type I synapses, which secrete glutamate as neurotransmitters, are responsible for excitatory synaptic transmission, whereas type II synapses use GABA and glycine as neurotransmitters for inhibitory synaptic transmission. At inhibitory synapses in the mammalian brain, GABA acts as a major neurotransmitter and is essential for maintaining the excitatory and inhibitory (E/I) ratio. Balancing between the excitatory synapse type and the inhibitory synapse type is also essential for normal brain function. From a neurophysiological perspective, The E/I balance means the relative activity for excitability and inhibitory synapse inputs. Integration of numerous neural circuits is important for rapid and normal neural information processing. Therefore, the proper E/I ratio is determined by the various types of inhibitory neurons present in the diverse brain region.

In the developing brain, inhibitory neurons with a high diversity of types detect other target neurons and form synapses with specific GABA receptors. Inhibitory synapse development is initiated through cooperative action with various classes of proteins (Figure 1B). Synaptic adhesion molecules regulate synaptic recognition and signal transduction, which are essential for synapse formation by physically connecting pre- and post-synaptic sites [69]. Neuroligin-2 (NL-2) has been studied to interact with scaffold proteins and other adhesion proteins and was the first adhesion molecule identified to be selectively localized at inhibitory synapses [70]. Neuroxin, which interacts with NL-2, is a cell surface molecule

and is important for presynaptic function. In an activity-dependent manner with specific post-synaptic ligands, endogenous neurexins are located at the presynaptic terminals of inhibitory synapses [71]. The class IV Semaphorin (Sema4D) exists as a factor that promotes inhibitory synaptic development, which works in conjunction with the receptor PlexinB1 [72, 73]. In a subset of interneurons, Tropomyosin-related kinase B (TrkB) is involved in inhibitory synaptic assembly by regulating the synaptic localization of gephyrin [74]. Equally, brain-derived neurotrophic factor (BDNF) as a ligand of TrkB regulates gephyrin clustering through mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathways [75]. In addition, fibroblast growth factor 7 (FGF7), which induces partially inhibitory synaptic differentiation, is known as a target-derived presynaptic factor. Dystroglycans present at inhibitory synapses act as ligands of neurexins and are regulated in an activity-dependent manner [76]. Although not essential for inhibitory synapse formation, glycosylation of dystroglycans at inhibitory synapses in the hippocampus is essential for homeostatic synaptic plasticity. During homeostatic scaling-up of inhibitory synapses, dystroglycans stabilize the inhibitory synapses by recruiting $GABA_A$ receptors [77, 78]. Another class of synaptic molecules, neurexophilin-1 with localized expression in subtypes of inhibitory neurons, are α-neurexin specific ligands. It regulates the short-term synaptic plasticity of inhibitory synapses and recruits presynaptic $GABA_B$ and postsynaptic $GABA_A$ receptors [79]. Additionally, Neurexophilin-1 and dystroglycan compete for binding of α-neurexin [80]. Synaptic molecules involved in inhibitory synaptic plasticity cooperatively drive activity-dependent redistribution of local microcircuits and maintain neural network activity.

Finally, as one of the intracellular factors regulating inhibitory synaptic development and function [81], Gephyrin is a representative synaptic scaffolding protein essential for inhibitory synaptic transmission and plasticity, and glycine and $GABA_A$ receptor clustering. It is regulated by phosphorylation of BDNF, cyclin-dependent kinase 5 (CDK5), and glycogen synthase kinase 3β (GSK-3β) in the posterior membrane. In addition, palmitoylation of gephyrin, which may affect clustering, suggests that multiple mechanisms regulate inhibitory synaptic development and gephyrin clustering [82].

A



(Pre-synaptic Neuron) (Post-synaptic Neuron)

B

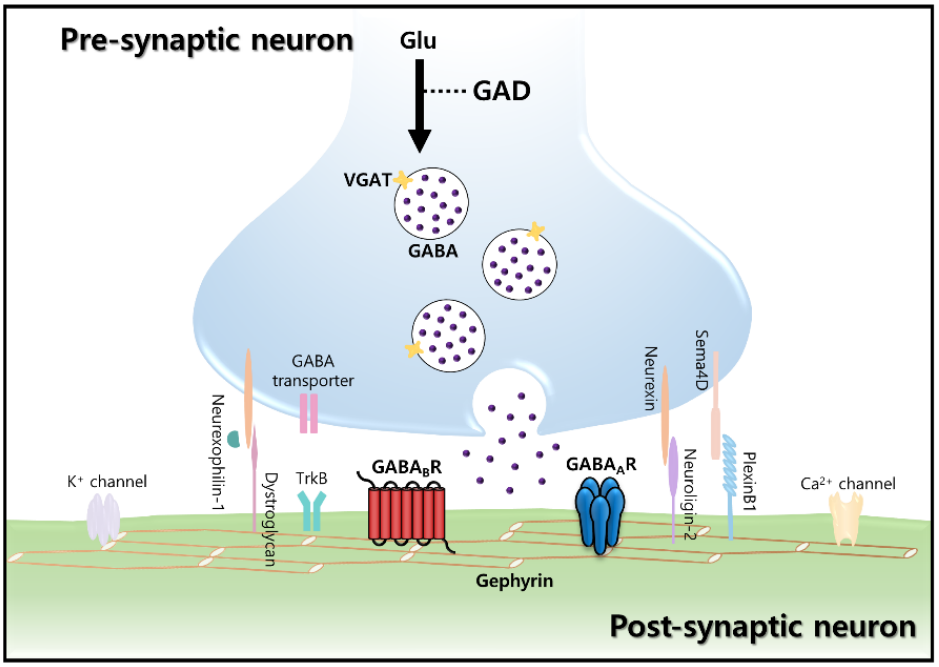


Figure 1. Schematic of the basic functional units of the nervous system and the major organizers of inhibitory synapses

(A) Illustration of neural signal transmission between two neurons with axons and synapses. The nervous system, including the brain, is all made up of neurons. Neurons that transmit signals in the nervous system vary in shape and size depending on the type, but have a similar basic structure. Neurons consist of a neuron cell body, dendrites, and axons. Neuronal cell body has a nucleus, cytoplasm, and many organelles that regulate life activities. The dendrites are connected to sensory receptors or the axon terminals of other neurons. The main function of dendrites is to receive incoming information. Axons that extend from the nerve cell body are responsible for transmitting information received by a neuron to the next neuron or reactor. When an electrical signal traveling along an axon reaches an axon terminal, a chemical signal (Neurotransmitter) is released from the axon terminal to transmit the signal to the dendrite of the next neuron. The neuron fires when enough neurotransmitter arrives, sending an electrical signal to the next axon, and so on until the message reaches the appropriate cell. Neurons can send nerve impulses in only one direction. In order for a nerve impulse to continue from a pre-synaptic neuron to a post-synaptic neuron, it must cross the synaptic cleft. (B) Illustration of major inhibitory synaptic organizers with synaptic adhesion and scaffold proteins localized to GABAergic inhibitory synapses. Overlapping marks indicate specific protein-protein interactions. The major inhibitory neurotransmitter in the central nervous system is γ-aminobutyric acid (GABA), which is converted from glutamate by glutamate decarboxylase (GAD). GABA is recruited into presynaptic vesicles through the action of the vesicular GABA transporter (VGAT). After membrane depolarization, GABA is released to synapse and binds to ionic $GABA_A$ receptors or metabolic $GABA_B$

receptors on the post-synaptic membrane. The $GABA_A$ receptor is a heteropentameric ligand-gated ion channel that reduces membrane excitability by selectively allowing the influx of Cl^- and HCO^{3-} ions. At least 19 subunit genes and heterologous $GABA_A$ receptors mediate most rapid synaptic inhibition. Metabolic $GABA_B$ receptors are G protein-coupled heterodimers of GABA-B1 and GABA-B2, which inhibit neurotransmitter release in post-synaptic neurons and induce cell membrane hyperpolarization. Subsequently, GABA dissociated from the receptor is reabsorbed by membrane GABA transporters localized to neurons and astrocytes.

The concept of synaptic plasticity implies adjusting the strength of synaptic connections between neurons. The Synaptic plasticity is central to information processing and plays an important role in enhancing cognitive processes in the brain nervous system. [83]. The long-term and short-term synaptic plasticity of inhibitory synapses varies according to the interneuron type and brain region involved [84, 85]. Inhibitory synaptic plasticity arises from differences in post-transcriptional deformation and lateral diffusion of GABA receptors, changes in the probability of GABA release, changes in the number of functional inhibitory synapses and the reversal potential of post-synaptic responses. The plasticity is generally important for experience-dependent learning and memory, neural circuit enhancement, and E/I balance [84]. Additionally, homeostatic synaptic plasticity is an essential device for maintaining a stable neural network in the activity of neurons in the face of various changes [86, 87].

As the cause of various brain-related diseases, the imbalance of E/I ratio and changes in synaptic plasticity can be related. Imbalances in the E/I ratio cause synaptic imbalances that underlie many brain disorders. A hallmark of brain disorders, including autism, schizophrenia, and epilepsy, is Synaptopathy [88]. During synaptic processes, synaptic adhesions and disruption of signal transduction can lead to abnormal neural network activity and degradation of certain neural circuits [89]. Dysfunction of key inhibitory synaptic proteins can lead to unique types of brain disorders, such as epilepsy, because the balance is tilted toward excitability. [90]. Ultimately, depending on the change in inhibitory synaptic plasticity, the pathological level of neural activity may vary. In many neuropsychiatric disorders, inhibitory synapses play a central role. Changes in the number of abnormal inhibitory synapses are highly correlated with the prevalence of several brain disorders,

including nerve damage, sleep disturbances, anxiety, autism spectrum disorders, Huntington's disease, and Parkinson's disease. In addition, plasticity of inhibitory synapses may be induced by excessive neural activity that may occur in pathological conditions such as seizures. In cranial neuropsychiatric disorders, the cause of inhibitory disorders may be decreased or overactivation of inhibitory synaptic plasticity [83, 91, 92].

1-4. Glutamate decarboxylases

The biosynthesis of GABA occurs by the decarboxylation of glutamate catalyzed by localized glutamate decarboxylase (GAD). GADs are widely present in the axon terminals of inhibitory neurons and in somatic cells and dendrites. The distribution of GADs throughout the cell is high in the Golgi complex and in the presynaptic cluster [93, 94]. A high correlation with GABA levels and subsequent GABAergic neurotransmission at inhibitory synapses is associated with the expression and activity of GADs [95-97]. Dysregulation of GAD has been implicated in a variety of neurological disorders, including representative schizophrenia and epilepsy. GAD is also involved in the development of non-neuronal tissues, including the palate and abdominal wall [98], and is also highly relevant in autoimmune diseases such as type 1 diabetes [99].

The two representative isoforms of GADs are GAD67 and GAD65, with molecular weights of 67 kDa and 65 kDa, respectively. The two isoforms are expressed in separate genes in GABAergic neurons of the brain and have different regulatory processes and molecular properties [100]. In most GABAergic cells, GAD isoforms are expressed simultaneously, but the expression rate differs according to brain developmental stages and brain regions. GAD67 is constitutively active, producing over 90% of basal level GABA, and is a brain protein required for the maturation and function of inhibitory circuits in the cerebral cortex [101]. In contrast, GAD65, which exists primarily in an inactive form, is transiently converted to an active form in response to dynamic GABAergic synaptic activation [102]. GABA synthesis in GAD65 fine-tunes and maintains GABAergic synaptic function during neuronal activity, and is focused on nerve endings for neurotransmission purposes. GAD67 is expressed at the highest level in the olfactory bulb, whereas GAD65 is

most strongly expressed in the striatum and is followed by the olfactory bulb [103].

Transcriptional levels of GAD67 and GAD65 are regulated through N-methyl-D-aspartate Receptor (NMDAR) and BDNF-TrkB activation. In addition, calcium influx following NMDAR activity contributes to the expression of GADs. Increased neuronal activity, in turn, enhances the activation of NMDAR and enhances the release of BDNF from excitatory neurons, thereby increasing the expression of GADs by activation of TrkB-RAS-ERK-CREB signaling [104].

2. Reelin

2-1. Structural features

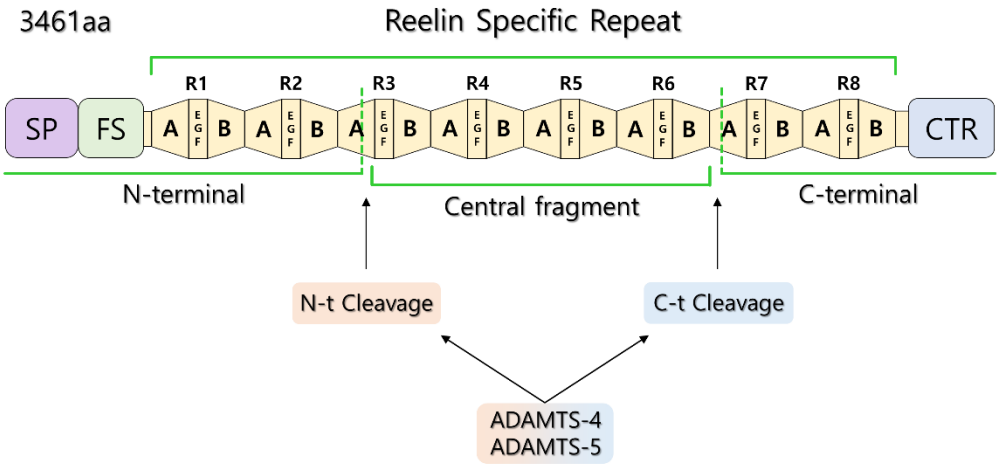
Reelin is an extracellular matrix glycoprotein that plays critical roles in the regulatory phase of brain wiring. Reelin regulates neuronal migration and stratification, dendritic arborization and synapse formation in the mammalian neocortex [105]. Reelin Gene (*reln*) encoded at 7q22.1 in humans is translated into a protein consisting of 3461 amino acids [106-108] . Reelin protein has approximately molecular weight of 388 kDa and its proteolysis is mediated by several proteases [109]. N- and C-terminal site cleavage is catalyzed by the extracellular matrix metalloproteinases ADAMTS-4 (A Disntegrin and Metalloproteinase with Thrombospondin motifs-4) [110] and ADAMTS-5 [111], whereas serine protease tissue plasminogen activator (tPA) can cleave Reelin at the C-terminal site [112, 113].

Structural features of Reelin include a signal peptide followed by an N-terminal sequence and a hinge region upstream from 8 Reelin repeats of 350-390 amino acids, each Reelin repeat consisting of two sub-repeats separated by an EGF motif. The C-terminus of the Reelin protein is very basically terminated with 33 amino acids. The epitope, also known as CR-50, is located near the N-terminus and consists of amino acids 230-346 of the Reelin glycoprotein (Figure 2). Epitopes that are formed *in vivo* are essential for the Reelin–Reelin electrostatic interaction to produce a soluble string-like homopolymer composed of up to 40 or more regularly repeating monomers [114].

Representative receptors for Reelin include apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) [115, 116]. Although the cell surface receptors VLDLR and ApoER2 are two members of the LDLR family, binding to Reelin

with similar affinity, these receptors are differentially expressed depending on organs, tissues and cell types [117]. In neurons, mainly ApoER2 is localized to the cell membrane of multipolar cells in the intermediate region. And VLDLR is localized to the leading region of neurons in the marginal zone [118].

The Reelin receptors of the LDLR gene family protein have a multi-domain structure in the extracellular region. The N-terminal LDLR class A (LA) module repeat region, known as the ligand binding domain, possesses major lipoprotein binding activity in the prototypical LDLR [119]. This LA module region and the central fragment containing the fifth and sixth Reelin repeats (R5-6) forms a complex [120-122]. Previous structure-guided alanine mutagenesis studies showed that Lys-2360 and Lys-2467 of R5-6 constitute the central binding site for the LDLR class A module of the receptor [120]. In addition, the Lys mutation of full-length Reelin also lost receptor binding and Dab1 phosphorylation activity [123]. Structurally, R5-6 of the central fragment contains an essential region for receptor binding. On the other hand, although receptor binding of the R5-6 fragment and subsequent Dab1 phosphorylation activity were confirmed, it was much less effective than full-length Reelin or artificial oligomerization in signal transduction [120, 123]. Therefore, central fragmentation and higher-order multimerization of Reelin can be very important structural features.



SP : Signal Peptide
FS : F-Spondin
CTR : C-Terminal Region
ADAMTS : A Disintegrin and Metalloproteinase with Thrombospondin motifs

Figure 2. Structural features and processing fragments of the extracellular matrix protein Reelin

Reelin is an extracellular matrix protein whose native domain begins with a signal peptide (SP) and F-spondin homology domain (FS). Eight bodies, R1 to R8, with Reelin-associated repeats, each separated by an epidermal growth factor (EGF)-like motif, consist of two subdomains (A and B). The C terminus is a small carboxy terminal region (CTR) of 33 amino acids and is positively charged. An essential function of the extracellular matrix protein is secretion, and the secreted Reelin is cleaved in two by metalloprotease activity to the N-terminal (Nt = N-R2), central (C = R3-R6) and C-terminal (Ct) produces three large fragments.

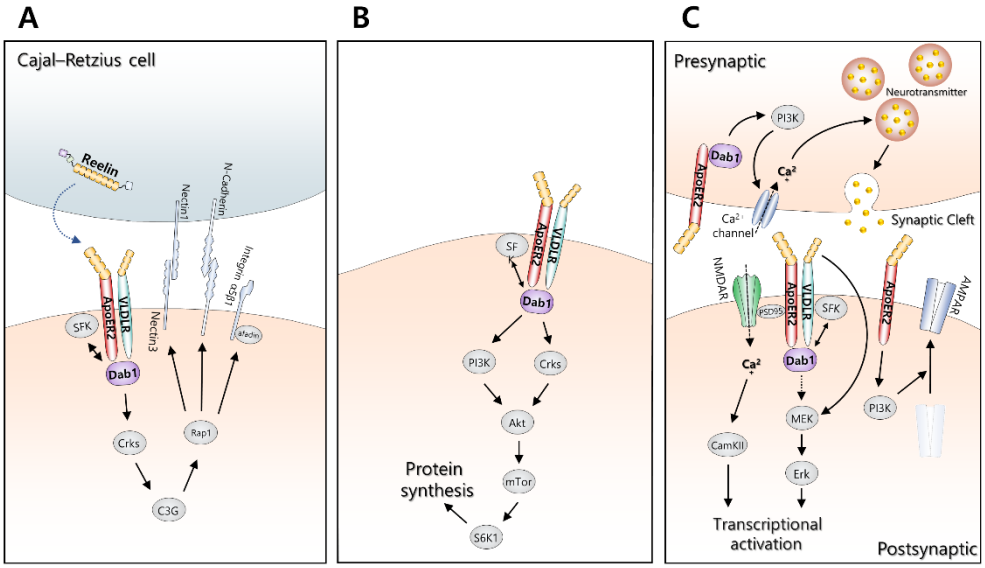
2-2. Signaling

Reelin signaling is initiated when Reelin and lipoprotein receptors (VLDL, ApoE) form a complex [124]. Calcium promotes the binding of Reelin to the receptor, but ApoE inhibits the formation of the complex [125]. Reelin binding induces oligomerization of Disabled-1 protein and lipoprotein receptor located in the cytoplasm [126]. Oligomerization is produced by the interaction of the phosphotyrosine kinase domain of Dab1 with the cytoplasmic tail sequence NPxY (Asp-Pro-any amino acid-Tyr) of the lipoprotein receptor [117, 127, 128]. The Dab1 receptor complex is phosphorylated at five tyrosine residues of Dab1 by Src and Fyn [129, 130]. Src and Fyn interact with tyrosine phosphorylated Dab1 via the SH2 domain [131]. Clustering of Dab1 via lipoprotein receptor induces tyrosine kinase activity and signaling cascade. The Dab1 adapter protein is essential-dependent on the activation of the Reelin signaling pathway. Tyrosine phosphorylation of Dab1 further induces activation of downstream signaling cascades for proper neuronal migration and layer development in the cerebral cortex [132]. The signaling cascade of Reelin on cortical development may modulate intrinsic biological activity at specific times.

During prenatal period (Figure 3A), Reelin regulates neuronal migration and cortical development through the Crk/C3G/Rap1 pathway. This signal transduction induces cell layer formation by controlling the functions of cell adhesion proteins including integrin α5β1, nectin3 and N-Cadherin [133, 134]. Glia-independent soma translocation of newborn neurons depends on the interaction between the translocating neurons and Cajal-Retzius cells and is regulated by Rap1 activation. Additionally, the small GTPase Rap1 in migrating neurons maintains N- cadherin on the plasma membrane, thereby regulating the direction of multipolar migration towards the pial surface. Thus, when neocortex is formed, Rap1 is

involved in several aspects of Reelin-induced migration [133].

Early postnatally (Figure 3B), Reelin activates the Crk adapter protein and the PI3K-Akt-mTOR pathway. Activation of this signaling pathway promotes protein synthesis, dendrite growth, and spinal development [135, 136]. In the late postnatal and adult brain (Figure 3C), The signaling activity of Reelin interacts with NMDAR and ApoER2 via PSD-95. It subsequently induces Ca^{2+} influx and CaM KII activation through NMDAR [137, 138]. Increased intracellular calcium increases phosphorylation and nuclear translocation of the transcription factor cAMP-response element binding protein (CREB). Neurotransmitter release (pre-synaptic) and the density of the ionic receptors NMDA and AMPA (post-synaptic) are regulated through the PI3K/Akt pathway [137, 139]. In addition, the receptors involved in the activation of the MEK-Erk1/2 pathway by Src/Fyn kinase are unknown [140, 141]. These signaling pathways induce synaptic activity and plasticity by promoting the expression of immediate-early genes involved in learning and memory [142].



During Prenatal Period | Early postnatally | Adult brain

Figure 3. Reelin signaling pathway in brain development

(A) Reelin secreted by Cajal-Retzius cells (CR) binds to lipoprotein receptors. Signaling is initiated by phosphorylation of tyrosine residues of Dab1 by the Src-family kinases (SFK) Fyn and Src. Through adhesion molecules, neurons induce migration through the Crk/C3G/Rap1 pathway. (B) After nerve migration, the main signal of Reelin is translated into dendrite development. Signaling mechanisms that influence dendrite development also include standard pathways controlling neuronal migration, including ApoER2/VLDLR, Dab1, SFK and Crk. The major pathway of Dab1 Downstream is the Mammalian Target Activation of Rapamycin (mTor) and S6 kinase 1 (S6K1) pathway that can regulate protein synthesis. (C) In the late postnatal stage, Reelin signaling involves additional signaling molecules such as CaM KII. Increased Ca^{2+} and Erk1/2 activation affect synaptic function through transcriptional activation. The PI3K/Akt pathway is mostly also involved in Reelin function, but not in early migration.

2-3. Functions

Reeler mice are spontaneous autosomal recessive mutant mice [106, 124]. According to a previous anatomical study of *reeler* mice, approximately reversal of the location of cortical plate neurons in the neocortex was observed [143]. In addition, it was found that the marginal zone was missing in the cerebral cortex, and all major cortical structures of the *reeler* rat brain were disrupted [109]. With these morphological features, the role of Reelin in the mechanism of brain structure formation through *reeler* mutant mice has been studied [144].

Reelin plays important roles in correct central nervous system development and function before and after birth [145]. Representatively, it has a role in neuronal migration that is essential for mammalian cerebral cortex formation. The main secretion of Reelin in the prenatal cerebral cortex is by Cajal-Retzius neurons in the marginal region [15]. Reelin induces radial migration of excitatory neurons generated in the ventricular or subventricular zone toward the cortical surface in an "inside-out" manner [146]. In this process, neurons generated at chronological stages each reach the top of the cortical plate in a distinct migration pattern. The earliest generated neurons migrate primarily to radial glia fiber-independent soma translocation [147]. Lately generated neurons begin to migrate and undergo transformation into multipolar neurons in preplate. It then transforms into a bipolar conformation suitable for a directional migration pattern to reach the top of the cortical plate [148]. Although full-length Reelin is predominantly present in the marginal zone, cleavage fragments of Reelin deficient in R3-6, N-R2 and N-R6 diffuse from the marginal zone into deep tissue to signal multipolar neurons [124]. When bipolar neurons reach the marginal zone, the most superficial layer of the cortex [148], Reelin induces dissociation from radial glial cell, leading to conversion to terminal soma translocation in a radial glia-independent

manner [148]. Soma translocation induction proceeds through the Crk/C3G/Rap1 pathway of Reelin signaling [134, 149]. In addition, neuronal migration is also related to temporal differences in Reelin receptor binding. [150]. Binding of Reelin to the ApoER2 receptor in newborn neurons induces stabilization of a key process that migrates to the marginal zone [151, 152]. After completion of migration, the Reelin/VLDLR complex is formed and endocytosis is induced. Eventually, a decrease in extracellular reelin causes a neuronal stop signal in the marginal zone [153, 154]. Reelin complexed with VLDLR and ApoER2 plays a variety of roles through signaling.

Reelin has a functional difference in excitatory and inhibitory neurons. Although the cytological function of Reelin signaling in glutamate excitatory neurons is concentrated, the effect on the migration of GABAergic neurons is limited. Except for certain subgroups, GABAergic neurons are similar to the inside-out positioning pattern of excitatory neurons in the cortical layer [155-158]. However, in ultrasound guided transplantation of MGE cells analysis, Dab1-deficient cortical GABAergic neurons migrated to their final destination and reached their normal target layer [159]. Also, in *Reeler* and *Dab1* mutant embryos, there were no significant differences from control mice as a result of analyzing the number and pathways of GABAergic neurons reaching the final destination. These results indicate that Reelin is not directly involved in the migration of GABAergic neurons [160]. Reelin expression, which was elevated immediately after birth, shows a decrease in mRNA levels, but Reelin expression can be maintained continuously in adult tissues.

During the postnatal period, Reelin expression is maintained by a small number of extant Cajal-Retzius cells, some GABAergic neurons of the cortex, and glutamatergic cerebellar neurons. Reelin produced by cortical GABAergic neurons has been identified in

a subpopulation of these cells expressing calretinin and calbindin [161, 162]. Layer I and II GABAergic neurons project to different cortical layers to secrete Reelin into the ECM [163]. The secreted Reelin surrounds and attaches to the dendritic shafts and spine of cortical pyramidal cells to play a role in dendritic spine regulation [135].

After the neuron reaches its destination , Reelin continues to regulate synaptic signaling pathways after neuron migration is complete to control dendritic spine structure and synaptic plasticity [144]. Reductions in dendritic tree complexity and dendritic spine density have been identified in neurons of juvenile and adult *reeler* mutant mice [164-166]. As such, previous studies confirmed the influence of Reelin on the development of dendrites and spine, which are important for neural circuit establishment [142]. Therefore, in mature neurons, Reelin signaling is important for maintaining neuronal structure.

In the adult CNS, Reelin is involved in regulation of synaptic function and neurotransmitter release [167]. Previous studies have confirmed that in *reeler* mice, synaptic signaling molecular levels are reduced as well as depletion of excitatory postsynaptic responses [135, 168-170]. And the addition of recombinant Reelin to hippocampal slices enhanced the long-term potentiation of hippocampus [141, 171, 172]. Reelin signaling at glutamatergic synapses regulates the expression and activity of presynaptic and postsynaptic AMPA and NMDA receptors. Long-term potential of the cortex and hippocampus is enhanced by Reelin-induced AMPAR insertion and NMDAR phosphorylation regulation [173, 174]. Also, in adult rodent studies, Reelin has been shown to modulate NMDA-type glutamate receptor activity through mechanisms involving Dab-1 and Src family kinase (SFK) [140]. Reelin signaling induces NMDA receptor activity and modulates calcium influx. Further downstream, the Crk and Dab-1 proteins appear to be involved in Reelin

signaling [132]. These results indicate that the Reelin-receptor complex controls Ca^{2+} entry through the NMDA receptor, thereby regulating synaptic plasticity in the post-synaptic signal transduction pathway [175].

As such, synaptic plasticity can be modulated through interactions between Reelin and other factors. The multi-scaffold protein Intersectin-1 (ITSN1) directly related to the Reelin canonical pathway has been identified [176]. Alterations of NMDA-organ enhancement in response to Reelin stimulation were expressed in mice lacking ITSN1. ITSN1 serves as a molecular bridge facilitating the interaction between VLDLR and the intracellular adapter Dab1. It promotes phosphorylation of the Reelin signal and downstream signaling as a potential aid in signal transduction [177].

There has been a variety of studies on the role of excitatory synaptic function and plasticity [174, 178]. However, the role of Reelin in GABAergic inhibitory synaptic activity has not yet been established. Recently, new synaptic functions of Reelin on GABA synapses and circuits were identified. In *reeler* haploinsufficient heterozygous mice, GABAergic transmission mediated by ionic $GABA_AR$ was altered without changing the action of GABA, resulting in a disruption of the excitation/inhibition balance [179]. It suggests that imbalances between brain network E/I ratios caused by abnormal Reelin signaling lead to neurological disorders such as Alzheimer's, schizophrenia and autism. Ultimately, various neurological disorders, including neurodegenerative disorders, are related to the function of Reelin [180-182].

According to a previous study, brain samples from schizophrenic patients had 50% reduced expression of Reelin protein and mRNA compared to normal controls [183]. A more recent study showed that schizophrenic patients in a control group had a 25-fold decrease in

Reelin protein expression in patients with increased *RELN* gene methylation levels [181]. Autism is another neurodevelopmental disorder characterized by deficits in cognition, communication, and social interaction. Reelin has been studied as an important potential biomarker of autism development [184]. Autism is also a complex and multifactorial disorder, and impaired function of Reelin may be one of the pathogenesis factors during neuronal maturation and movement [182]. Also, Reelin signaling receptors, which are involved in neuronal migration and cortical layers during embryonic brain development, not only signal transduction of Reelin, but also regulate the synthesis and degradation of Aβ, and may inhibit tau hyperphosphorylation, thus affecting Alzheimer's disease (AD) [185, 186]. Therefore, Reelin is a signaling protein that properly regulates tau phosphorylation, which is involved in the pathogenesis of Alzheimer's disease, and there is a correlation between Reelin protein expression and β-amyloid peptide (Aβ) [187, 188]. These results suggest that Reelin may be involved in the pathogenesis of neurological diseases through neurotransmission and connectivity deficits during development [189].

3. Psoralidin

Psoralidin is a natural compound isolated from the seeds of *Psoralea corylifolia*. Several studies have found that Psoralidin has multiple properties, including antioxidant, anti-osteoporotic, anti-inflammatory and anti-tumor effects [190-192]. In previous studies, Psoralidin-mediated intracellular effects and structural synaptic modifications on excitatory neurons suggest that Psoralidin has a specific role as an NMDA receptor modulator [193]. It has also been reported that Psoralidin has potent antidepressant-like properties mediated through the monoamine neurotransmitter and the hypothalamic-pituitary-adrenal (HPA) axis system. These evidences suggest a potential effect of the natural compound Psoralidin on neurotransmitter production. Despite its diverse roles and potential therapeutic effects, little is known about the action of Psoralidin on GABAergic neurons. In this study, we investigated the effect of Psoralidin on inhibitory neurotransmission-related genes expression and the development of inhibitory synapses in primary cortical or hippocampal neurons.

We discovered that Psoralidin reduced the level of GADs through transcriptional regulation in GABAergic neurons. The reduced expression of GADs by Psoralidin was not mediated by NMDA receptor, a major known pathway for GADs regulation. In addition, Psoralidin attenuated the formation of inhibitory synapses in primary hippocampal neurons.

Ⅱ. STUDY AIMS

1. To study the effect of Reelin on the PI3K signaling pathways in individual excitatory and GABAergic neurons.
2. To examine the effect of Reelin on the dendrite growth of GABAergic neurons
3. To investigate whether Reelin affects the development of inhibitory synapses in primary neurons
4. To study the expression of genes that are related to inhibitory synapses by Reelin.
5. To study the expression pattern of Dab1 in GABAergic neurons.
6. To study the influence of a natural compound Psoralidin on the expression of glutamate decarboxylases and the development of inhibitory synapses in primary neurons.

Ⅲ. MATERIALS AND METHODS

1. Reagents and antibodies

The primary antibodies used in this study were rabbit anti-phospho-S6 235/236 (1:3000 for Western blotting, 1:1000 for immunofluorescence, Cell Signaling, Danvers, MA, USA, #4858, #2211), rabbit anti-phospho-S6 240/244 (1:5000 for Western blotting, 1:1000 for immunofluorescence, Cell Signaling, Danvers, MA, USA, #5364), rabbit anti-phospho-Akt (1:5000 for Western blotting, 1:1000 for immunofluorescence, Cell Signaling, Danvers, MA, USA, #4060), rabbit anti-Akt (1:5000, Cell signaling, Danvers, MA, USA, #4691), chicken anti-Map2 (1:30,000, Abcam, Cambridge, UK, ab5392), mouse anti-gephyrin (1:1000, Synaptic systems, Goettingen, Germany, 147-021), mouse anti-gephyrin (1:3000, Synaptic systems, Goettingen, Germany, 147-111), rabbit anti-VGAT (1:3000 for Western blotting, 1:1000 for immunofluorescence, Synaptic Systems, Goettingen, Germany, 131 002), mouse anti-GAD67 (1:3000 for Western blotting, 1:1000 for immunofluorescence Millipore, Temecula, CA, USA, MAB5406), anti-GAD65 (1:3000, Abcam, Cambridge, UK, ab26113), somatostatin (1:100, Santacruz, Oregon, USA, sc-55565), and mouse anti- β-actin-HRP (1:50,000, Sigma, St. Louis, MO, USA, A3854). The secondary antibodies were HRP-conjugated anti-rabbit and anti-mouse (Invitrogen, Rockford, IL, USA, G21040, G21234) antibodies conjugated to Alexa Fluor 488 goat anti-chicken (Invitrogen, A11039), Alexa Fluor 555 goat anti-mouse (Invitrogen, Eugene, OR, USA, A21422), and Alexa Fluor 647 goat anti-rabbit (Invitrogen, Eugene, OR, USA, A21244).

2. Primary neuronal culture

The cerebral cortices and hippocampi were dissected from the brain of ICR mouse (embryonic day 15.5) and digested with 0.25% trypsin (Gibco, Grand Island, NY, USA)–DNase I (Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ for 20 min; trypsin was inactivated using fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The dissociated neurons were placed on poly-L-lysine (Sigma, St. Louis, MO, USA, P5899) precoated plates and cultured in neurobasal medium supplemented with 2% B27 supplement, Glutamax, and penicillin–streptomycin (Gibco, Grand Island, NY, USA). The cells were cultured at 37 ℃ in a saturated atmosphere containing 95% air and 5% CO_2, and the medium was replaced with fresh medium a day after isolation. Afterward, half of the medium was replaced every 3–4 days. All animals used in this study were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee at Chosun University. Because culture hippocampal neurons are more homogenous in their morphology and biophysical properties compared to cortical neurons [194-199], we used hippocampal neurons to examine dendrite growth and inhibitory synapse formation on excitatory neurons to reduce experimental variability.

3. Production of recombinant Reelin and semi-purification

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA, SH30243.01) with 10% fetal bovine serum and transfected with mock (control) or *Reln* cDNA construct pCrl, kindly provided by G. D'Arcangelo [106], using Transit (Mirus, Madison, WI, USA, 61044894). The medium of the transfected cells was replaced with serum-free medium after transfection at 24 h and collected for 2 days. The supernatants were centrifuged for the removal of cell debris and concentrated using Amicon

Ultra-15 filters (Merck, Carrigtwohill, Ireland, UFC903008) at 2680× g for 20 min prior to addition to neuronal cultures as described previously [164]. Primary neurons were replenished with Reelin-containing concentrates, referred to as Reelin-rich protein supplementation.

4. RNA isolation and RT-PCR analysis

Total RNA was obtained from primary neurons using a GeneJET RNA Purification Kit (Thermo Scientific, Vilnius Lithuania, K0732) according to the manufacturer's instructions. The total RNA (0.5–1 μg) was reverse-transcribed to generate cDNA using the iScriptTM cDNA Synthesis Kit (bio-rad, Hercules, CA, USA, 1708891). The mRNA quantification was performed using a StepOne real-time PCR system (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK, 4309155). Transcript-specific primers used in the quantitative RT-PCR were: Gad67, F: 5'-CACAGGTCACCCTCGATTTTT- 3', R: 5'-ACCATCCAACGATCTCTCTCATC-3', PrimerBank ID: 31982847a1; Gad65, F: 5'-TCCGGCTTTTGGTCCTTC-3', R: 5'-ATGCCGCCCGTGAACTTTT-3', PrimerBank ID: 6679925a1; Neuroligin1, F: 5'-GGTACTTGGCTTCTTGAGCAC-3', R: 5'-AAACACAGTGAT-TCGCAAGGG-3', PrimerBank ID: 28972598a1; Neuroligin2, 5'-TGTCATGCTCAGCGCAGT-AG-3', 5'-GGTTTCAAGCCTATGTGCAGAT-3', PrimerBank ID: 33989614a1; Semaphorin 4D, 5'-CCTGGTGGTAGTGTTGAGAAC-3', 5'-GCAAGGCCGAGTAGTTAAAGAT-3', PrimerBank ID: 7305471a1; glyceraldehyde 3-phosphate dehydrogenase (Gapdh), F: 5'-AGGTCG-GTGTGAACGGATTTG-3', R: 5'-TGTAGACCATGTAGTTGAGGTCA-3', PrimerBank ID: 9055212a1F. GAPDH was used as a reference gene for normalization. The

relative quantification of the mRNA was calculated using the Pfaff1 method [200]. .

5. Western blot analysis

Primary cortical neurons were lysed at designated times with Radioimmunoprecipitation assay (RIPA) buffer. The protein extracts were separated using 6% or 8% SDS-PAGE gels and transferred to a 0.22 μm nitrocellulose (NC) membrane. The membrane was incubated in 5% skin milk blocking buffer for 1 h at room temperature and then with a primary antibody at 4 ℃ overnight. After washing with Tris-buffered saline and Tween 20 (TBST), a secondary antibody was supplemented to the membrane for 1 h at room temperature. Membranes were developed with Western blot ECL solution (Neutronex, Goryeong, South Korea, NXECL-2011). Total Akt, Erk, or β-actin were used as loading controls.

6. Immunofluorescence

Primary hippocampal neurons, grown on glass coverslips coated with poly-L-lysine, were fixed in 4% paraformaldehyde (PFA)–phosphate buffered saline (PBS) for 15 min and then permeabilized by 0.1% Triton X-100–PBS for 10 min. After incubation with 10%normal goat serum (NGS) blocking buffer for 1 h at room temperature, the neurons were incubated with primary antibody at 4 ℃ overnight. After washing with PBS, the neurons on the coverslip were incubated with secondary antibodies for 1 h at room temperature. The neurons were washed with PBS 3 times for 5 min and then were mounted on positively charged glass slides with mounting media (Thermo Scientific, Kalamazoo, MI, USA, 9990402). The neurons were imaged using a confocal laser microscope (Nikon, A1+, Japan).

7. Dendrite analysis and quantification of gephyrin–VGAT-positive clusters

The ImageJ program and NeuronJ plugin were used to trace dendrites. The density, area, and intensity of gephyrin or gephyrin–VGAT-positive clusters were measured in primary of secondary dendrites of excitatory hippocampal neurons. The background intensity of each channel was subtracted and the imageJ function Adjust/Threshold was applied to each image under the same conditions to quantify the cluster size. We analyzed the total lengths of the dendrites of 30 to 90 cells in each group, while 30 to 40 dendritic fields were randomly selected from pyramidal neurons in each experiment. All analyses were performed by blinded experimenters with regard to treatment.

8. Statistical analysis

All statistical data and graphs are expressed as mean (standard deviation, SD). After analysis using one-way analysis of variance (ANOVA), statistical significance was assessed according to the experiment. Mann-Whitney U test, Kruskal-Wallis test, and Dunn's comparison test and Newman-Keuls multiple comparison test were used for comparison between group means. Results were averaged from multiple experiments (n) consisting of multiple wells for 3 different cultures, as indicated in the figure legend. Results were considered statistically significant when the p-value was less than 0.05.

Ⅳ. RESULTS

Part. I Reelin affects signaling pathway of a group of inhibitory neurons

1. Reelin does not phosphorylate Akt or S6 protein in numerous inhibitory neuron

Primary cortical or hippocampal neurons isolated from the brains of mouse embryos are composed of mostly excitatory neurons and some inhibitory neurons (Figure 4A). The protein extract was used for Western blotting, regardless of the cell type, but individual neurons were visualized with a confocal microscope using immunofluorescence staining of cells. Western blot analysis showed that Reelin-rich protein supplementation phosphorylated Akt and a downstream effector, ribosomal S6 protein, within 20 min after the supplementation in 5-day *in vitro* (DIV) primary cortical neurons (Figure 4B). The relative levels of Akt phosphorylated at serine 473 and of S6 protein phosphorylated at serine 235/236 or serine 240/244 were 1.46 (0.09), 1.40 (0.26), and 1.17 (0.14) compared to control, respectively (controls = 1.00 (0.07), 1.00 (0.14), and 1.00 (0.05), respectively) (Figure 4C). Since inhibitory neurons make up approximately 10–20% of all neurons in primary neuron cultures, the levels of phosphorylated signaling proteins presented by Western blotting do not indicate the distinct response of inhibitory neurons to Reelin. To investigate whether the Reelin signaling pathway is stimulated in individual inhibitory neurons, an immunofluorescence assay was performed on primary cortical neurons using antibodies against GAD67, a marker of inhibitory neurons; Map2, a dendritic marker of

neurons; and phosphorylated Akt at serine 473. Both GAD67- and Map2-positive neurons were considered inhibitory neurons, while GAD67-negative–Map2-positive neurons were considered excitatory neurons. Interestingly, the intensity of phosphorylated Akt in the soma or dendrites of excitatory neurons significantly increased in response to Reelin-rich protein supplementation, but not in those of inhibitory neurons (Figure 4D, E). The relative intensity levels of phosphorylated Akt in response to Reelin-rich protein supplementation in the soma of excitatory and inhibitory neurons were 1.32 (0.72) and 0.90 (0.57), respectively, compared to the controls (1.00 (0.48) and 0.85 (0.47), respectively). These results were also observed in primary hippocampal neurons (Figure 4F), where the relative intensity level of phosphorylated Akt in response to Reelin-rich protein supplementation was 2.22 (1.27) compared to the controls (1.00 (0.63)) in excitatory neurons and 1.58 (1.47) compared to control (1.02 (0.97)) in inhibitory neurons. In addition, we further investigated whether or not the downstream signaling molecule, ribosomal S6 protein, of the PI3K/Akt pathway was affected by Reelin in inhibitory neurons. In accordance with Akt phosphorylation, Reelin-rich protein supplementation-induced phosphorylation of S6 at serine 235/236 was confirmed in excitatory neurons but not in inhibitory neurons (Figure 5A, B). The phosphorylation levels of S6 in both excitatory and inhibitory neurons were completely suppressed by the PI3K inhibitor LY294002, regardless of Reelin supplementation (Figure 5B); however, the levels of phosphorylated S6 were significantly increased in excitatory neurons in response to Reelin-rich protein supplementation after treatment of PI3K inhibitor, which suggests the involvement of other signaling pathways.

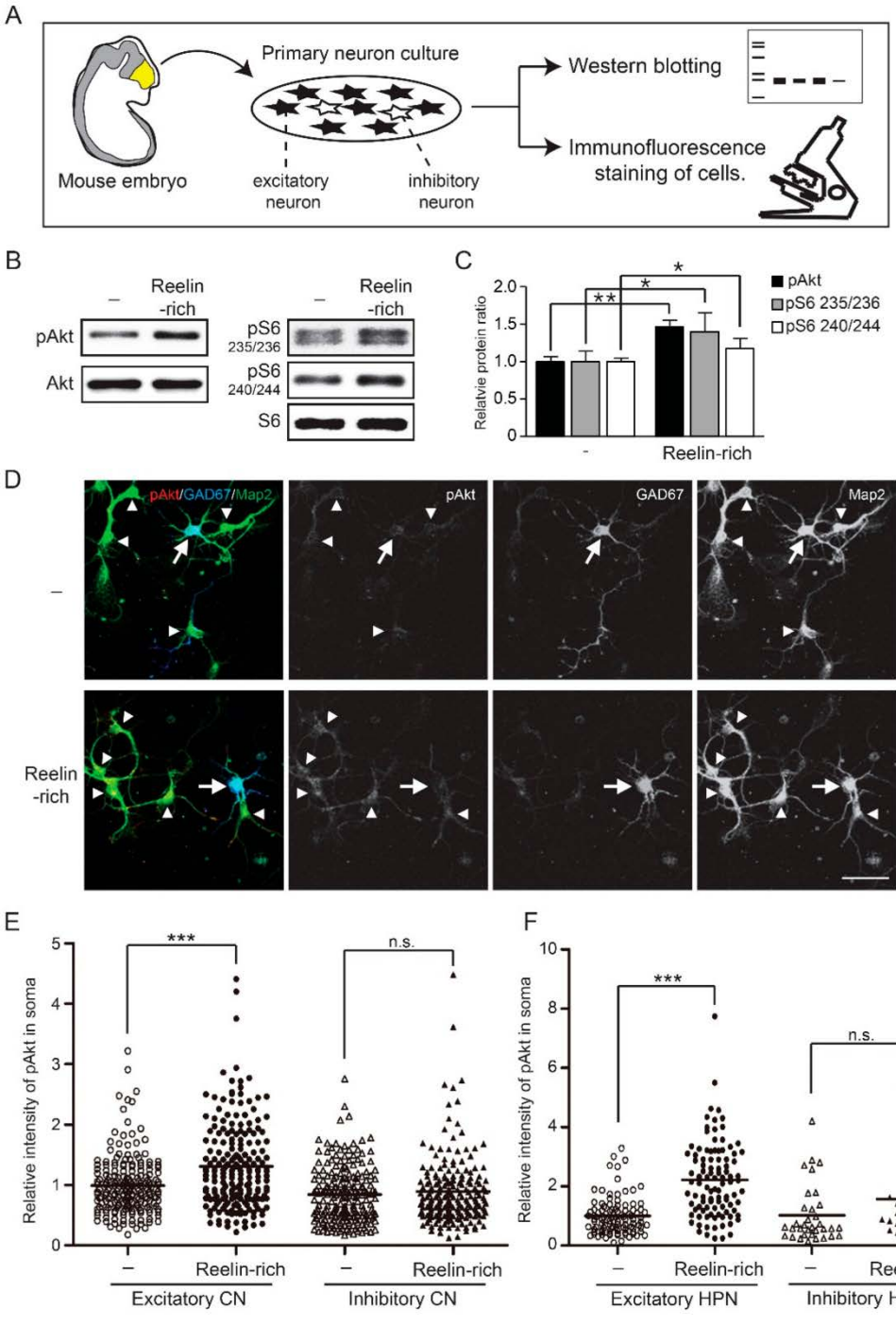


Figure 4. Phosphorylation of Akt and ribosomal S6 protein by Reelin-rich protein supplementation in individual inhibitory neurons

(A) Schematic diagram of the neuronal culture and methodology. Primary cortical or hippocampal neurons isolated from the brain of mouse embryos are composed of mostly excitatory neurons and some inhibitory neurons. The protein extract was used for western blotting, regardless of the cell type, but individual neurons were visualized with a confocal microscope using immunofluorescence staining of cells. (B) Five-day *in vitro* cortical neurons were supplemented with Reelin-rich protein for 20 min and assayed using western blotting. The levels of phosphorylated Akt and S6 were significantly increased by Reelin-rich protein supplementation. (C) The results were averaged from three individual experiments and analyzed using the Mann–Whitney U test. (D) Five-day *in vitro* cortical neurons were supplemented with Reelin-rich protein for 20 min and imaged by confocal microscopy using Map2 (neuron marker), GAD67 (inhibitory neuron marker), or phospho-Akt antibodies. GAD67-positive inhibitory neurons are indicated with arrows and GAD67-negative excitatory neurons with arrowheads. A significant increase in the level of phosphorylated Akt was shown in GAD67-negative–Map2-positive excitatory neurons, but not in GAD67-positive inhibitory neurons. (E) The fluorescence intensity of the soma was quantified and the sample distribution of individual cortical neurons is shown (number of excitatory neurons = 200, number of inhibitory neurons = 200). (F) Fluorescence intensity quantification and sample distribution of individual hippocampal neurons (number of excitatory neurons = 100, number of inhibitory neurons = 33–34). The results were analyzed using the Kruskal–Wallis test followed by Dunn's comparison test. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n.s., not significant. Scale bar, 50 μm.

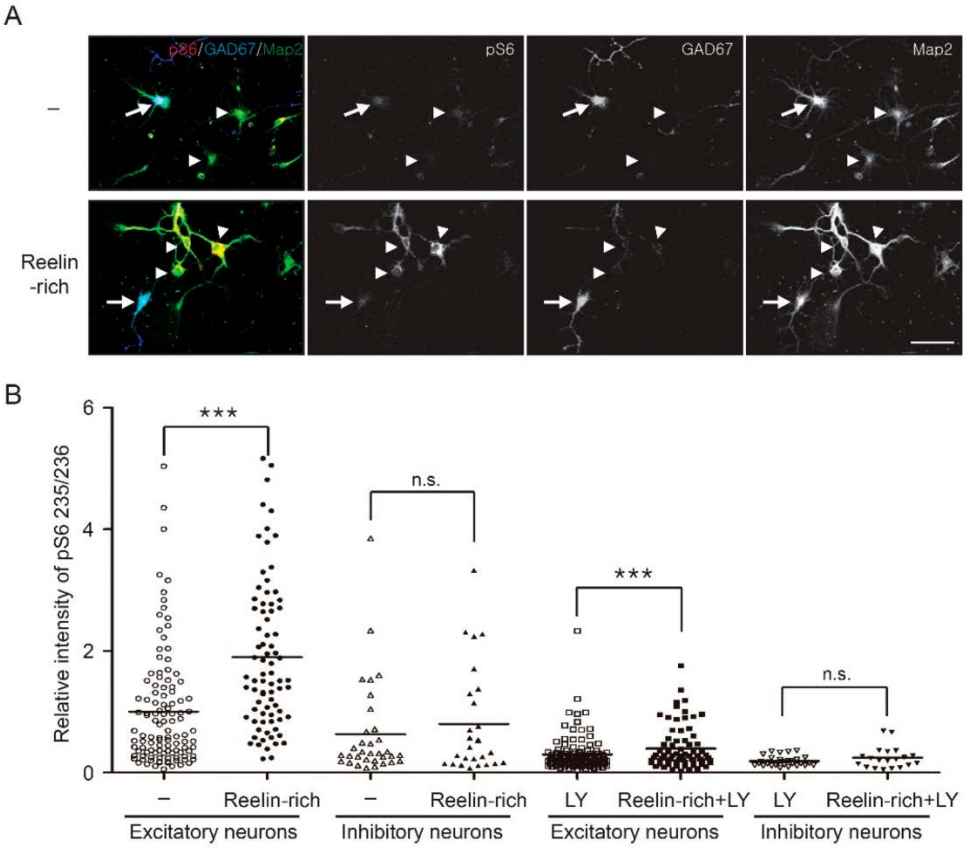


Figure 5. Phosphorylation status of ribosomal S6 protein, a downstream molecule of the PI3K/Akt pathway, by Reelin-rich protein supplementation in individual inhibitory neurons

(A) Five-day *in vitro* hippocampal neurons were supplemented with Reelin-rich protein for 20 min and imaged using confocal microscopy using Map2, GAD67, or phospho-S6 (serine 235/236) antibodies. GAD67- positive inhibitory neurons are indicated with arrows and GAD67-negative excitatory neurons with arrowheads. (B) The fluorescence intensity of the soma was quantified and the sample distribution of individual neurons is shown (number of excitatory neurons = 75–119, number of inhibitory neurons = 20–33). A significant increase in the levels of phosphorylated S6 was shown in GAD67-negative–Map2-positive excitatory neurons, but not in GAD67-positive inhibitory neurons. S6 phosphorylation in both excitatory and inhibitory neurons was completely suppressed under the PI3K inhibitor LY294002 (10 μM), regardless of Reelin-rich protein supplementation. The results were analyzed using the Kruskal–Wallis test followed by Dunn's comparison test. Note: *** $p < 0.001$; n.s., not significant. Scale bar, 50 μm.

2. Reelin-Rich Protein Supplementation Induces Phosphorylation of S6 in Somatostatin-Positive Inhibitory Neurons

GABAergic inhibitory neurons are heterogenous and can be classified by their morphology, electrophysiological properties, molecular identities, and neurochemical properties. In the classification of neurochemical properties, parvalbumin (PV) and somatostatin (SST)-expressing inhibitory neurons represent two broad and rarely overlapping subtypes in the neocortex and hippocampus [201, 202]. Since PV is not sufficiently expressed in developing neurons [203], and because several antibodies used in this study did not detect endogenous parvalbumin in the immunofluorescence assay, we analyzed SST-positive inhibitory neurons to study the subtype-specific response of inhibitory neurons to Reelin. In SST-positive inhibitory neurons, Reelin-rich protein supplementation significantly phosphorylated S6 at serine 235/236 (Figure 6A, B). The relative intensity levels of phosphorylated S6 at serine 235/236 in response to Reelin-rich protein supplementation were 1.80 (1.18) and 2.10 (1.23) in SST-negative and SST-positive neurons, respectively (controls: 1.00 (0.63) and 1.15 (0.88), respectively). Since GAD67-positive inhibitory neurons and SST-expressing interneurons made up approximately 13.5% and 3.3% of all neurons in our culture, respectively, we presumed that Reelin may not activate the PI3K/Akt/S6 signaling pathway in PV-expressing inhibitory neurons, which is the major subtype of inhibitory neurons.

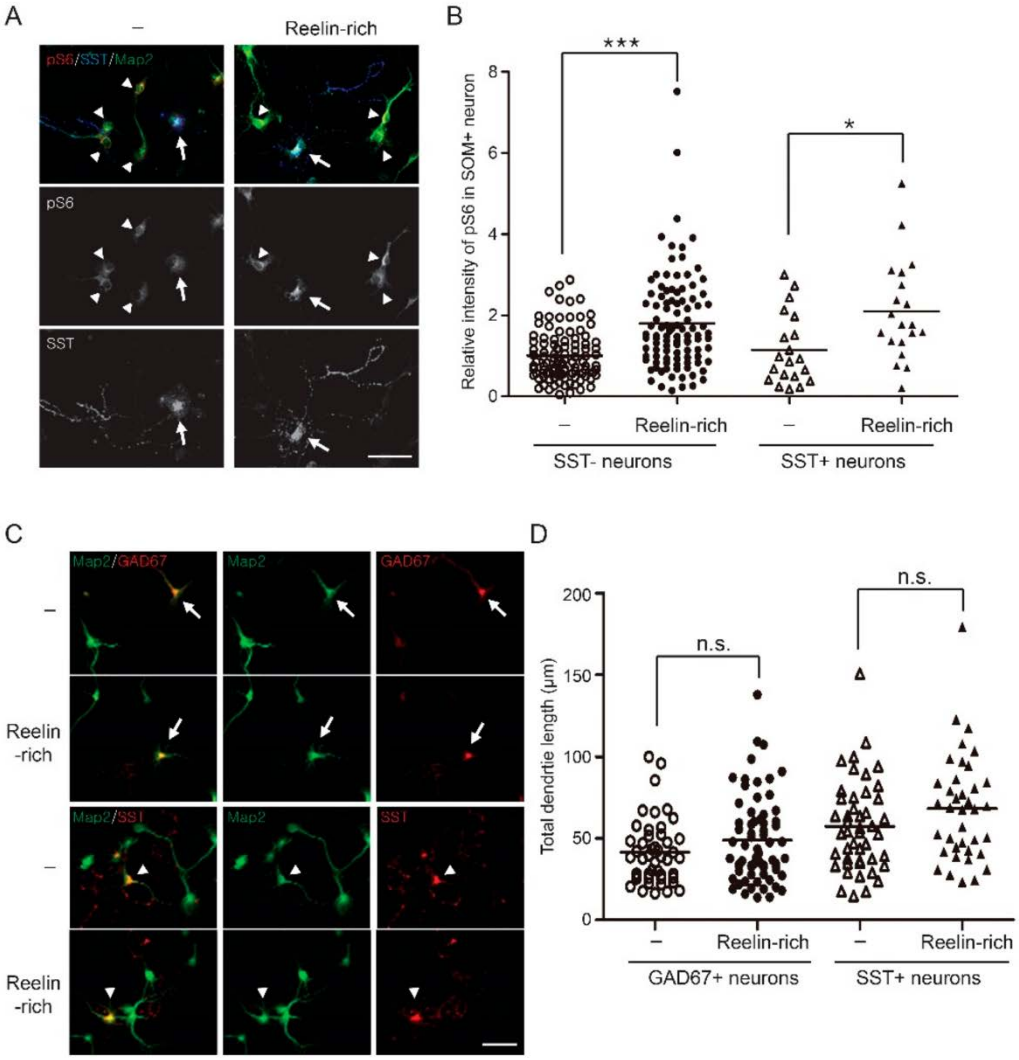


Figure 6. Reelin-rich protein-induced phosphorylation of S6 without dendrite outgrowth effect in SST-positive inhibitory neurons

(A) Five-day *in vitro* hippocampal neurons were supplemented with Reelin-rich protein for 20 min and imaged by confocal microscopy using Map2, GAD67, or phospho-S6 (serine 235/236) antibodies. Somatostatin-positive inhibitory neurons are indicated with arrows and somatostatin-negative excitatory neurons with arrowheads. (B) The fluorescence intensity of the soma was quantified and the sample distribution of individual SST-positive inhibitory neurons is shown (number of SST-negative neurons = 99–102, number of SST-positive inhibitory neurons = 20). (C) Four-day *in vitro* hippocampal neurons were supplemented with Reelin-rich protein for 24 h and imaged by confocal microscope using Map2, GAD67, or somatostatin antibodies. GAD67-positive inhibitory neurons are indicated with arrows and somatostatin-positive inhibitory neurons with arrowheads. (D) Fluorescence intensity quantification and sample distribution of individual hippocampal neurons (number of GAD67-positive inhibitory neurons = 47–67, number of SST-positive inhibitory neurons = 38–46). No significant differences in dendrite outgrowth were observed between most inhibitory neurons and SST-expressing neurons. The results were analyzed using the Kruskal–Wallis test followed by Dunn's comparison test. Note: * $p < 0.05$, *** $p < 0.001$; n.s., not significant. Scale bar, 50 μm.

3. Reelin-rich protein supplementation does not affect dendrite outgrowth of inhibitory neurons in primary cultures

Since dendrite outgrowth of immature neurons is reduced in *Reln*-deficient mice and rescued by Reelin supplementation [165], and because Reelin has a tropic effect on dendrite outgrowth through the PI3K/Akt pathway [204], we studied whether Reelin- rich protein supplementation enhances dendrite outgrowth of inhibitory neurons. We supplemented Reelin-rich protein to 4 DIV hippocampal neurons for 24 h and performed an immunofluorescence assay to measure total dendrite lengths; however, no significant differences in dendrite outgrowth were observed between most inhibitory neurons and SST-expressing neurons in our culture conditions (Figure 6C, D).

4. Reelin-rich protein supplementation reduces the density of inhibitory synapses

Inhibitory synapses located on the dendrites of pyramidal neurons are important for dendritic integration [205]. To study whether Reelin affects the development of inhibitory synapses, we analyzed inhibitory synapses in 12 DIV hippocampal neurons using immunofluorescence assay and confocal microscopy. We examined dendritic inhibitory synapses in hippocampal excitatory neurons for morphological studies because of their better homogeneity than cortical neurons [194, 198, 199]. Gephyrin, an inhibitory postsynaptic marker, and VGAT, an inhibitory presynaptic marker, were used to measure the density of inhibitory synapses. The thickness of the primary or secondary dendrites used for analysis was approximately 2 μm. After 12 h of Reelin-rich protein supplementation, the densities of gephyrin- or VGAT-positive clusters were unchanged (Figure 7A, B); however, the densities of co-localized gephyrin- and VGAT-positive clusters decreased significantly (Figure 7A, C). The average density of gephyrin–VGAT clusters per 10 μm of dendrite with Reelin-rich protein supplementation was 1.05 (0.52) (control, 1.35 (0.68). Western blot analysis showed that the expression levels of inhibitory synapse-related proteins, including GAD67, GAD65, gephyrin, and VGAT, were unchanged 12 h after Reelin-rich protein supplementation (Figure 7D). In addition, the mRNA levels of genes, including GAD67, GAD65, Semaphorin4D, Neuroligin1, and Neuroligin2, were not altered (Figure 7E). These data suggest that Reelin influences the development of inhibitory synapses on excitatory neurons without directly activating the PI3K/Akt/S6 pathway in numerous inhibitory neurons.

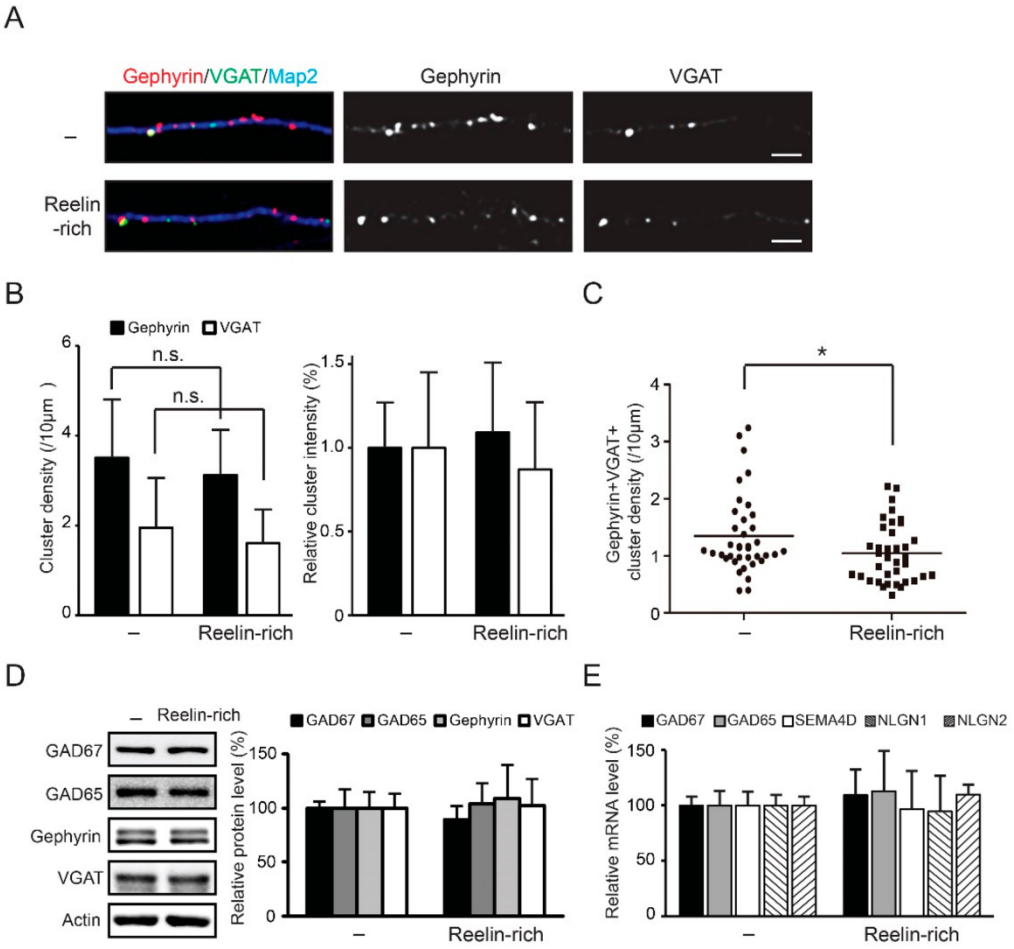


Figure 7. Reduced density of inhibitory synapses by Reelin-rich protein supplementation without changes in expression of inhibitory synapse-related proteins

(A) Twelve-day *in vitro* hippocampal neurons were supplemented with Reelin-rich protein for 12 h and imaged using immunofluorescence followed by confocal microscopy. Gephyrin-positive or VGAT-positive clusters were seen on the dendrites of Map2-positive excitatory neurons. (B, C) The numbers and fluorescence intensity levels of synaptic gephyrin-positive or VGAT-positive clusters were quantified and the sample distribution of both gephyrin- and VGAT-positive clusters is shown. Each density of gephyrin- or VGAT-positive clusters did not change, but the density of both gephyrin- and VGAT-positive clusters decreased significantly. The results (number of dendritic fields = 37–39) were analyzed using the Mann–Whitney U test. (D) Twelve-day *in vitro* cortical neurons were treated with Reelin-rich protein for 12 h and assayed using western blot analysis. The expression levels of inhibitory synapse-related proteins were not changed in response to Reelin-rich protein supplementation. The results were averaged from four individual experiments. (E) Twelve-day *in vitro* cortical neurons were treated with Reelin- rich protein for 3 h and mRNA contents were quantified using quantitative real time-PCR. The transcripts levels of inhibitory synapse-related genes were not changed in response to Reelin-rich protein supplementation. The results were analyzed using the Mann–Whitney U test. Note: * $p < 0.05$ and n.s., not significant. Scale bars, 5 μm.

5. Expression profile of endogenous Dab1 in developing neocortex and primary neurons

Disabled-1 (Dab1) is an essential intracellular adapter protein for reelin signaling. Dab1 is phosphorylated at tyrosine residues by the Src-family kinases (SFK) Fyn and Src when Reelin binds to the lipoprotein receptor [142]. The three major domains of Dab1 are an N-terminal Protein Interaction (PI) and Phospho-Tyrosine Binding (PTB) domain in complex with the Reelin receptor, an internal tyrosine-rich region, and a C-terminal serine and threonine-rich region [117] [206]. The tyrosine-rich region consists of five tyrosine residues (Y185, Y198, Y200, Y220, Y232). At least three of the tyrosine residues that are phosphorylated in response to Reelin stimulation are Y198, Y220, and Y232 [207]. The phenotypes of *reeler* and Dab1-/- mice are characterized by extensive defects in cell layer formation [208]. This phenotype is similar in mice expressing a mutant Dab1 form in which all five tyrosine residues are substituted, demonstrating that Dab1 tyrosine phosphorylation is a key factor in Reelin signaling [209-212]. Tyrosine phosphorylated Dab1 recruits various Src Homology 2 (SH2) domain-containing proteins, including the p85 regulatory subunit of phosphatidylinositide 3 kinase (PI3K) [212]. Phosphorylated Dab1 undergoes mechanisms including ubiquitination by E3 ubiquitin ligase and degradation by the proteasome system, whereby Dab1 signaling is rapidly downregulated [210]. For precise neuronal positioning in developing neocortex, Dab1 phosphorylation triggers various downstream signaling cascades involved in the activation and termination of Reelin signaling and results in cytoskeletal remodeling [142]. Also, several studies using Dab1 knockout mice showed that Dab1 is involved in the regulation of synaptic plasticity as well as in dendrite development

and synapse formation after birth.

To ensure the expression of Dab1 in individual neurons including GABAergic neurons, we conducted immunofluorescence assay using commercial Dab1 antibodies. polyclonal Dab1 antibody (AB5840) produced by Millipore and monoclonal Dab1 antibody (sc-271136) produced by SantaCruz were tried in this study. When screened with Dab1 knockout mouse brain tissue, the Millipore Dab1 antibody was not specific for endogenous Dab1 expression (data not shown here). SantaCruz Dab1 antibody showed excellent endogenous Dab1 expression in primary cortical neurons using western blotting (Figure 8A). In neurons supplemented with Reelin for 6 h, the level of Dab1 was reduced to 66.1% compared to controls, which is expected due to degradation of Dab1 via the proteosome pathway as negative signaling of Reelin [206]. Also, when using a confocal microscope, overexpression of pCAX Dab1-GFP (Addgene #310139) in HEK293T cells localized the GFP signal and was visualized by the SantaCruz Dab1 antibody (Figure 8B). However, when it was used in primary cortical neurons to visualize endogenous Dab1, Reelin-induced degradation of Dab1 was not demonstrated at all (Figure 8C). We therefore, concluded that the SantaCruz sc-271136 Dab1 antibody was not specific for endogenous Dab1 when using immunofluorescence staining of cells. We planned to order custom Dab1 polyclonal antibody for further studies.

To produce custom Dab1 polyclonal antibodies, we used pCAX-Dab1 (Addgene #30139, mouse gene) as a template plasmid of PCR cloning technique. The fragment amplified using PCR with Dab1 primers containing restriction enzyme sequences EcoR1 or BamH1 for selective amplification of the Dab1 gene corresponding to residues 107 - 243 [128] (Figure 9A). The amplified Dab1 fragment was inserted to plasmid pGEX-6p-3, which was used for

the expression of GST-Dab1 fragment fusion protein in BL21 competent *E. coli*. After purification and deletion of GST of GST-Dab1 fragment, it was injected into rabbits for immunization aided by AbClon Inc..

The custom ordered polyclonal Dab1 antibodies were used for detecting endogenous Dab1 expression in primary cortical neurons using western blotting. In neurons treated with Reelin for 6 h, the level of Dab1 was apparently reduced compared to mock supplement controls when using purified Dab1 antibody #1 (antibodies from the first rabbit) and serum Dab1 antibody #2 (from the second rabbit) (Figure 10A, B) due to degradation of Dab1 via the proteosome pathway as negative signaling of Reelin [206]. Also, overexpression of pCAX Dab1-GFP (Addgene #310139) in HEK293T cells localized the GFP signal and was visualized by the serum Dab1 antibody #1 (Figure 10C). Further experiments using via immunofluorescence and confocal microscopy may show the endogenous expression of Dab1 in individual neurons.

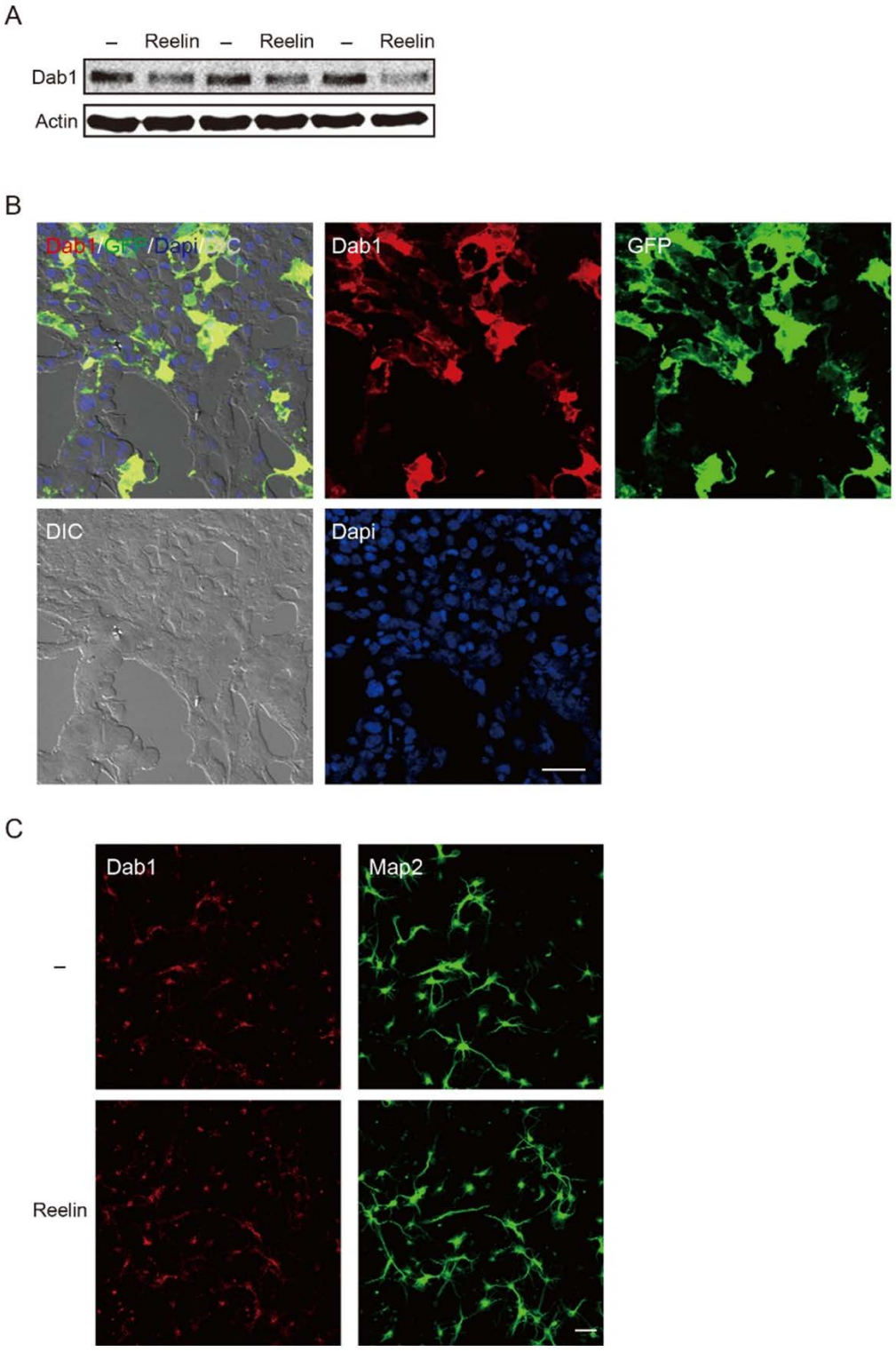
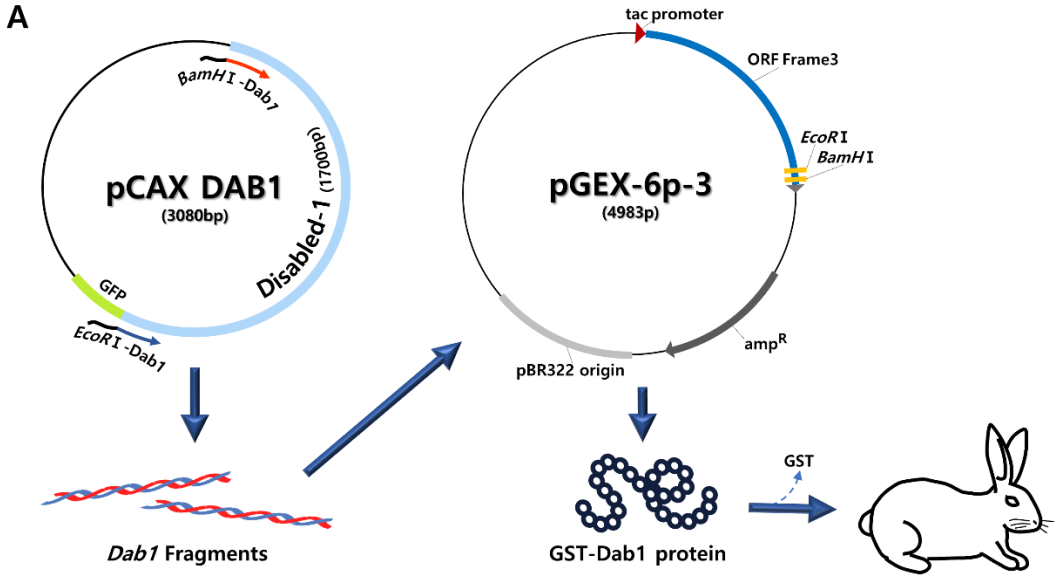


Figure 8. Commercial Dab1 antibodies are not suitable for detection of endogenous Dab1 in immunofluorescence

(A) Endogenous Dab1 expression was detected by SantaCruz, sc-271136, Dab1 antibody using western blotting analysis. Dab1 level was reduced in Reelin supplementation in 6 h. (B) Overexpression of Dab1 tagged with GFP using pCAX Dab1-GFP plasmid was immunostained with the SantaCruz Dab1 antibody and we found that the signal from the antibodies was colocalized with GFP autofluorescence. (C) Reelin-induced degradation of Dab1 in 6 h was not demonstrated in immunofluorescence analysis with the SantaCruz Dab1 antibody. Scale bars, 50μm.



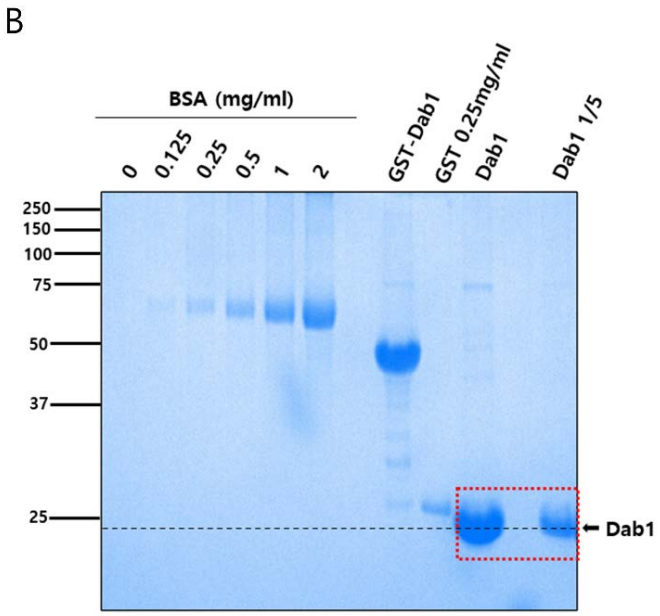
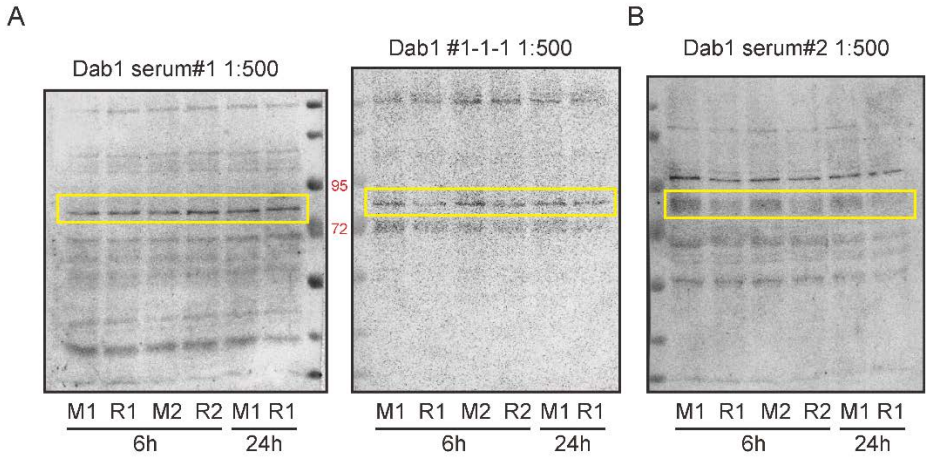


Figure 9. Schematic diagram of Dab1 specific antibody cloning and fragment expression

(A) The selected Dab1 fragment region amplified from pCAX-Dab1-GFP (Addgene #30139, mouse gene) using PCR was inserted to plasmid pGEX-6p-3 for the production of GST-Dab1 fragment fusion protein. The plasmid was transformed in BL21 competent E. coli and GST-Dab1F was induced. After purification of the GST-Dab1 fragment using glutathione sepharose 4B beads (GE healthcare), GST was removed for the injection into rabbit. (B) The purity and quantity of the purified GST-Dab1F and Dab1F was confirmed by SDS-PAGE separation followed by Coomassie brilliant blue staining. Rabbit polyclonal antibodies against the mouse Dab1 fragment were obtained by immunizing rabbits aided by AbClon Inc.



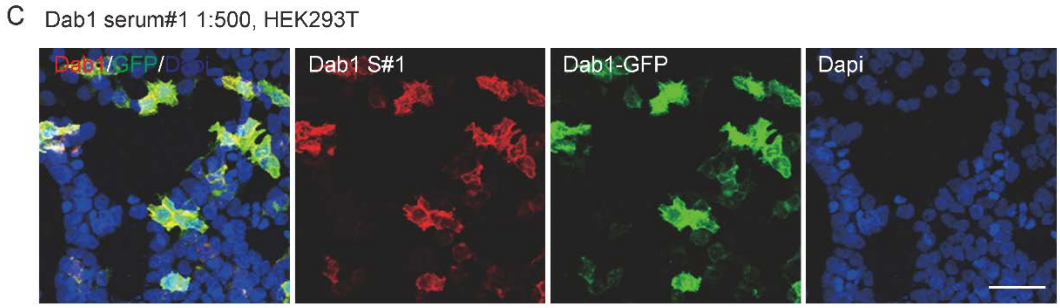


Figure 10. Dab1 protein detection using custom polyclonal antibody

(A, B) Endogenous Dab1 expression in primary cortical neurons was detected using western blotting analysis with custom polyclonal Dab1 antibodies. After 6 h of Reelin supplementation in neurons, Dab1 levels decreased when using purified Dab1 antibodies. (C) Utilizing pCAX Dab1-GFP (Addgene #310139), overexpression of GFP-tagged Dab1 was immunostained with a custom polyclonal Dab1 antibody, and it was confirmed that the signal from the antibody overlaps with the GFP autofluorescence signal. Scale bar, 50 μm.

Part. II Psoralidin decreases the expression of Glutamate decarboxylases and attenuate inhibitory synapse development

1. Psoralidin reduces the expression of GAD67 via transcriptional regulation in primary cortical neurons.

To explore how Psoralidin affects the expression of GADs, we initially treated 7 DIV primary cortical neurons with Psoralidin at the dose of 1, 5, and 10 μM for 24 hours, and analyzed protein levels using western blotting. Psoralidin significantly decreased the levels of GAD67, GAD65, and gephyrin in concentration-dependent manner, whereas the level of VGAT did not change. The relative levels of GAD67 were 84.7 (20.0), 60.7 (11.3), and 48.9 (20.0) at 1, 5, and 10 μM, respectively, compared to control (controls = 100 (20.9)) (Figure 11A, B). The decrease in GAD67 was appeared as early as 6 hours after Psoralidin treatment (Figure 11C) and mRNA level of GAD67 was also significantly reduced at 12 hours after the treatment. The mRNA levels of GAD67 were 0.59 (0.18) at 5 μM and 0.54 (0.28) at 10 μM Psoralidin compared to control (controls = 1.00 (0.07)) (Figure 11D).

To visualize the expression of GAD67 in individual GABAergic inhibitory neuron after Psoralidin treatment, immunofluorescence was performed on primary cortical neurons using antibodies against GAD67, and Map2, a dendritic marker of neurons. Both GAD67 and Map2 positive neurons were considered GABAergic inhibitory neurons. As shown in the western blot analysis above, the GAD67 intensity in GABAergic neurons was significantly reduced under 24 h of Psoralidin treatment ($60.2 \pm 35.6\%$, $p < 0.05$, Student's t-test, Figure 11E and F), while Map2 intensity was unaltered.

Together, this data demonstrated that Psoralidin reduced the expression of GADs in a concentration-dependent manner. The reduced expression of GADs was attributed to transcriptional suppression.

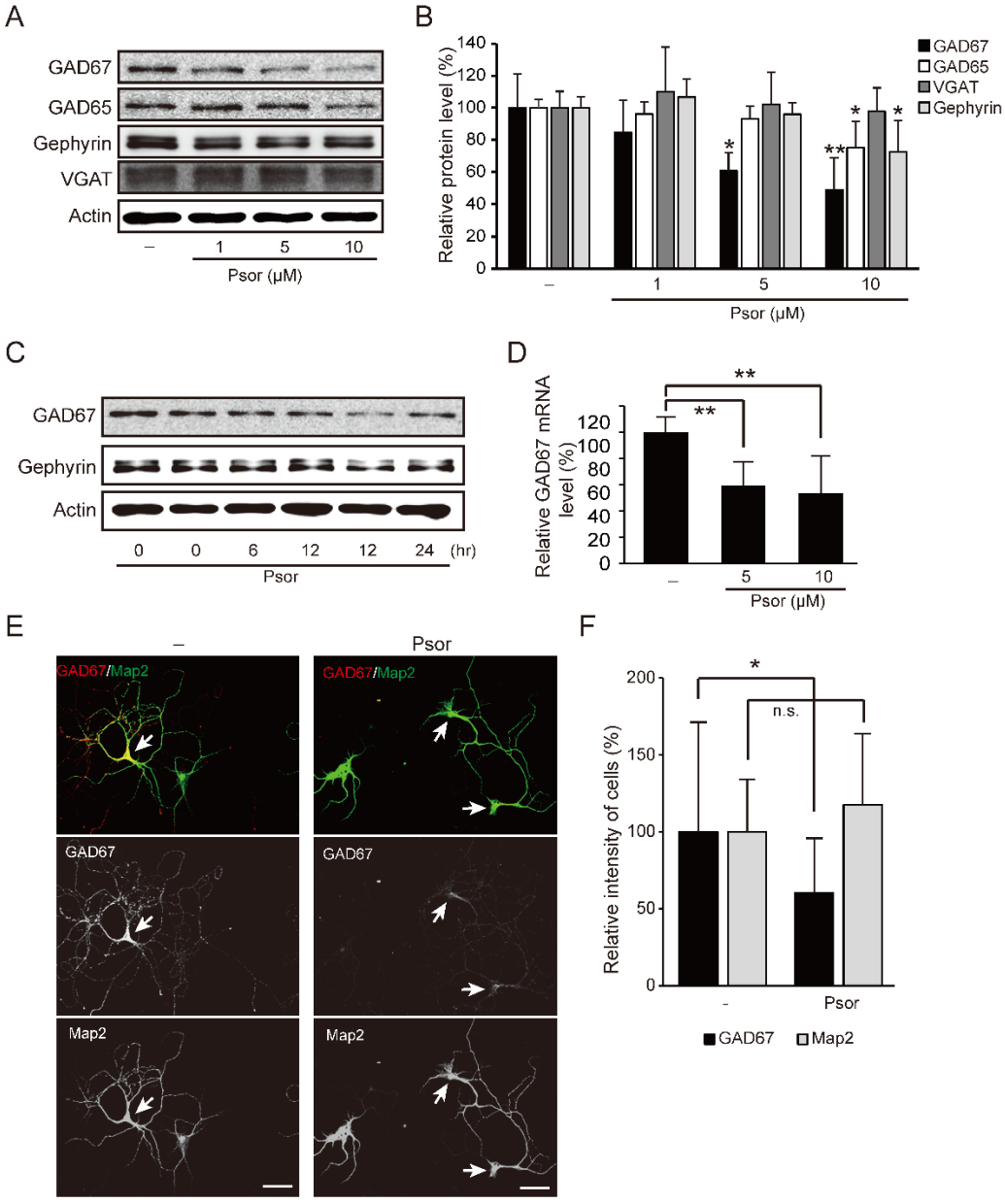


Figure 11. Reduction of glutamate decarboxylase (GAD) expression in primary cortical neurons by Psoralidin

(A, B) 7 Day *in vitro* (DIV) primary cortical neurons were treated with Psoralidin at specific concentrations (1, 5 and 10 μM) for 24 h followed by western blotting for analysis. It significantly reduced the levels of GAD67, GAD65 and gephyrin. However, the levels of VGAT were not changed by Psoralidin. Results were averaged from at least 3 individual experiments and t-tests were performed using Excel. (C) 7 DIV primary cortical neurons were treated with 10 μM Psoralidin at set times (0,6,12 and 24 h) and analyzed by western blot. GAD67 decreased from 6h after Psoralidin treatment. (D) mRNA levels of GAD67 were quantified using quantitative real-time PCR and analyzed using t-test. The mRNA level of GAD67 was also significantly decreased after 12 h of treatment, compared to the control group (control = 1.00 (0.07)) 5 μM Psoralidin was 0.59 (0.18), and 10 μM was 0.54 (0.28). (E) 13 DIV hippocampal neurons were treated with Psoralidin for 24 h. Individual GABAergic inhibitory neurons were imaged by confocal microscopy using the Map2, GAD67 antibody. (F) Quantification of images showing that the fluorescence intensity of GABAergic inhibitory neurons was reduced by Psoralidin treatment. The fluorescence level of GAD67 was significantly decreased in Map2-positive and GAD67-positive neurons. Quantification was analyzed by t-test. The results were analyzed using One-way analysis of variance followed by Newman-Keuls multiple comparison test. *, $p < 0.05$; **, $p < 0.01$, and n.s., not significant. Scale bars, 50um.

2. Decrease in the expression of GAD67 by Psoralidin is not mediated by the NMDA receptor

The expression of GAD67 is transcriptionally regulated by several modulators including glutamate, BDNF, and certain cannabinoids [213, 214]. In particular, binding of glutamate to NMDA receptors has an ambivalent effect on GAD expression in GABAergic neurons. Physiological levels of glutamate released following normal neuronal activity promote the expression of GADs [215], whereas glutamate-mediated excitotoxic insults in neurons causes transcriptional downregulation of *Gad1* and *Gad2* genes [216, 217]. Using western blot analysis, we confirmed that the decrease in the level of GAD67 induced by 20 μM extracellular glutamate for 10 minutes was significantly alleviated by pre-treatment of 10 μM CPP, a NMDA receptor antagonist 12 h after the glutamate exposure (Figure 12A, B and C). The protein levels of GAD67 were 26.2 ± 14.4% at 20 μM glutamate and 83.6 ± 9.8% at 20 μM glutamate pretreated with CPP compared to control (controls = 100 ± 12.7%). It was also shown that the reduction of GAD67 induced by reactive oxygen species, 100 μM hydrogen peroxide or mitochondrial toxin, 20 nM rotenone was mediated by the NMDA receptor (Figure 12A, B, and C). The protein levels of GAD67 were 87.4 ± 20.4% at 50 μM hydrogen peroxide and 61.6 ± 14.8% at 100 μM hydrogen peroxide pretreated with CPP compared to control (controls = 100 ± 10.4%). The protein levels of GAD67 were 105.8 ± 12.7% at 10 nM rotenone and 61.5 ± 10.6% at 20 nM rotenone pretreated with CPP compared to control (controls = 100 ± 8.7%). To investigate whether the NMDA receptor is implicated in Psoralidin-induced repression of GAD67, we pretreated NMDA receptor antagonists, CPP at 10 μM, or MK801 at 1 μM for 30 min and then applied 10 μM of Psoralidin for 12 h to 5

or 6 DIV primary cortical neurons. We found that the decreased level of GAD67 by Psoralidin was not restored at all by the pre-treatment of the NMDA receptor antagonists (Figure 12D, E). The relative protein levels of GAD67 were 57.4 ± 18.9% at 10 μM Psoralidin and 38.9 ± 23.8% at 10 μM Psoralidin pretreated with MK801 compared to control (controls = 100 ± 8.5%). Based on studies that the suppression of GAD67 is mostly mediated by the NMDA receptor [218-221], discovery of the cellular mechanism of Psoralidin-induced GAD67 downregulation will reveal novel pathways for regulating GAD67 expression. Therefore, several chemical modulators were used to screen potential inhibitors of Psoralidin-induced GAD67 repression. However, none of the modulators, including a proteasome inhibitor, a calpain inhibitor, and a CAMK2 inhibitor, were able to reverse the inhibition of GAD67 by Psoralidin. GADs can be cleaved via proteasomal degradation pathway [222, 223] or via μ-calpain activated by increase in intracellular Ca^{2+} concentration [224, 225]. However, A proteasome inhibitor, MG132, or a calpain inhibitor, ALLN was not able to reverse the inhibition of GAD67 by Psoralidin in this study (Figure 12F). It has been reported that Psoralidin binds to estrogen receptors and activates the classic estrogen receptor signaling pathway [226]. Therefore, we used tamoxifen to study whether estrogen receptors are implicated in reduced expression of GAD by Psoralidin. However, tamoxifen did not reverse the effect of Psoralidin on GAD67 expression (data not shown here). Since higher methylation status of *Gad1* promoter has been reported to be related with *Gad1* mRNA [227, 228], we also used hypomethylating agent, 5-Azacytidine, for the inhibitor screening, but it did not change the expression pattern of GAD67 induced by Psoralidin at all (data not shown here). Also, we found that an autophagy inhibitor, chloroquine, did not affect to the expression of GAD67.

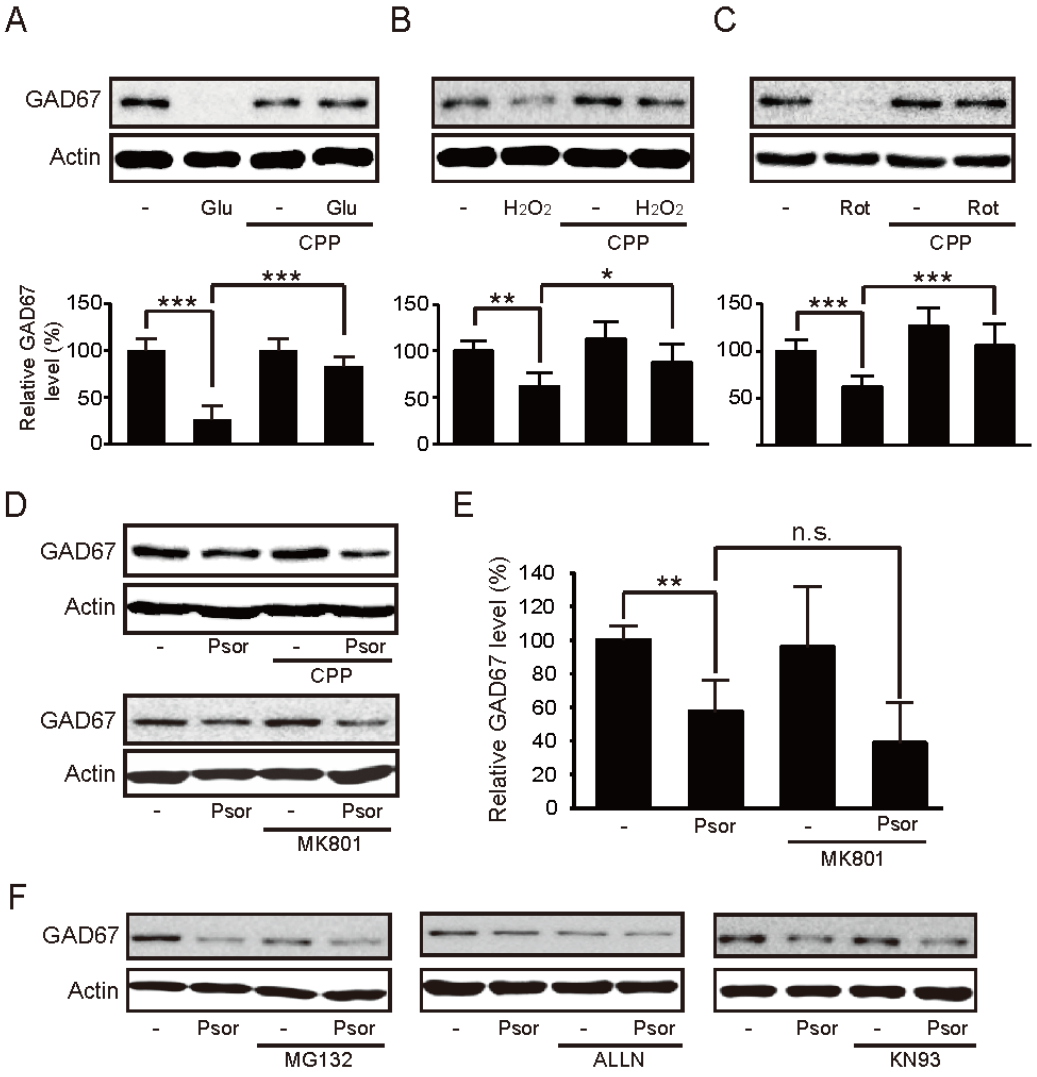


Figure 12. Posralidin-induced reduction of GAD67 was not mediated by the N-methyl-D-aspartate (NMDA) receptor

(A, B and C) 7 days *in vitro* cortical neurons were treated with 20 μM glutamate, 100 μM hydrogen peroxide, or 20nM rotenone for 12 h, pre-treated with or without 10 μM CPP. The relative levels of GAD67 were measured using western blot analysis. The decrease in GAD67 level was significantly alleviated by pre-treatment of CPP. (D, E) 5 or 6 DIV primary cortical neurons were pre-treated with NMDA receptor antagonist, 10 μM CPP or 10 μM MK801 for 30 min, followed by 10 μM of Psoralidin for 12 h. The decreased level of GAD67 by Psoralidin was not restored at all by the pretreatment of the NMDA receptor antagonists. (F) Proteasome inhibitor, MG132, Calpain inhibitor, ALLN, and CAMK2 inhibitor, KN93, were not able to reverse the inhibition of GAD67 by Psoralidin. The results were analyzed using One-way analysis of variance followed by Newman-Keuls multiple comparison test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, and n.s., not significant.

3. Psoralidin reduces the density of inhibitory synapses

To study whether Psoralidin affects the development of inhibitory synapses, inhibitory synapses of 13 days *in vitro* hippocampal neurons were treated with 10 uM Psoralidin for 24 h and were analyzed using immunofluorescence and confocal microscopy. Antibodies against gephyrin, an inhibitory postsynaptic marker, and MAP2, a dendrite marker were used to visualize the morphological differences of inhibitory synapses. The area and strength of the gephyrin cluster on secondary dendrites in neurons remained unchanged after the addition of Psoralidin for 24 h (Figure 13A). However, the density of gephyrin-positive inhibitory synapses was decreased significantly (Figure 13B). This suggests that Psoralidin affects the formation of inhibitory synapses.

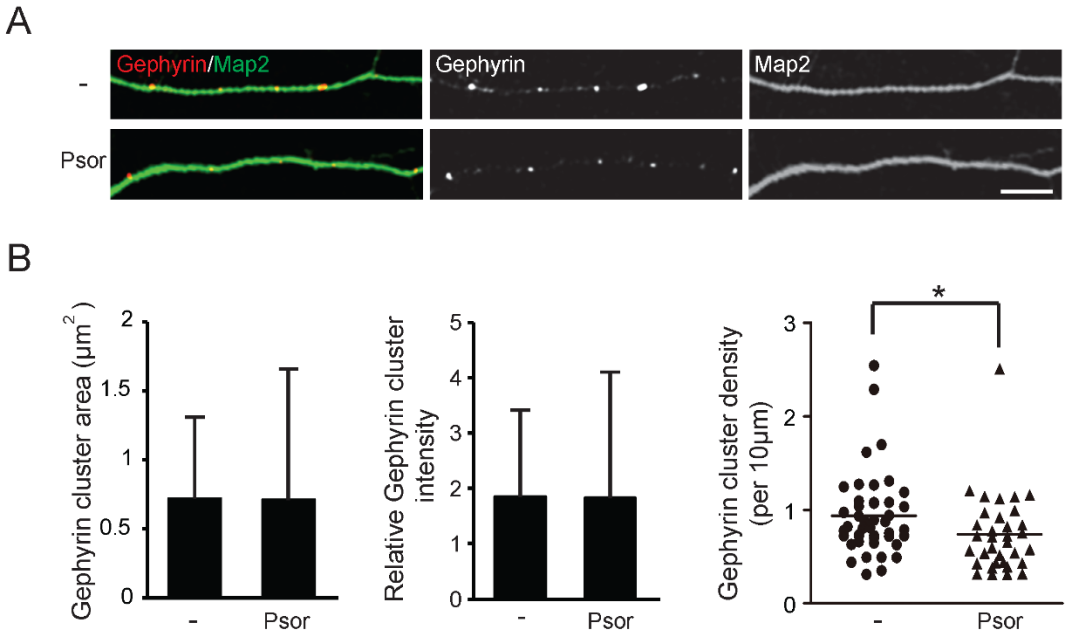


Figure 13. Reduction of inhibitory synaptic densities by Psoralidin

(A) 13 days *in vitro* hippocampal neurons were treated with Psoralidin at 10 μM for 24 h. Map2 and gephyrin antibodies were used to visualize inhibitory synapses using immunofluorescence and a confocal microscope. Gephyrin clusters were measured on secondary dendrites of neurons. (B) Quantification of synaptic gephyrin clusters. The area and strength of the gephyrin cluster on dendrites remained unchanged after the addition of Psoralidin for 24 h. The density of gephyrin-positive inhibitory synapses was decreased significantly. The results were analyzed using Student's t-test. *, $p < 0.05$. Scale bars, 10um.

V. DISCUSSION

Reelin is a critical protein in migration of excitatory neurons during cortex formation; however, it has been reported that it does not directly affect the migration and positioning of inhibitory neurons [229, 230]. Since Reelin is involved in several postnatal processes in the development and function of excitatory neurons, such as dendrite outgrowth, spine formation, and synaptic plasticity [164, 165, 204, 231], it is interesting to study Reelin and the functions of inhibitory neurons. Reelin plays several roles through the activation of the adaptor protein Dab1 and the subsequent PI3K/Akt signaling pathway for dendrite and spine development in excitatory neurons [164, 165, 204].

The canonical Reelin signaling pathway examined using western blotting does not represent the responses of individual neurons to Reelin. Since inhibitory neurons make up approximately 10–20% of all neurons, the distinct responses of inhibitory neurons to stimuli can be masked by the responses of excitatory neurons in western blotting. In this study, an immunofluorescence assay was used to examine Reelin- induced activation of intracellular signaling in individual inhibitory neurons. We found that Reelin-rich protein supplementation did not phosphorylate Akt and S6 protein in most inhibitory neurons. Inhibitory neurons are heterogenous and may respond to Reelin in different ways, depending on the subgroups. In this study, SST-expressing interneurons significantly phosphorylated Akt and S6 under Reelin-rich protein-supplemented conditions, similar to excitatory neurons. It is shown for the first time in this work that Reelin has a differential effect on signaling pathways in certain groups of inhibitory neurons. Because studies on subgroup-specific inhibitory neurons during cortex development are rare, neuronal migration and maturation

of SST-expressing neurons related with Reelin needs to be investigated further *in vivo*. In addition, SST-expressing interneurons have been reported to contribute to hippocampal memory by regulating mTORC1, which is part of an upstream signaling complex involving S6 [232] ; thus, mTORC1 is an interesting target for studying the roles of Reelin and SST-expressing interneurons in learning and memory. Furthermore, since SST-expressing interneurons made up about a quarter of the inhibitory neurons in our culture conditions, we presume that Reelin may not activate the PI3K/Akt/S6 signaling pathway in PV-expressing interneurons, the major subtype of inhibitory neurons.

In this study, we used Reelin-rich protein supplementation. Due to difficulties purifying Reelin, Reelin-conditioned medium has been used to examine the function of Reelin *in vitro* or ex vivo [204, 231, 233]; however, the conditioned medium might have several limitations, including other secreted proteins, degradation, and differing protein levels compared to the control medium. Previous studies have elucidated the new role of purified full-length Reelin in the activation of intracellular signaling pathways, such as MAPK/ERK1/2 [138, 234], which remains unchanged with the Reelin-conditioned medium; therefore, it could be interesting to further investigate the function of Reelin with purification in inhibitory neurons.

Since Dab1 is critical for the activation of the PI3K/Akt/S6 signaling pathway by Reelin, and because its distribution in tissue is restricted [235, 236], it is necessary to investigate the expression of Dab1 in inhibitory neurons to elucidate the cellular mechanisms behind its function; however, due to the limited availability of commercial Dab1 antibodies against mouse species for immunocytochemistry, it is not easy to directly determine the cell specific expression of Dab1 at this point.

Single-neuron transcriptome analyses have shown that in adult mice, the expression patterns of proteins upstream of the Reelin signaling pathway, Dab1, VLDLR, and ApoER2, do not differ between excitatory and inhibitory neurons [237]; however, at postnatal day 0, the transcripts of Dab1 were much lower than those of excitatory neurons, but not those of VLDLR and ApoER2 [238] (http://zylkalab.org/datamousecortex, accessed on: 1 June 2020). Immunofluorescence using the custom ordered Dab1 antibodies may show the expression of Dab1 in individual neurons in further studies.

Despite the lack of response of most inhibitory neurons to Reelin, Reelin-rich protein supplementation reduced the density of inhibitory synapses in excitatory neurons without altering the expression of inhibitory synapse-specific proteins in this study. Previous studies have shown that glutamate receptor activity is required for the development of GABAergic synapses in early developing neurons through interactions between excitatory and inhibitory synaptic machinery [239-242]. Given that Reelin stimulates the formation of dendritic spines and excitatory neurotransmission [165, 231, 234], Reelin may affect the development of inhibitory synapses by modulating glutamatergic activity without direct stimulation of the Akt/S6 signaling pathway in inhibitory neurons. Recent studies have also shown that Reelin reduces the function of presynaptic $GABA_B$ receptors in glutamatergic excitatory neurons [243], and that *Reln* haploinsufficiency leads to immature GABAergic synaptic transmission [179]. This study revealed neuron-specific responses of Reelin, as well as a new function of Reelin in GABAergic inhibitory synapse development, which contributes to the cellular mechanisms underlying Reelin-associated neurological disorders.

Ⅵ. FUTURE DIRECTIONS

1. Differential expression of Reelin signaling machineries according to the type of neurons

The expression of Reelin is spatiotemporally regulated [244]. In the prenatal period, it is produced and secreted from Cajal-Rezius cells in the marginal zone of developing cortex [124, 245]. In postnatal period, Reelin is secreted by groups of inhibitory neurons scattered in the neocortex and hippocampus [124], affecting differentiation and function of neurons. With differential expression and roles of Reelin, distinct cellular machineries may be involved in brain development and function. Since Reelin has shown the differential activation of downstream signaling molecule of PI3K in GABAergic neurons in this study, the expression of the distinct proteins in Reelin signaling pathway such as VLDLR/ApoER2, and Dab1 needs to be investigated depending on the groups of GABAergic neurons and developmental periods. GABAergic inhibitory neurons are highly heterogenous and can be classified into electrophysiological, morphological, and molecularly distinct classes that play highly diverse and specialized roles in shaping network outputs [39].

GABAergic inhibitory neurons constitute between 12% and 25% of all neocortical neurons depending on brain regions. The expression of *Reln* mRNA was found at different rates depending on the subtypes of GABAergic neuron [171] [246]. Most Somatostatin-positive neurons express *Reln* mRNA [247]. On the other hand, *Reln* was expressed in 63% of neuropeptide Y-positive neurons (NPY), 50% of pavalbumin (PV)-positive neurons, and 27% of Vasoactive Intestinal Peptide (VIP)-positive neurons [248]. Since Reelin is

expressed in Cajal-Rezius cells in marginal zone of developing neocortex and has been shown that Reelin does not directly affect the migration of most inhibitory neurons, molecular machineries responding to Reelin does not present or work in migrating GABAergic neurons [249]. However, given that GABAergic neurons are highly diverse and Reelin activates PI3K-Akt-S6 signaling pathway especially in Somatostatin-positive neurons in this study, prenatal behaviors of certain group of GABAergic neurons in developing neocortex can be affected by Reelin. For this study, migration pattern or differentiation of Somatostatin-positive GABAergic neurons can be investigated in GABAergic neuron-specific deletion of *Reeler* mice. After birth, Reelin begin to be secreted from inhibitory neurons and several studies has shown that Dab1, a key adaptor protein in Reelin signaling [129, 134, 165, 209, 212, 250], is present in GABAergic neurons in adult mouse. These studies propose that Reelin's activity in GABAergic neurons may correlated with the time of Reelin secretion from the GABAergic neurons. In addition, because inhibitory synapse formation on the dendrite of excitatory neurons was affected by Reelin in this study, further studies are needed to be done whether Dab1 and its downstream signaling complexes are involved in the development of inhibitory synapses.

2. Finding novel regulatory pathways of GADs expression using chemical modulators

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter, a basic regulator of neural networks during mammalian neurogenesis. GABA signaling regulates neuronal proliferation, differentiation, migration, and neural network wiring in neurodevelopment [251, 252]. Since intracellular GABA levels and GABAergic neurotransmission are correlated with GADs level and activity in neural networks [253, 254], it is important to study the cellular mechanisms that regulates GABA levels. GABA is synthesized from glutamate by the action of glutamate decarboxylases (GAD67 and GAD65) and the coenzyme Pyridoxal Phosphate (PLP) [255].

In this study, we discovered that Psoralidin, a natural compound isolated from the seeds of *Psoralea corylifolia*, reduced the expression of GAD67 and GAD65 through transcriptional regulation in primary GABAergic neurons. The expression of GADs is transcriptionally up-regulated by synaptic transmission mediated by physiological level of glutamate, or neurotropic factor, BDNF [256-258], whereas the transcriptional down-regulation of GADs is mostly seen under glutamate-mediated excitotoxic insults as shown in this study [259] (Figure 12A). Mitochondrial or oxidative stress induces neuronal damage and apoptosis, triggering extra-synaptic glutamate release. In accordance with this, hydrogen peroxide or rotenone induced decrease in GAD67 level, which was alleviated by NMDA receptor antagonist, as shown in this study, (Figure 12B). It has been reported that the survival rate of medium-high concentration of 10 μM Psoralidin was 71.9% in primary cortical neurons [260, 261], which approximately 30% of all neurons were damaged and

extra-synaptic glutamate might be released. Still, reduced expression of GADs by Psoralidin was not mediated by NMDA receptor at all, a major known pathway for GADs regulation [262, 263] . Since we assumed that the discovery of cellular mechanisms of Psoralidin-induced GAD67 down-regulation will reveal novel pathways for regulating GAD67 expression, several chemical modulators were used to screen potential blockers of Psoralidin-induced GAD67 repression. GADs can be cleaved via proteasomal degradation pathway [222, 223] or via μ-calpain activated by increase in intracellular Ca^{2+} concentration [224, 225]. However, using a proteasome inhibitor, MG132, and a calpain inhibitor, ALLN was not able to reverse the inhibition of GAD67 by Psoralidin in this study. It has been reported that Psoralidin binds to estrogen receptors and activates the classic estrogen receptor signaling pathway [226]. Therefore, we used tamoxifen to study whether estrogen receptors are implicated in reduced expression of GAD by Psoralidin. However, tamoxifen did not reverse the effect of Psoralidin on GAD67 expression (data not shown here). Since higher methylation status of *Gad1* promoter has been reported to be related with *Gad1* mRNA [227, 228], we also used hypomethylating agent, 5-Azacytidine, for the inhibitor screening, but it did not change the expression pattern of GAD67 induced by Psoralidin at all (data not shown here). Also, we found that an autophagy inhibitor, chloroquine, did not affect to the expression of GAD67. Further inhibitors or agonists will be screened

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ABSTRACT

The Role of Reelin and Psoralidin in GABAergic Neurons

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The central nervous system generates various neural circuits and regulates circuit activity for rapid and accurate information processing. The excitation and inhibition of neural networks are regulated by the balanced activity of excitatory and inhibitory neurons. In the cerebral cortex, excitatory neurons constitute approximately 80% of total neurons and mainly secrete the neurotransmitter, glutamate. Glutamate binds to the glutamate receptor on the post-synaptic membrane, induces depolarization, and promotes action potential generation in the corresponding neuron. On the other hand, inhibitory neurons, which make up 20% of neurons, mainly secrete Gamma-aminobutyric acid (GABA) that binds to GABA receptors in the post-synaptic membrane. It controls the action potential by inducing hyperpolarization of the target nerve cell membrane potential. The functional disorder of excitatory or inhibitory neurons causes an imbalance in the excitation and inhibition of the

neural network, causing several neurological disorders such as autism, schizophrenia, and depression.

Reelin is a secretory protein involved in a variety of processes in forebrain development and function, including neuronal migration, dendrite growth, spine formation, and synaptic plasticity. *RELN* is a candidate gene for several psychiatric disorders, such as schizophrenia, major depression, and autism spectrum disorder. Intracellular signaling pathways activated by Reelin have been investigated in accordance with its various roles. Reelin binds to apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) to activate C3G/Rap1 for neuronal migration and phosphoinositide 3-kinase (PI3K)/Akt/mTOR for dendrite and spine development. In addition, Reelin has been reported to be involved in synaptic plasticity through the activation of MAPK/ERK1/2 via an unknown receptor. While most of Reelin's functions focus on excitatory neurons and excitatory synapses, little is known about its effects on inhibitory neurons. In this study, we investigated the phosphatidylinositol 3-kinase/Akt pathway of Reelin in primary cortical and hippocampal neurons. Individual neurons were visualized using immunofluorescence to distinguish inhibitory neurons from excitatory neurons. Reelin-rich protein supplementation significantly induced the phosphorylation of Akt and ribosomal S6 protein in excitatory neurons, but not in most inhibitory neurons. In somatostatin-expressing inhibitory neurons, one of major subtypes of inhibitory neurons, Reelin-rich protein supplementation induced the phosphorylation of S6. Subsequently, we investigated whether or not Reelin-rich protein supplementation affected dendrite development in cultured inhibitory neurons. Reelin-rich protein supplementation did not change the total length of dendrites in inhibitory neurons *in vitro*. and, we examined the development of inhibitory synapses in primary hippocampal

neurons and found that Reelin-rich protein supplementation significantly reduced the density of gephyrin–VGAT-positive clusters in the dendritic regions without changing the expression levels of several inhibitory synapse-related proteins. To investigate the mechanism of selective neuronal response to Reelin, the spatio-temporal expression of Dab1, a key adaptor protein of Reelin intracellular signaling, are being examined using custom-ordered Dab1 antibody and immunofluorescence.

Next, the expression mechanism of glutamate decarboxylases (GADs), an essential protein for the synthesis and secretion of GABA, was investigated using Psoralidin to study the factors affecting the function of inhibitory neurons. Psoralidin is a natural compound isolated from the seeds of *Psoralea corylifolia*, and various properties including antioxidant and anti-inflammatory effects have been reported. In this study, the effect of Psoralidin on inhibitory neurotransmission and synaptic-related gene expression in cortical neurons and hippocampal neurons was examined. As a result, Psoralidin decreased the expression of GAD67 and GAD65 proteins in primary neurons. The reduction in GAD expression caused by Psoralidin was not mediated by NMDA receptors, a major known pathway for GADs regulation. Also, Psoralidin delayed the development of inhibitory synapses in primary hippocampal neurons.

In short, this study analyzed the role of Reelin on the neuron type-specificsignaling process and inhibitory synapse development in primary neurons. In order to investigate the mechanism of neuron-selective Reelin signaling, spatio-temproal Dab1 expression need to be studied further. Also, the molecular mechanism of Psoralidin-induced reduction of glutamate decarboxylase needs to be elucidated further. These studies will contribute to elucidating the factors related to the response and function of inhibitory neurons and to

understanding the molecular mechanisms of neurological brain diseases resulting from the imbalance of excitation/inhibition of neural networks.

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Generation of *Sestrin2* knockout cells using CRISPR/Cas9 system

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Abstract - Sestrin2 is a stress-responsive protein whose expression is induced by oxidative or energy stress to regulate various cellular functions including cell survival and energy homeostasis. Sestrin2 plays an important role in balancing cellular redox system via suppressing reactive oxygen species and in regulating adenosine monophosphate-activated protein kinase-mammalian target of rapamycin signaling for optimal cell growth and survival. Although Sestrin2 has been known to be involved in alleviating cell injury and death under a variety of stress and pathologic conditions, molecular mechanism and novel functions of Sestrin 2 needs to be further elucidated. Here, we generated *Sestrin2* knockout HEK-293FT cell line using CRISPR/Cas9 gene editing technique. The cell line might be utilized for multiple studies to elucidate pathophysiological roles of Sestrin2. It would be also possible to screen novel compounds to modulate cell survival in a Sestrin2-dependent manner.

Key words: CRISPR/Cas9, Sestrin2, cell death

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Full Length Article

Hypoxia regulates the level of glutamic acid decarboxylase enzymes and interrupts inhibitory synapse stability in primary cultured neurons

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ABSTRACT

Gamma-aminobutyric acid (GABA) is the main neurotransmitter of inhibitory synaptic transmission, which is critical for oscillatory activity and synchronization of neurons in neural networks. GABA is synthesized by glutamic acid decarboxylase (GAD) enzymes in the inhibitory neuron and, thus, the deregulation of GAD enzymes and subsequent change of GABAergic activity are involved in various neurological and neuropsychiatric diseases. Under hypoxic conditions, neurons undergo neuropathological alterations which can be subtle or severe. Many studies have focused on the alteration of excitatory neurons by hypoxic injury, while inhibitory neuronal changes have not been well determined. Here, we demonstrated that hypoxic conditions decrease the expression of inhibitory neuron-related proteins, including GAD enzymes, through transcript downregulation and proteasomal degradation. Hif-1α induction and glutamate release under hypoxic conditions were implicated in the mechanism of GAD enzyme level reduction. Surprisingly, these conditions altered the density and size of inhibitory synapses, which was irreversible by reoxygenation, but was mediated by glutamate activity. Our findings suggest that potential implication of the compositional and structural alterations of inhibitory neuron in the pathogenesis of various hypoxic injuries.

1. Introduction

The interaction between excitatory neurons and inhibitory interneurons regulates the function of neural networks in the brain through chemical synapses. In the synapses, excitatory synaptic transmission is mediated by the release of glutamate, whereas inhibitory synaptic transmission is mediated by gamma-aminobutyric acid (GABA). Neurons acquire oscillatory activity and synchronization through the function of inhibitory neurotransmission in these circuits, which is implicated in cognitive processes such as information transfer, perception, motor control, and memory. Thus, the compositional and structural alteration of GABAergic circuits influences the balance of excitation/inhibition, which is associated with neurological and neuropsychiatric diseases such as autism spectrum-related disorders, schizophrenia, and epilepsy (Chao et al., 2010; Cline, 2005; Curley et al., 2011; Kaila et al., 2014; Lisman, 2012; Rubenstein, 2010).

GABA, the main neurotransmitter for inhibitory neurotransmission in the cerebral cortex, is synthesized by two distinct glutamic acid decarboxylase enzymes, GAD67 and GAD65 in GABAergic neurons (Ji et al., 1999; Martin and Rimvall, 1993). GAD67 contributes to the production of over 90% of the basal level of GABA throughout the neuron, which is required for the maturation and function of inhibitory circuits in the cerebral cortex (Asada et al., 1996; Esclapez et al., 1994), while GAD65 is transiently activated to supply GABA during dynamic GABAergic synaptic activity (Choi et al., 2002; Hensch et al., 1998; Kaufman et al., 1991; Patel et al., 2006; Tian et al., 1999). The level of GAD67 and GAD65 can be modulated under various neuropathological conditions by glutamate neurotoxicity or BDNF/TrkB (Aguado et al., 2003; Baptista et al., 2010; Betley et al., 2009; Monnerie and Le Roux, 2007; Sanchez-Huertas and Rico, 2011). Thus, various pathological conditions, altering the quantity and function of GAD67 or GAD65, may affect GABAergic synapse formation and transmission, resulting in modification of the circuits of neural networks.

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ORIGINAL PAPER

Psoralidin Stimulates Expression of Immediate-Early Genes and Synapse Development in Primary Cortical Neurons

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Abstract

Upon synaptic stimulation and glutamate release, glutamate receptors are activated to regulate several downstream effectors and signaling pathways resulting in synaptic modification. One downstream intracellular effect, in particular, is the expression of immediate-early genes (IEGs), which have been proposed to be important in synaptic plasticity because of their rapid expression following synaptic activation and key role in memory formation. In this study, we screened a natural compound library in order to find a compound that could induce the expression of IEGs in primary cortical neurons and discovered that psoralidin, a natural compound isolated from the seeds of *Psoralea corylifolia*, stimulated synaptic modulation. Psoralidin activated mitogen-activated protein kinase (MAPK) signaling, which in turn induced the expression of neuronal IEGs, particularly Arc, Egr-1, and c-fos. *N*-methyl-D-aspartate (NMDA) receptors activation and extracellular calcium influx were implicated in the psoralidin-induced intracellular changes. In glutamate dose–response curve, psoralidin shifted glutamate EC_{50} to lower values without enhancing maximum activity. Interestingly, psoralidin increased the density, area, and intensity of excitatory synapses in primary hippocampal neurons, which were mediated by NMDA receptor activation and MAPK signaling. These results suggest that psoralidin triggers synaptic remodeling through activating NMDA receptor and subsequent MAPK signaling cascades and therefore could possibly serve as an NMDA receptor modulator.

Keywords Psoralidin · NMDA receptor modulator · Immediate-early genes · Synaptic plasticity · Learning and memory

Abbreviations

IEGs	Immediate-early genes
MAPK	Mitogen-activated protein kinase
NMDA	*N*-methyl-D-aspartate
ANOVA	Analysis of variance

Introduction

The induction of long-term potentiation (LTP) and long-term memory formation requires rapid synthesis of mRNAs and proteins, which is stimulated by glutamate binding to its receptors and consequent activation of downstream signaling cascades, including Ras-MAPK-ERK1/2 [1–5]. Genes responsible for such proteins, called immediate-early genes (IEGs), have been extensively studied because of their short and rapid transcription in response to synaptic activation and their role in synaptic plasticity and memory formation [6]. While some neuronal IEGs, such as c-fos, Egr-1 and Egr-3 act as transcription factors by regulating the transcription of specific genes involved in structural plasticity, others, such as Arc, Homer-1a, Bdnf and Narp act as effectors as they directly promote plastic changes [7]. In the hippocampus the expression of Arc, c-fos, and Egr-1 are increased rapidly after neuronal activity and during hippocampus-dependent learning processes [8–10]. Mice with c-fos null mutations lacked structural plasticity associated with motor skill learning in the kindling model of epilepsy [11–13]. Arc is abundant in the postsynaptic density and interacts

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Journal of Mushrooms 한국버섯학회지 Research Article

해조류 기반 엘리시터 처리에 의한 꽃송이버섯의 GABA 함량 증가 및 흥분성 신경세포의 수상돌기 발달 억제

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Enhanced GABA content from sodium alginate-induced *Sparassis latifolia* influences dendrite development in primary cortical neurons

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ABSTRACT: *Sparassis latifolia* is a fungus abundant in β-glucan and amino acids and is highly valued as a medicinal mushroom. Among amino acids, γ-aminobutyric acid (GABA) is a free amino acid and has biological effects, such as increase/decrease of hypertension, improvement of cerebral blood flow, and prevention of dementia. In this study, biological elicitors were used to increase bioactive substances as a biofortification method. Sodium alginate extracted from seaweed (*Sargassum horneri, Sargassum fulvellum, Sargassum fusiforme*) were used as the elicitor. The levels of β-glucan and GABA in the mycelium and fruiting body grown by adding the elicitor to the medium were investigated. Addition of sodium alginate positively affected GABA production and negatively affected the β-glucan production in these fungi. Sodium alginates extracted from *S. fulvellum* induced the highest increase in GABA in the mycelium and fruiting bodies. Moreover, we investigated the effects of the extracts from mycelium and fruiting bodies on dendrite development in primary cortical neurons. We found that the extract from the fruiting bodies of sodium alginate treated fungi with increased levels of GABA inhibited the dendrite outgrowth of excitatory neurons, but not inhibitory neurons.

KEYWORDS: *Sparassis latifolia,* Elicitor, GABA, β-Glucan, Primary cortical neurons

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서 론

기억력은 급변하는 현대 생활에서 중요성이 점차적으로 증대되고 있으며, 사회적으로 학습량이 많은 청소년에서부터 노년에 이르기까지 주요 관심 대상이 되고 있다. 이러한 기억력의 저하는 사회적으로 문제가 되고 있으며, 학습과 기억력 감퇴증상이 일차적으로 나타나는 치매 질환은 65세 이상인 사람들의 약 10%, 80세 이상의 노인들에서는 약 50% 이상이 발병하는 것으로 추정되고 있다 (Naveh-Benjamin *et al.*, 2004). 이러한 현상은 노인인구가 빠르게 증가함에 따라 더 급속하게 증가할 것으로 예

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REVIEW

The regulation of glutamic acid decarboxylases in GABA neurotransmission in the brain

Seong-Eun Lee[1] · Yunjong Lee[2] · Gum Hwa Lee[1]

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Abstract Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter that is required for the control of synaptic excitation/inhibition and neural oscillation. GABA is synthesized by glutamic acid decarboxylases (GADs) that are widely distributed and localized to axon terminals of inhibitory neurons as well as to the soma and, to a lesser extent, dendrites. The expression and activity of GADs is highly correlated with GABA levels and subsequent GABAergic neurotransmission at the inhibitory synapse. Dysregulation of GADs has been implicated in various neurological disorders including epilepsy and schizophrenia. Two isoforms of GADs, GAD67 and GAD65, are expressed from separate genes and have different regulatory processes and molecular properties. This review focuses on the recent advances in understanding the structure of GAD, its transcriptional regulation and post-transcriptional modifications in the central nervous system. This may provide insights into the pathological mechanisms underlying neurological diseases that are associated with GAD dysfunction.

Keywords Glutamic acid decarboxylase · GABA · Neurotransmission · Inhibitory neuron · Synaptic plasticity

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GADs in GABA neurotransmission

Neural circuits and networks are based on the interconnection of neurons via synapses. The release of neurotransmitters at the synapse controls the activity of postsynaptic neurons, which are either excitatory or inhibitory. Principally, excitatory synaptic transmission is mediated by glutamate, while most inhibitory transmission is mediated by gamma-aminobutyric acid (GABA) in the brain. GABA functions through binding to specific transmembrane receptors, called $GABA_A$ or $GABA_B$ receptor, clustered on the plasma membrane of the synapse. The binding of GABA to GABA receptors causes the opening of ion channels specific for chloride or potassium ions, typically resulting in a decrease in the transmembrane potential. Thus, GABA reduces the postsynaptic activation and the frequency of action potential firing, and further controls the oscillation and synchronization of neural activities (Ambrad Giovannetti and Fuhrmann 2019). Since GABA plays a key role in the balance of excitation/inhibition (E/I), it is also generally involved in brain plasticity-related processes like learning, memory, and locomotion (Kullmann et al. 2012; Rozycka and Liguz-Lecznar 2017). GABAergic dysfunction is associated with various neurological disorders such as autism spectrum disorders, schizophrenia, and epilepsy as well as neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (Chao et al. 2010; Lisman 2012; Kaila et al. 2014; Ambrad Giovannetti and Fuhrmann 2019; Milosevic et al. 2019). In addition to the function of GABA as a neurotransmitter, GABA also acts as a trophic factor for the regulation of proliferation, neuroblast migration, dendrite growth, and synapse formation during early development (LoTurco et al. 1995; Varju et al. 2001; Represa and Ben-Ari 2005).

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Enhanced phosphorylation of S6 protein in mouse cortical layer V and subplate neurons

Seong-Eun Lee[a], Seojin Hwang[a], Sang-Gun Ahn[b], Gabriella D'Arcangelo[c] and Gum Hwa Lee[a]

The mammalian neocortex is composed of six major layers of neurons. Each group of neurons in the cortical layers has distinct characteristics based on the expression of specific genes and connectivity patterns of neural circuits. Neuronal subtype transition and regional identity acquisition are established by temporal cues and interaction between several transcription factors during neurogenesis. The impairment of cortical lamination or neural circuits results in a wide range of neurodevelopmental disorders such as autism, schizophrenia, and certain forms of childhood epilepsy. Despite continuous efforts to classify neurons with the aid of genetic and epigenetic analyses, the neuron-specific properties associated with post-transcriptional modification remain unclear. In the present study, the distribution of phosphorylated S6-positive layers across the neocortex was examined using several layer markers. The development of pS6 S235/236 layers in layer V and the subplate was spatiotemporally regulated in the mouse brain. In addition, enhanced phosphorylation of ribosomal protein S6 in Ctip2-positive layer V neurons *in vivo* was sustained under in-vitro conditions using a culture of primary cortical neurons. *NeuroReport* 31: 762–769

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Keywords: layer V, neocortex lamination, ribosomal protein S6, subplate

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Introduction

The mammalian neocortex is composed of six major cellular layers (I–VI). In early cortical development, principal neurons are generated from a pool of progenitors through asymmetric or symmetric divisions in the ventricular or subventricular zone. The neurons then migrate to their final destination for proper positioning and subsequent neural circuit formation. Subtype transition and regional identity acquisition are established based on temporal cues and interaction between several transcription factors during neurogenesis [1]. The impairment of cortical lamination or neural circuits may result in a wide range of neurodevelopmental disorders such as autism, schizophrenia, and certain forms of childhood epilepsy [2–4].

Neurons positioned within a distinct cortical layer are characterized by the expression of specific genes and connectivity patterns of neural circuits [5–7]. Recent advances in molecular biology, including large-scale gene expression analysis and single-cell omics, has profoundly improved our understanding of neuronal heterogeneity. However, despite continuous efforts to classify neurons with the aid of genetic or epigenetic analyses, subtype-specific properties associated with post-transcriptional modifications remain unclear. In recent studies, a band of neurons with strongly phosphorylated S6 (pS6) ribosomal protein were detected in cortical layer V [8,9]; however, distinct characteristics of the neurons have not been determined. In the present study, the spatial distribution of pS6 S235/236-positive neurons, the developmental time-course of their expression in the mouse neocortex, and the expression of additional cellular markers were examined in detail. In addition, primary cortical neurons were isolated to show that the enhanced phospho-S6 levels were conserved under neocortical architecture-free in-vitro conditions.

Materials and methods

Tissue preparation

Whole brains were isolated from embryonic day (E) 13.5, 15.5, and 17.5, or postnatal day (P) 0 and 3. The brains were immersed in perfusion solution containing 4% paraformaldehyde (PFA) in PBS overnight at 4°C. Next, the brains were cryoprotected in 30% sucrose in PBS at 4°C overnight. Brains were mounted onto a sliding microtome using optimal cutting temperature (Tissue-Tek) and sectioned in the coronal plane into slices 25–50-μm thick. The 2–5 days *in vitro* (DIV)-dissociated cortical neurons were fixed in 4% PFA/PBS for 15 min.

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Article

Reelin Affects Signaling Pathways of a Group of Inhibitory Neurons and the Development of Inhibitory Synapses in Primary Neurons

Seong-Eun Lee and Gum Hwa Lee *

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Abstract: Reelin is a secretory protein involved in a variety of processes in forebrain development and function, including neuronal migration, dendrite growth, spine formation, and synaptic plasticity. Most of the function of Reelin is focused on excitatory neurons; however, little is known about its effects on inhibitory neurons and inhibitory synapses. In this study, we investigated the phosphatidylinositol 3-kinase/Akt pathway of Reelin in primary cortical and hippocampal neurons. Individual neurons were visualized using immunofluorescence to distinguish inhibitory neurons from excitatory neurons. Reelin-rich protein supplementation significantly induced the phosphorylation of Akt and ribosomal S6 protein in excitatory neurons, but not in most inhibitory neurons. In somatostatin-expressing inhibitory neurons, one of major subtypes of inhibitory neurons, Reelin-rich protein supplementation induced the phosphorylation of S6. Subsequently, we investigated whether or not Reelin-rich protein supplementation affected dendrite development in cultured inhibitory neurons. Reelin-rich protein supplementation did not change the total length of dendrites in inhibitory neurons in vitro. Finally, we examined the development of inhibitory synapses in primary hippocampal neurons and found that Reelin-rich protein supplementation significantly reduced the density of gephyrin–VGAT-positive clusters in the dendritic regions without changing the expression levels of several inhibitory synapse-related proteins. These findings indicate a new role for Reelin in specific groups of inhibitory neurons and the development of inhibitory synapses, which may contribute to the underlying cellular mechanisms of *RELN*-associated neurological disorders.

Keywords: reelin; inhibitory synapse; GABAergic neurons; Akt phosphorylation

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1. Introduction

Reelin is a large glycoprotein that is secreted by Cajal-Retzius cells in the marginal zone during cortex formation in the fetal brain and is then released from a group of inhibitory neurons after birth [1,2]. The Reelin gene (*Reln*) was first discovered as a regulator of neuronal migration during neocortex formation [1,3]. Further studies have elucidated its role in synaptic plasticity, as well as dendrite development and spine formation for excitatory neurotransmission [4–7]; thus, *RELN* is a candidate gene for several psychiatric disorders, such as schizophrenia, major depression, and autism spectrum disorder [3,8–10]. Intracellular signaling pathways activated by Reelin have been investigated in accordance with its various roles. Reelin binds to apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) to activate C3G/Rap1 for neuronal migration and phosphoinositide 3-kinase (PI3K)/Akt/mTOR for dendrite and spine development [3]. In addition, Reelin has been reported to be involved in synaptic plasticity through the activation of MAPK/ERK1/2 via an unknown receptor [4,5,11]. While most of Reelin's functions focus on excitatory neurons and excitatory synapses, little is known about its effects on inhibitory neurons.

감사의 글

2017년... 오랜 진로 고민 끝에 결심한 대학원 진학과 실험실 생활 시작, 그리고 2022년 불안한 마음에 보이지 않던 졸업을 앞두고, 이 학위논문이 무사히 끝맺음을 지을 수 있기까지 도움주신 많은 분들께 진심을 담아 감사의 인사 올립니다.

저의 부족하고 서툴렀던 학위과정내내 가장 가까이에서 흥미로운 연구주제와 세심한 가르침으로 연구에 대한 열정을 키워 주신 이금화 교수님께 깊은 감사드립니다. 감사한 마음을 글로 표현할 순 없지만, 교수님께서 베푸신 은혜와 말씀하셨던 연구자의 덕목들을 잊지 않고 앞으로 연구자의 길을 걸어갈 때 꼭 지키며 실천하겠습니다. 존경하는 교수님께 언제나 행복한 일만 가득하시길 진심으로 바라며 기도하겠습니다. 끊임없는 격려와 애정 가득한 관심을 보내주신 기성환 교수님 지칠 때면 큰 힘이 주셔서 감사합니다. 저의 부족한점에 대해서 현실적인 조언과 공감 해주시는 최홍석 교수님께 감사를 드립니다. 그리고 바쁘신 와중에도 미흡한 제 논문을 심사해주시고 문제점과 미비점을 잡아 주신 이연종 교수님, 김규민 교수님 따뜻한 위로와 격려를 보내주셔서 정말 감사합니다.

실험실 생활을 시작할 때 옆에서 다정하게 대해준 유일한 실험실 선배이자 동생 서진아, 나이 많은 실험실후배 적응시켜주느라 고생 많았어 정말로 고마워. 학위 과정동안 실험기기 이용할 때면 많은 도움 줬던 지현아, 매번 밝게 웃는 얼굴로 친절하게 알려줘서 고마웠어, 그리고 항상 긍정적인 마인드로 모범이 되어준 삼석아, 고민 있거나 힘들 때마다 많은 도움을 줘서 너무 고마웠다. 친동생처럼 아낌없는 충고와 지원으로 챙겨준 경록이형, 해맑게 열렬한 응원을 보내준 태현이, 사소한 궁금증 있을 때 언제든지 답변해주던 범기와 도성이에게 고맙다는 말 전합니다.

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그리고 세상에서 가장 소중하고 우리 이동우 장로님과 우리 김정숙 권사님, 학위과정 동안 철부지 아들을 끝까지 믿음으로 지켜 봐주시고, 한없는 사랑과 희생으로 저를 이끌어 주심에 감사의 말씀드립니다.
언제나 건강하고 화목한 가정안에서 즐거운 일 가득한 날들만 보내길 소망해요. 엄마 아빠 사랑해요.

지면으로 미처 다 말씀드리지 못했지만, 저를 아끼고 격려해주신 모든 분들께 진심으로 감사한 마음을 전합니다. 초심을 잃지 않고, 더욱 정진하는 연구자 이성은으로 성장해 나가도록 노력하겠습니다.