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석사학위 논문

Expression of Immediate-early genes and Modulation of Synaptic Plasticity by Psoralidin

조선대학교 대학원

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초대 신경 세포에서 소랄리딘에 의한 즉각조기발현유전자의 발현과 시냅스 가소성의 조절

2018 년 8 월 24 일

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ABBREVIATIONS

DMSO	Dimethylsulfoxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
Psor	Psoralidin
MAPK	Mitogen-activated protein kinase
ERK1/2	Extracellular signal-related kinase1/2
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
NMDAR	N-methyl-D-aspartate receptor
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
IEGs	Immediate-early genes
LTP	Long-term potentiation
LTD	Long-term depression
Egr-1	Early growth response protein 1
Arc	Activity-regulated cytoskeleton-associated protein
c-fos	AP-1 transcription factor subunit C
PSD 95	Postsynaptic density protein 95
VGLUT 1	Vesicular glutamate transporter 1
MAP 2	Microtubule-associated protein 2
PI3-kinase	Phosphatidylinositol 3-kinase

국 문 초 록

초대 신경 세포에서 psoralidin 에 의한 즉각 조기발현유전자의 발현과 시냅스 가소성의 조절

황 서 진

지 도 교 수: 이 금 화

약학과

조선대학교 대학원

시냅스 가소성은 자극에 반응하여 시냅스의 강도를 조절하거나 새로운 시냅스의 형성 또는 제거를 통하여 신경세포들 사이의 연결을 변형시키는 과정이다. 이러한 시냅스 가소성의 장기간 지속 형태인 Long term potentiation 과 Long term depression 은 동물의 행동학적인 면에서 기억 형성이나 학습에 기본이 되는 요소로 알려져 있다. 흥분성 시냅스에서의 신경 전달 물질인 glutamate 는 시냅스를 활성화 시키고, NMDA 와 AMPA 수용체에 결합하여 시냅스 변형을 유도하는 신호 전달 과정과 여러 가지의 후속 effector 들을 조절한다. 시냅스 가소성의 조절을 통한 기억 형성 과정에서 빠른 mRNA 와

단백질의 합성이 요구되는데, immediate-early genes (IEGs)은 시냅스의 활성에 빠르게 반응하여 발현되기 때문에 시냅스 가소성과 기억 형성 과정에서 IEGs 의 역할이 중요하다고 알려져 있다. 본 연구에서는 *psoralea corylifolia* 의 씨앗으로부터 분리된 천연 화합물인 psoralidin 의 IEGs 의 발현과 시냅스 가소성의 조절에서의 영향에 대해 연구하였다.

먼저, 대뇌 피질 신경세포에서 psoralidin 이 Erk1/2 의 인산화를 통한 Mitogen-activated protein kinase signaling pathway 를 활성화 시키고 Arc, c-fos 그리고 Egr-1 과 같은 IEGs 의 발현을 유도하는 것을 확인하였다. 또한, NMDA receptor 의 활성화와 세포 밖의 칼슘이온의 유입이 이러한 과정에 영향을 주는 것을 확인하였다. 다음으로, psoralidin 이 해마의 신경세포에서 NMDA receptor 를 통하여 흥분성 시냅스의 density, size, area 를 증가시키는 것을 확인하였다.

결과적으로, psoralidin 은 MAPK 와 NMDA receptor 활성화를 통한 시냅스의 변형과 신경세포의 IEGs 의 발현을 유도한다. 이러한 시냅스 기능을 조절하는 새로운 물질의 발견은 약물 표적의 제시와 병리학적 기전을 제시함으로 신경 발달 장애 및 신경 퇴행성 장애와 같은 중추 신경계 질환의 치료제 개발을 위해 중요하다.

I. INTRODUCTION

The neurons are functional units in the central nervous system, communicating each other by forming a neural network. In the neural network, activated neurons transmit electrical signals to other neurons by releasing neurotransmitter into a small gap between the communicating neurons, which is called synapse. Based on the types of neurotransmitters released into the synaptic cleft, synapse can be excitatory or inhibitory for the generation of action potential on the postsynaptic neuron. Also, each synapse undergoes structural modification upon stimuli to strengthen or weaken the connection to rewire the neural networks, which is termed synaptic plasticity. As long-lasting forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) have been proposed as a cellular and molecular mechanism to understand learning and memory in both invertebrate and vertebrate nervous systems [1]. LTP is a long-lasting increase in synaptic transmission via persistent strengthening of synapses, whereas LTD is a long-lasting decrease in synaptic transmission because of weakening of synaptic strength [1, 2]. Therefore, the structural and functional modification of synapses has been studied to elucidate the correlations between synaptic plasticity and memory formation.

Glutamate is one of the major neurotransmitter at excitatory synapses in the central nervous system and binds to its postsynaptic receptor including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate receptors (NMDARs). Upon binding of glutamate, AMPAR is opened for the influx of cations to induce postsynaptic depolarization. The membrane depolarization and glutamate binding to NMDAR cause calcium influx, which initiates LTP through controlling several

processes including aggregation, endocytosis, and trafficking of AMPARs [3-6] and through triggering multiple intracellular downstream signals such as Ras-MAPK-ERK1/2 and p38 pathways that subsequently induce rapid synthesis of specific mRNAs and proteins required for synaptic plasticity [7, 8]. Therefore, the postsynaptic modifications which are modulated by NMDARs and its downstream products have been proposed as the molecular basis of synaptic plasticity [9, 10].

The induction of LTP and long-term memory formation requires rapid synthesis of mRNAs and proteins, which is driven by glutamate binding to its receptors and consequent activation of downstream signaling pathways as mentioned above [11]. Genes responsible for such proteins, called immediate-early genes (IEGs), has been focused because of its short and rapid transcription in response to synaptic activation and its roles in synaptic plasticity and memory formation [12]. Some neuronal IEGs, such as c-fos, Egr-1 and Egr-3, regulate the transcription of specific genes as a transcription factor and some including Arc, Homer-1a, Cox-2 and Narp promote directly plastic changes as an effector [13]. In the hippocampus, a center of memory formation, the expression of Arc, c-fos, and Egr-1 are increased rapidly after neuronal activity and during hippocampus-dependent learning processes [14-16]. c-fos triggers the expression of other genes involved in structural plasticity for long-term synaptic plasticity. Mice with c-fos null mutations lacked structural plasticity associated with motor skill learning in the kindling model of epilepsy [17-19]. Arc is abundant in postsynaptic density and interacts with postsynaptic cytoskeletal proteins involved in AMPARs endocytosis or in actin stabilization [20]. Arc knock-out mice showed normal short-term memory but impaired long-term memory for several learning tasks [21, 22], indicating that Arc is important for long-term synaptic plasticity

and memory formation.

Given increasing demands for therapeutics in disorders of the central nervous system such as neurodevelopmental disorders and neurodegenerative disorders, finding new modulators of synaptic function is important to investigate pathologic mechanisms and suggest biological targets for drug discovery. Through a wide screening of natural compounds, we found out that psoralidin modulates synaptic function in primary cortical neurons and hippocampal neurons. Psoralidin is a natural compound isolated from the seeds of *Psoralea corylifolia*. Several studies have revealed that psoralidin has diverse effects including anti-oxidant, anti-osteoporosis, anti-inflammatory, anti-apoptotic and anti-tumor effect [23-25]. The anti-tumor action of psoralidin at high dosage has been investigated in various cancer cell lines such as androgen-independent prostate cancer cells, human lung cancer cell, and breast cancer cells [25, 26]. Despite various implications and potential therapeutic usage of psoralidin, it has not been demonstrated the effects of psoralidin in the central nervous system. Here, we studied the role of psoralidin in synaptic plasticity-related signaling pathways and gene expression, and synaptic structural modification in primary cortical or hippocampal neurons. We discovered that psoralidin induced the activation of MAPK signaling pathway via Erk1/2 phosphorylation and the expression of IEGs mRNA such as Arc, Egr-1, and c-fos. These phenomenons were initiated by the activation of NMDAR, but not AMPAR, and extracellular calcium influx. Furthermore, psoralidin increased the density of excitatory synapses in primary hippocampal neurons, which were mediated by NMDAR. This study demonstrates that psoralidin triggers synaptic remodeling through the MAPK activation and the induction of neuronal IEGs suggesting a novel role of psoralidin as a NMDAR modulator.

II. MATERIALS AND METHODS

1. Reagents and Antibodies

The primary antibodies were rabbit anti-phospho-Erk1/2 (Cell Signaling, #9101), rabbit anti-Erk1/2 (Cell Signaling, #9102), rabbit anti-phospho-Akt (Cell Signaling, #4060), chicken anti-Map2 (Abcam, ab5392), mouse anti-PSD 95 (Santa Cruz Biotechnology, sc-32290), rabbit anti-VGLUT1 (Synaptic Systems, 135 302) and mouse anti- β-actin-HRP (Sigma, A3854). The secondary antibodies were HRP-conjugated anti-rabbit and anti-mouse (Invitrogen, G21040, G21234), antibodies conjugated to AlexaFluor 488 goat anti-chicken (Invitrogen, A11039), AlexaFluor 555 goat anti-mouse (Invitrogen, A21422) and AlexaFluor 647 goat anti-rabbit (Invitrogen, A21244). MK-801 (M107), PD 0325901 (PZ0162), LY294002 (L9908), Thiazolyl Blue Tetrazolium (M2128), CNQX (C239), nifedipine (N7634) and EGTA (E3889) were purchased from Sigma-Aldrich. BAPTA-AM (B1205) was purchased from Invitrogen.

2. Primary neuronal culture

Cerebral cortices and hippocampi were dissociated from the brain of embryonic day (E) 15.5 ICR mice. The dissociated cerebral cortices and hippocampi were digested with 0.25% Trypsin (Gibco) /DNase I (Sigma) at 37°C for 20 minutes. Trypsin was inactivated using Fetal Bovine Serum. Cells were placed on poly-L-lysine (Sigma) pre-coated plates and cultured in Neurobasal medium supplemented with 2% B27 supplement, Glutamax, and penicillin/streptomycin (Invitrogen). The neurons were maintained at 37°C in a saturated atmosphere containing 95 % air and 5 % CO_2, changing medium completely a day after of

isolation and then changed the half of the medium with fresh medium every 3-4 days.

3. MTT assay

To analyze cytotoxicity, 5-6 days in vitro (DIV) neurons were plated at a density of 0.5 x 10^6 cells/well in 12 well plates were incubated with Psoralidin (1, 5, 10 μM) at 37°C for 24 hour. After treatment, viable cells were stained with MTT at 0.3 mg/mL and incubated at 37°C for 2 hours. The media were then removed and any formazan crystals produced in the wells was dissolved with the addition of 500 μL of dimethyl sulfoxide (DMSO). Absorbance at 550 nm was measured using an enzyme-linked immunosorbent assay microplate reader (spectra MAX, Molecular Device, Sunnyvale, CA). Cell viability was defined relative to the untreated control [i.e., viability (% control) = 100 x (absorbance of treated sample) / (absorbance of control)].

4. RNA isolation and RT-PCR analysis

Total RNA was extracted using GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. To generate cDNA, total RNA (0.5-1 μg) was reverse-transcribed using iScript^{TM} cDNA Synthesis Kit (Bio-rad). The mRNA quantification was performed by StepOne real-time PCR system (Applied Biosystems) using a SYBR Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems). Transcript-specific primers used in the quantitative RT-PCR were: Arc, F: 5'-AAGTGCCGAGCTGAGATGC-3', R: 5'-CGACCTGTGCAACCCTTTC-3', Egr-1, F: 5'-TCGGCTCCTTTCCTCACTCA-3', R: 5'-CTCATAGGGTTGTTCGCTCGG-3', c-Fos, F: 5'-CGGGTTTCAACGCCGACTA-3', R: 5'-TTGGCACTAGAGACGGACAGA-3', GAPDH, 5'-AGGTCGGTGTGAACGGATTTG-3', R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene for normalization. The relative quantification of the mRNA was calculated using the Pfaff1 method [26].

5. Western Blot Analysis

Cortical neurons were lysed with Radioimmunoprecipitation assay buffer (RIPA). The protein were separated using 8 % SDS-PAGE gels and transferred to a 0.22-um nitrocellulose (NC) membrane. The membrane was incubated in 5% skim milk blocking buffer for 1 hour at room temperature and then, was incubated with primary antibody at 4°C overnight. After washing with Tris-buffered saline and Tween 20, a secondary antibody was added to the membrane for 1 hour at room temperature. Membrane were developed with western blot ECL solution (Supex). β-actin was used as immunoblotting controls. More than three separate experiments were performed with different lysates to confirm the changes in the protein levels.

6. Immunofluorescence

Dissociated hippocampal neurons were grown on glass coverslips coated with poly-L-lysine. The neurons were fixed in 4 % paraformaldehyde (PFA)/phosphate buffered saline (PBS) for 15 minutes, and then permeabilized by 0.1 % Triton X-100/PBS for 10 minutes. The neurons were incubated with 10 % normal goat serum (NGS) blocking buffer for 1 hour at room temperature. The neurons were incubated with mouse anti-PSD95, and rabbit anti-VGLUT1, and chicken anti-MAP2 antibody at 4°C overnight. After washing with PBS, the neurons were incubated with secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 647 or AlexaFluor 555 (Invitrogen) for 1 hour at room temperature. The neurons were washed with

PBS 3 times for 5 minutes and then were mounted on positively charged glass slides with mounting media (Thermo Scientific, 9990402). The neurons were imaged using a Nikon A1 confocal laser microscope system.

7. Quantification of PSD 95/VGLUT1-positive clusters

The density, area, and intensity of VGLUT1-positive PSD 95 clusters were measured from images of secondary dendrites of excitatory hippocampal neurons using the ImageJ program and NeuronJ plugin. The background intensity of each channel was subtracted and the function Adjust/Threshold in ImageJ was applied to each image with the same condition for quantifying cluster size. Fifty to one hundred dendritic fields were analyzed from randomly selected pyramidal neurons in each experiment. Density was calculated (mean±s.e.m.) as the number of clusters per 100 um^2 of dendritic length. All analyses were performed by an experimenter blinded with respect to treatment.

8. statistical analysis

Results are presented the data as mean ± standard deviation (SD), and analyzed using Student's t-test of ANOVA, followed by the Dunnett's post-hoc tests or Newman-Keuls' post-hoc tests, as appropriate per experiment. The results were averaged from multiple experiments (n), which were several wells over 3 different cultures, as indicated in the figure legends. The results were considered statistically significant when the *p* values were less than 0.05.

III. RESULTS

1. Identification of modulators that induce the expression of immediate-early genes by screening natural compounds in primary cortical neurons.

To find synaptic modulators which activate MAPK pathway to induce the expression of immediate-early genes, we screened a natural compound library (Fig. 1A). First, we screened 436 natural compounds in a human neuroblastoma cell line, SH-SY5Y, which is used as *in vitro* models for neuronal function and differentiation, by assaying the activity of MAPK signaling pathway via Erk1/2 phosphorylation. Also, it has been reported that glutamate receptors are present in several neuroblastoma cell lines [28, 29]. We treated the SH-SY5Y cells with each compound at 5 μM for 30 minutes and then, lysed the samples with RIPA buffer. We analyzed the level of phospho-Erk1/2 using western blot analysis. In this experiement, we found that 86 compounds increased the level of phospho-Erk1/2 more than three times as much as control treatment in SH-SY5Y cells (Fig. 1B). To investigate whether 86 compounds increase the activity of Erk1/2 in primary cortical neurons, we conducted the western blot analysis after treating each of the 86 compounds at 5 μM for 30 minutes in 5-6 DIV primary cortical neurons. As a result, we found that 41 selected primary active compounds increased the activity of Erk1/2 in primary cortical neurons (Fig. 1C). In respond to synaptic activation and consequent signaling cascade activation such as MAPK or PI3K, immediate-early genes (IEGs) are rapidly transcribed and might play an important role in synaptic plasticity and long-term memory formation [30-33]. We treated 7 DIV primary cortical neurons with the 41 selected actives at 5 μM for 1 hour, to examine the transcripts level of neuronal IEGs such as Arc and Egr-1 using quantitative RT-PCR. Thus, we finally determined that 19 active compounds induced the activation of MAPK signaling pathway and the expression of

neuronal IEGs in primary cortical neurons (Fig. 1D).

Considering supply and properties of the active compounds, we chose one final active compound, psoralidin, to study further for the modulation of synaptic plasticity. Psoralidin is a coumestan analogue isolated from the seeds of *Psoralea corylifolia* and has various biological activities such as anti-inflammatory, anti-oxidant, anti-osteoporosis, and anti-cancer effects [23-25] (Fig. 2). Despite potential biomedical activities and a recent report about the effect of total prenylflavonoids including psoralidin from *psoralea corylifolia* on age-related cognitive deficits, any study has not been performed for the role of psoralidin in the modulation of brain function. The distribution of psoralidin in central nervous system has been described that psoralidin might be absorbed slowly into plasma after oral administration, but could be accumulated longer in the central nervous system due to its long half-life [34]. Here, we investigated the effect of psoralidin in the activation of synaptic plasticity-related signaling pathway, genes and further synapse structural modification *in vitro* using primary mouse cortical and hippocampal neurons.

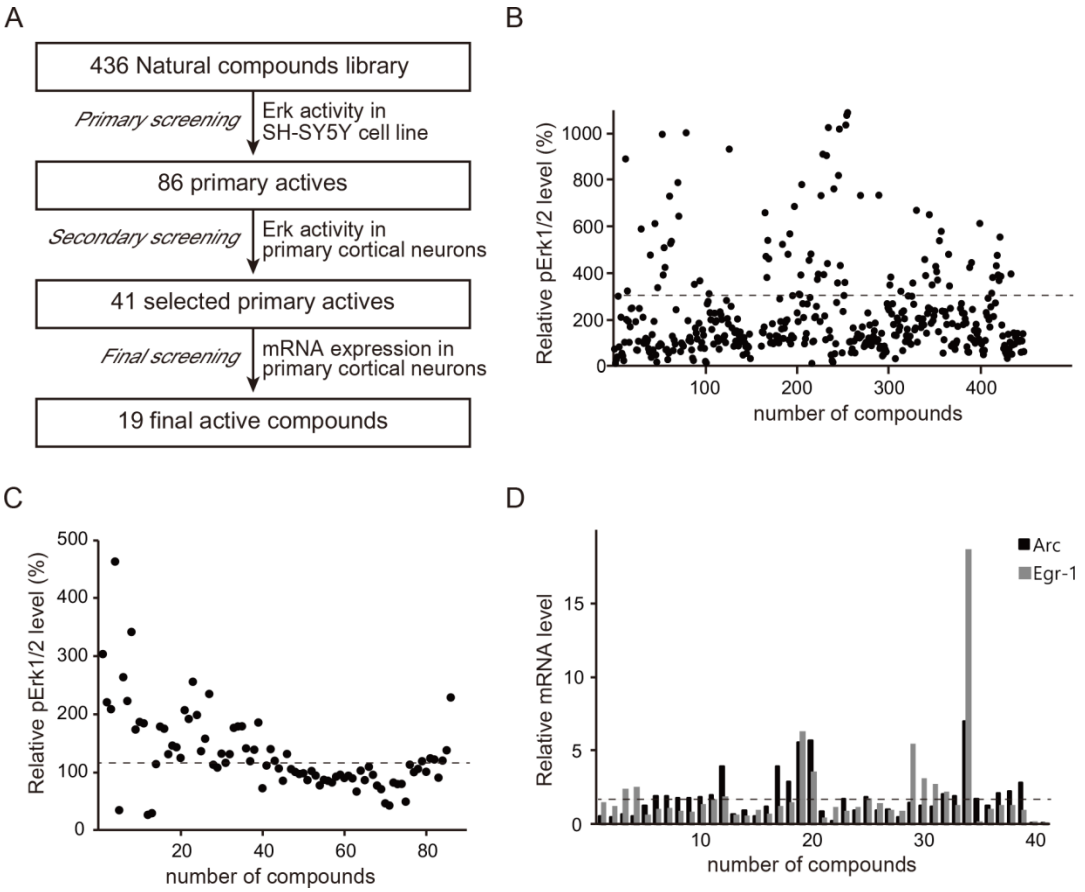
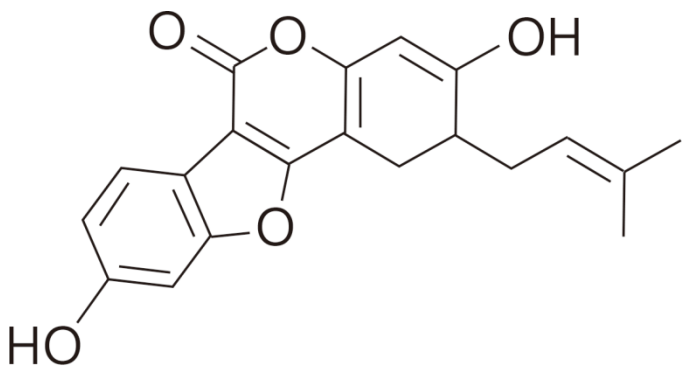


Figure 1. Identification of active compounds that increase the activation of Erk and induce the expression levels of IEGs by screening.

(A) Screening process in SH-SY5Y cell lines and primary cortical neurons. (B) SH-SY5Y cells were treated with natural compounds, at 5 μM and assayed by western blot analysis. 86 compounds increase the activity of Erk1/2 in SH-SY5Y cells. (C) Five to six DIV primary cortical neurons were treated with 5 μM primary active compounds and assayed by western blot analysis. 41 compounds increase the activity of Erk1/2 in primary cortical neurons. (D) Seven DIV primary cortical neurons were treated with 5 μM selected primary active compounds and the mRNA contents were analyzed using quantitative RT-PCR. Arc and Egr-1 transcripts of 19 final active compounds were increased.



Psoralidin

Figure 2. Structure of psoralidin.

Psoralidin is a coumestan analogue isolated from the seeds of *Psoralea corylifolia*.

2. Psoralidin increases the activity of Erk1/2 and the expression of IEGs in primary cortical neurons.

To explore how psoralidin increase the activity of Erk1/2 signaling proteins in primary cortical neurons, we performed western blot analysis using specific antibodies including phospho-Erk1/2, total-Erk1/2 and actin for normalization. We treated 5-6 DIV primary cortical neurons with 1, 5, 10, 20 μM of psoralidin for 30 minutes and then, lysed the samples with RIPA buffer. We found that the levels of phospho-Erk1/2 in primary cortical neurons were significantly increased in a concentration-dependent manner by psoralidin (Fig. 3A and 3B). Psoralidin resulted in about 1.7-2 fold increase in Erk1/2 phosphorylation at 5 μM (Fig. 3B and 3D). Since psoralidin has been reported for its anti-cancer effect at high dosage in various cancer cell lines [25, 26], we performed MTT assay and for further analysis, determined the concentration of psoralidin as 5 μM at which psoralidin showed profound Erk1/2 activation without affecting neuron's survival (Supplementary Fig. 1, cell survival at 5 μM of psoralidin, 96.6 ± 10.6%). The level of phospho-Erk1/2 by 5 μM of psoralidin was highest by 30 minutes after psoralidin treatment (Fig. 3C and 3D) and got lowered to basal level by 12 hours after the treatment (data not shown). However, the phosphorylation level of Akt at Serine473 was never elevated at 5 μM of psoralidin during 12 hours after treatment (Fig. 3A, 3B, 3C, and 3D). At higher dose (10 μM or 20 μM) psoralidin even slightly decreased the level of phospho-Akt at Serine473 (Fig.1A and 1B, 10 μM, 87.6 ± 6.5%; 20 μM, 82.3± 11.5%). To investigate whether activation of Akt by psoralidin is masked by basal synaptic activities, we pre-treated the cortical neurons with 20 μM CNQX and 10 μM nifedipine, which block non-NMDA-type glutamate receptor and L-type voltage-gated calcium channels. However, 5μM of psoralidin didn't affect the level of phospho-Akt in CNQX and nifedipine-pretreated cortical neurons

(Supplementary Fig.2). These results indicate that psoralidin activates specifically Erk1/2 signaling cascade unlike NMDA and other neurotropic factors such as BDNF or Reelin [35-37], and also suggest that the activated Erk1/2 signaling pathway by 5 μM psoralidin might not interact with PI3K pathway.

To examine how psoralidin affects the expression of IEGs, we treated 7DIV primary cortical neurons with psoralidin at 1, 5, 10 μM for 1 hour, and analyzed the mRNA level of several IEGs using quantitative RT-PCR. While transcripts levels of some neuronal IEGs such as Bdnf or Homer1a were not changed, Arc, Egr-1 and c-fos mRNA levels were significantly increased in a concentration-dependent manner when treated with psoralidin (Fig. 3E). The relative transcripts levels compared to DMSO treatment as control were Arc, 200 ± 79%, 219 ± 104%, 182 ± 74%; Egr-1, 166 ± 77%, 352 ± 80%, 398 ± 151%; c-fos, 342 ± 298%, 550 ± 392%, 754 ± 669% at 1, 5, 10 μM, respectively.

Together, our data demonstrate that psoralidin increase the activity of Erk1/2 via phosphorylation, but not Akt phosphorylation, in a concentration-dependent way with maximal increase by 30 minutes. Also, the expression of neuronal IEGs including Arc, c-fos and Egr-1 was significantly induced by psoralidin, which proposes that psoralidin might stimulate activity-dependent IEGs expression with Erk1/2 activation *in vitro*.

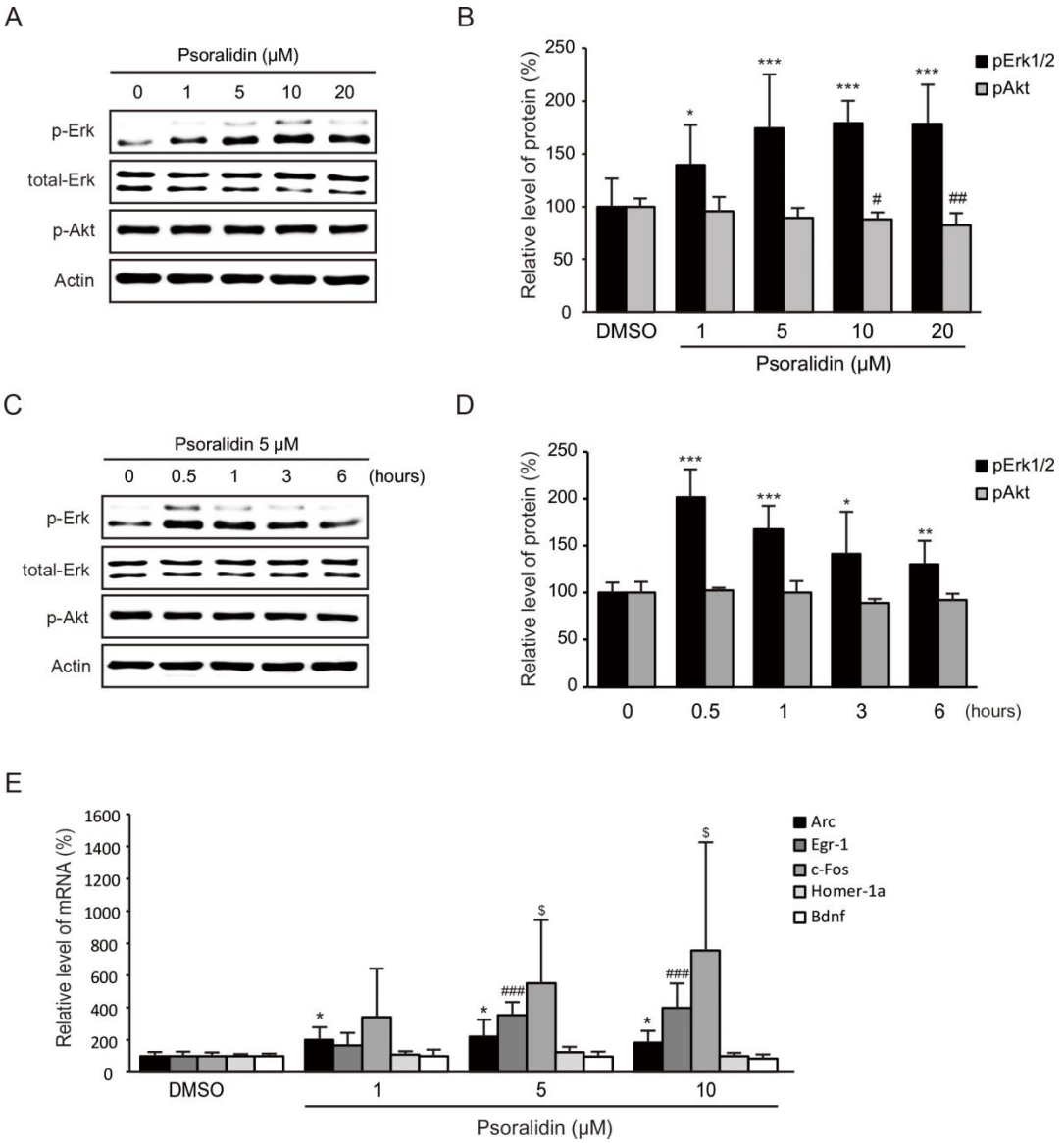


Figure 3. Increase in the level of phospho-Erk1/2 and in the expression of IEGs transcripts by psoralidin.

(A) Five to six days in vitro (DIV) primary cortical neurons were treated with psoralidin at the designated concentrations (1, 5, 10 μM) for 30 minutes and assayed using western blot analysis. The levels of Erk1/2 phosphorylation were increased in a concentration dependent manner. However, the levels of phospho-Akt were not increased by psoralidin. (B) The results were averaged from at least three individual experiments and analyzed using one-way ANOVA, followed by Dunnett's post-hoc tests (n=4). (C) Five to six DIV primary cortical neurons were treated with 5 μM psoralidin at the designated times (0.5, 1, 3, 6 hours) and analyzed by western blot. The level of Erk1/2 phosphorylation was maximized at 30 minutes, and, the levels were decreased over time. (D) The results were averaged from at least three individual experiments and analyzed using one-way ANOVA, followed by Dunnett's post-hoc tests (n=4). (E) Seven DIV primary cortical neurons were treated with psoralidin at the designated concentrations (1, 5, 10 μM) for 1hour and the IEGs mRNA contents were analyzed using quantitative RT-PCR. Arc, c-fos and Egr-1 transcripts were significantly increased by psoralidin, while, Bdnf and Homer1a transcripts were not elevated by psoralidin. Error bars represent standard deviation (SD). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and # $p< 0.05$; ##$p < 0.01$; ###$p < 0.001$, \$ $p < 0.05$; \$\$ $p < 0.01$; \$\$\$ $p < 0.001$.

Supplementary Data

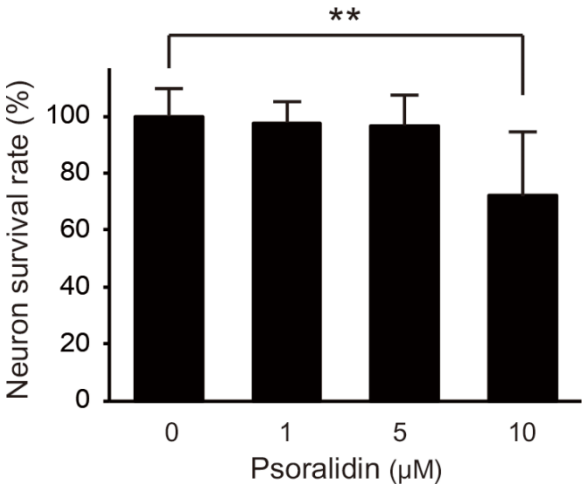


Figure 1. Survival rate of primary cortical neurons treated with psoralidin.

No significant change of survivals was seen upto 5 μM of psoralidin. However, the survival rate of cortical neurons was reduced significantly when treated with 10μM of psoralidin (71.9 ± 22.4% compared to DMSO treatment). The result was analyzed by student's t-test. Error bars represent standard deviation (SD). **, $p < 0.01$.

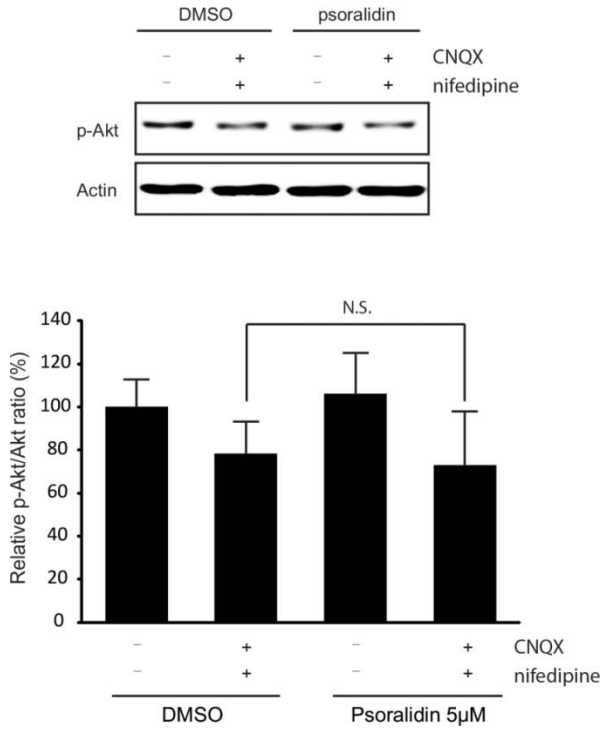


Figure 2. No change of Akt activation at Serine 473 by psoralidin under reduced basal synaptic activities.

Primary cortical neurons were pre-treated with 20 μM CNQX and 10 μM nifedipine for 30 minutes, which block non-NMDA-type glutamate receptor and L-type voltage-gated calcium channels. Psoralidin at 5μM didn't increase the level of phospho-Akt in CNQX and nifedipine-treated cortical neurons. The result was analyzed by student's t-test. Error bars represent standard deviation (SD). N.S., nonsignificant.

3. Implication of NMDA receptor activation and extracellular calcium influx in the IEGs expression and MAPK signaling pathway activation by psoralidin.

To study whether MAPK pathway or PI3-kinase pathway is implicated in the Erk1/2 activation by psoralidin, we pre-treated MEK inhibitor, PD0325901, at 10 μM or PI3-kinase inhibitor, LY294002, at 10 μM for 30 minutes and then, applied 5 μM psoralidin for 30 minutes to the 5-6 DIV primary cortical neurons. Using western blot analysis, we found that the increased level of Erk1/2 phosphorylation by psoralidin was completely abolished by PD0325901, but was not significantly reduced by LY294002 (Fig. 4A and 4B). Also, we found out that the expression of IEGs such as Arc, c-fos and Egr-1 induced by psoralidin was totally blocked by pre-treatment of PD0325901 (Fig. 4G).

To demonstrate whether activation of NMDA receptor is implicated in Erk1/2 activation and IEGs expression by psoralidin, we pre-treated NMDA glutamate receptor antagonist, MK-801, at 1 μM for 30 minutes, followed by treatment of 5 μM psoralidin for 30 minutes. Western blot analysis showed that the increment of Erk1/2 phosphorylation by psoralidin was significantly reduced when MK-801 was pre-treated (Fig. 4C and 4D). These data was in accordance with the data from quantitative RT-PCR that psoralidin-induced IEGs expression was abolished by MK-801 pre-treatment (Fig. 4H). Since the activation of NMDA receptors cause the influx of extracellular calcium ions to initiate intracellular downstream signaling cascade, we assayed whether the influx of calcium ion is necessary for psoralidin-induced Erk1/2 activation. Using EGTA or BAPTA-AM which chelates extracellular calcium ions or intracellular calcium ions, respectively, we performed western blot analysis to see the level of phosphorylated Erk1/2. The up-regulated level of phospho-Erk1/2 caused by psoralidin was

abolished in the presence of EGTA in culture medium at 2 mM for 30 minutes (Fig. 4E and 4F), but not in the presence of BAPTA at 10 μM.

Taken together, our data demonstrate that psoralidin increase the activation of Erk1/2 and the expression of IEGs through NMDA receptor activation followed by calcium influx. Thus, psoralidin may work as an agonist or a modulator of NMDA receptor activity to influence synaptic activities.

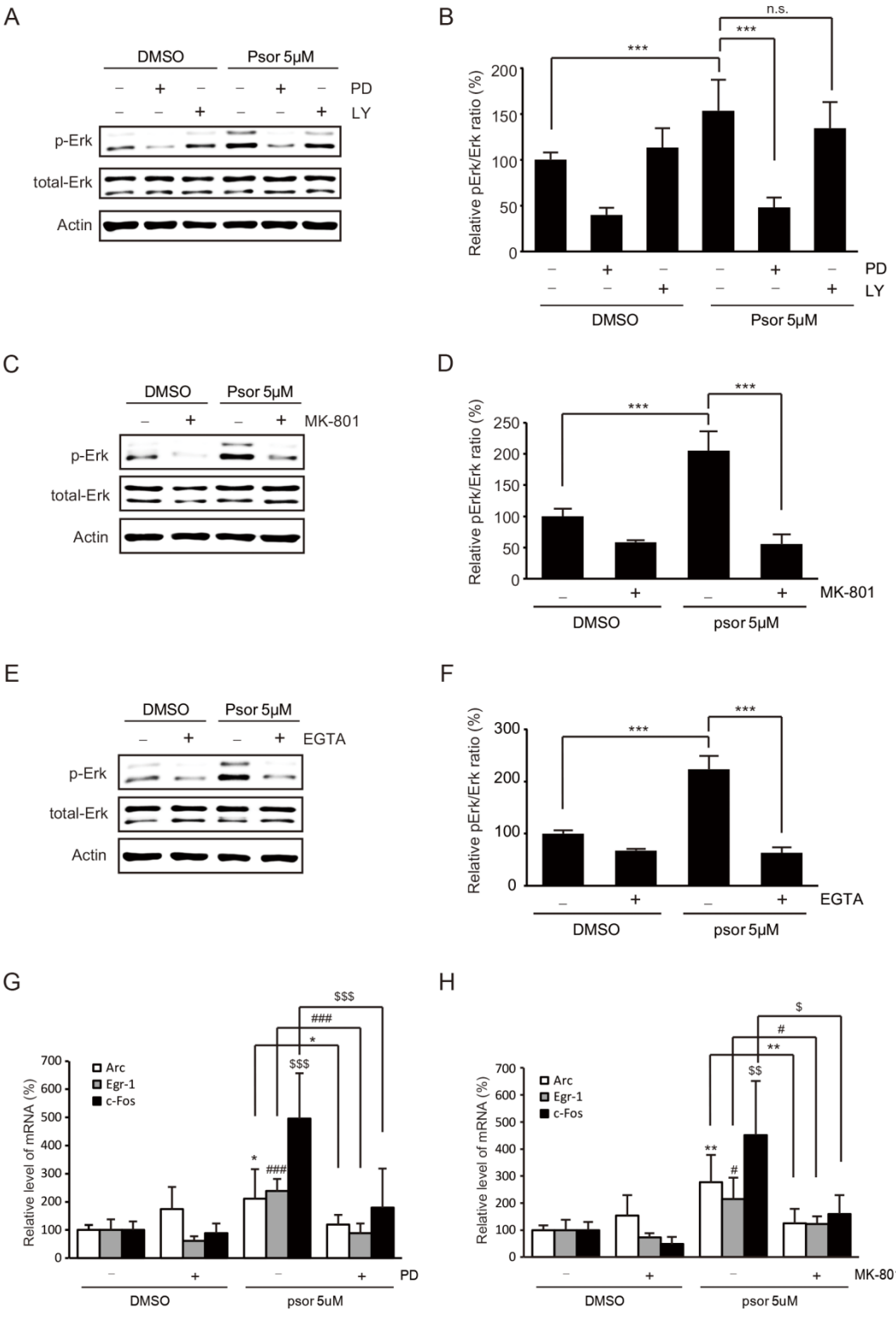


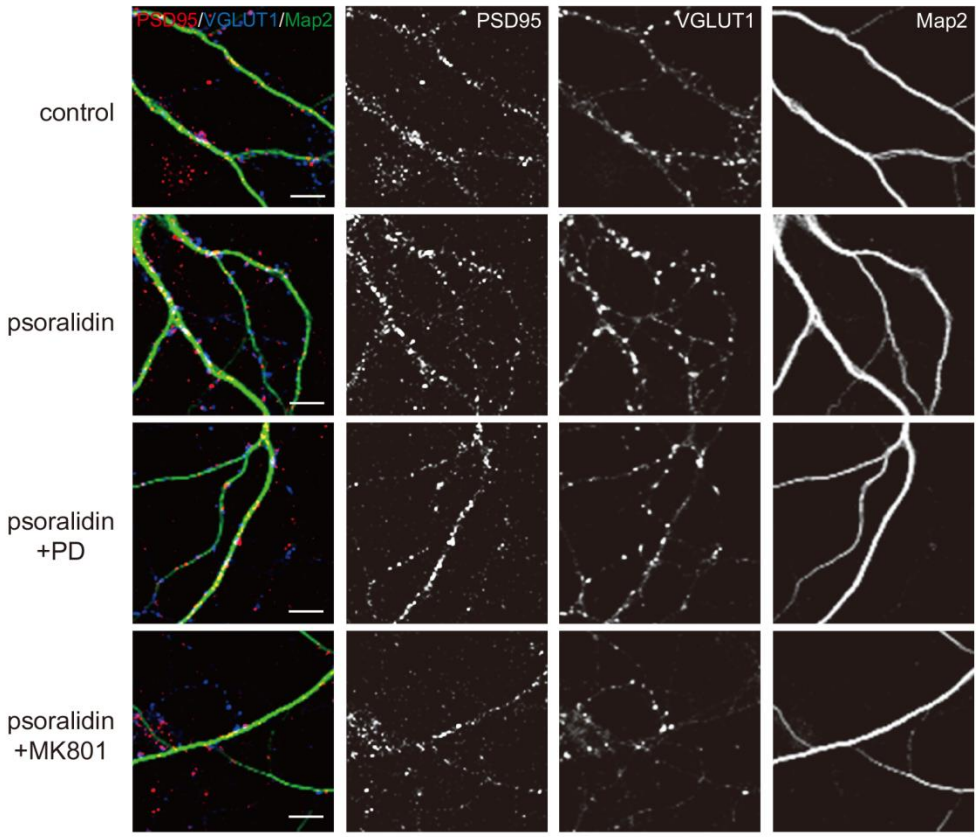
Figure 4. NMDA receptor- and extracellular calcium influx-mediated MAPK pathway activation and IEGs expression by psoralidin.

(A) The increment of Erk1/2 phosphorylation by psoralidin was significantly reduced in 5-6 DIV primary cortical neurons pre-treated with PD0325901 10 μM, but not with LY294002 10 μM. (B) The results were averaged from four individual experiments and analyzed using Student's *t*-tests (n=4). (C) The increased level of phospho-Erk1/2 by psoralidin was significantly reduced in 5-6 DIV primary cortical neurons when pre-treated with MK-801 at 1 μM. (D) The results were averaged from four individual experiments and analyzed using Student's *t*-tests (n=4). (E) The increment of Erk1/2 phosphorylation by psoralidin was significantly reduced in 5-6 DIV primary cortical neurons pre-treated with EGTA 2 mM. (F) The results were averaged from three individual experiments and analyzed using Student's *t*-tests (n=3). (G) The expression of IEGs such as Arc, c-fos and Egr-1 induced by psoralidin was totally blocked in 7 DIV primary cortical neurons when pre-treated with PD0325901 10 μM. (H) Psoralidin-induced IEGs expression was significantly reduced in 7 DIV primary cortical neurons when pre-treated with MK-801 1 μM. Error bars represent standard deviation (SD). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, $ $p < 0.05$; $$ $p < 0.01$; $$$ $p < 0.001$ and n.s. nonsignificant.

4. Structural modification of excitatory synapses by psoralidin.

Since the activation of NMDAR and the expression of IEGs are critical for the modulation of synaptic plasticity, we investigated the effect of psoralidin on the structural alteration of excitatory synapses using immunofluorescence and confocal imaging system. We visualized the excitatory synapses using antibodies of postsynaptic density marker, postsynaptic density protein 95 (PSD95); presynaptic marker, vesicular glutamate transporter 1 (VGLUT1); and dendrite marker, MAP2. We treated 16-17 DIV primary hippocampal neurons cultured on glass coverslips in a 24 well plate with 5 μM of psoralidin for 6, 12, 24 hours. Since cultured hippocampal neurons have higher homogeneity in morphology and biophysical properties than cortical neurons [38-44] and hippocampi are the main place to form learning and memory, we used hippocampal neurons for analysis of synaptic structure. We quantified both VGLUT1 and PSD95-positive clusters on MAP2-positive secondary dendrites and further analyzed their area and intensity. Surprisingly, the density of the clusters were significantly increased by psoralidin (Fig. 5A and 5B), which was maximized at 6 hours of treatment and then reversed to basal level by 24 hours (data not shown). To study whether NMDA receptor and MAPK pathway is implicated in the synaptic modification by psoralidin, we pre-treated MK-801 or PD0325901 at 1 μM each for 30 minutes and then, treated neurons with 5 μM of psoralidin for 6 hours. The structural changes of excitatory synapse caused by psoralidin were abolished completely by pre-treatment of MK-801 or PD0325901. Together, our results suggest that psoralidin induces structural alteration of excitatory synapses, which is mediated by the activation of NMDA receptor and MAPK signaling pathway.

A



B

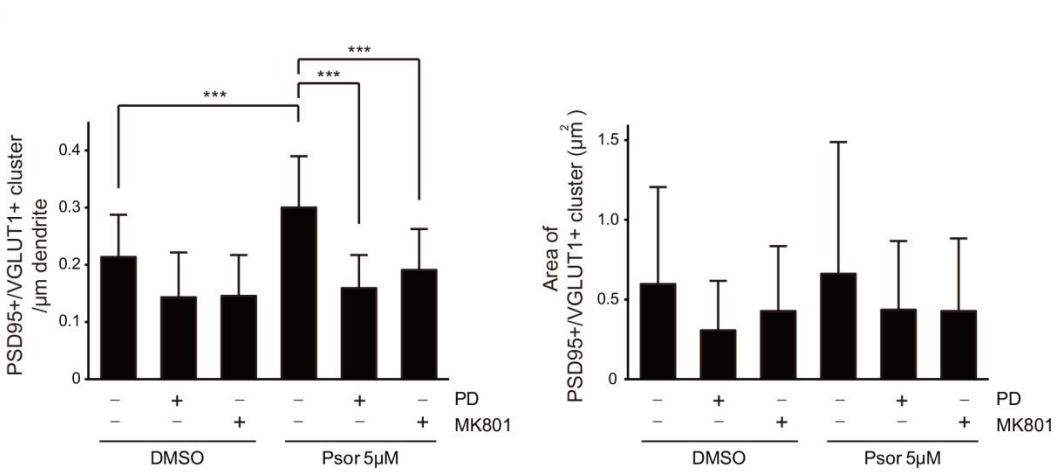


Figure 5. The structural modification of excitatory synapses by psoralidin.

(A) Representative images taken using immunofluorescence and confocal microscopy after treatment done for 6 hours in sixteen to seventeen DIV hippocampal neurons. Excitatory synapses were seen on Map2-positive neurons using antibodies against to PSD95 and VLUT1. B. Quantification of synaptic PSD95-positive and VLUT1-positive clusters. The cluster density was significantly increased by psoralidin treatment. These alterations were abolished by PD98059, a MEK inhibitor, and by MK801, NMDA glutamate receptor antagonist. Results (number of dendritic fields = 20–45) were analyzed using one-way ANOVA, followed by Dunnett's post-hoc tests. Error bars represent standard deviation (SD). *** $p < 0.001$. Scale bars, 10 mm.

IV. DISCUSSION

Long-lasting forms of synaptic plasticity (LTP and LTD) are key aspects of learning and memory. Activation of NMDA receptor regulates the expression of immediate-early genes (IEGs) required to maintain LTP and to form long-term memory through the activation of intracellular signaling pathways [45]. The structural and functional alteration of synapses due to the changes of signaling pathways through NMDA receptor is associated with a variety of pathologic conditions including epilepsy, schizophrenia, and Alzheimer's disease [46-51]. Especially, functional deficiency of NMDA receptor in inhibitory neurons has been reported to be involved in the pathogenesis of schizophrenia [52, 53]. Thus, the discovery of new compounds that regulate NDMA receptor activity or synaptic function might be important to elucidate the pathological mechanisms and to develop new therapeutics of the related neurological disorders. In this study, we discovered the role of psoralidin for the first time in the modulation of synaptic plasticity through the regulation of NMDA receptor activity and expression of IEGs in primary cortical neurons. LTP in hippocampal and cortical neurons requires the activation of Erk1/2 [54-56] and the activation of Erk1/2 by calcium influx through NMDA receptor results in IEGs expression [57]. We demonstrated that treatment of psoralidin significantly increased the phosphorylation level of Erk1/2 and induced the expression of neuronal IEGs including Arc, Egr1 and c-fos in primary cortical neurons in concentration-dependent manner. The change in the activation of the intracellular signaling protein and synaptic plasticity-related gene transcription by psoralidin was implicated by calcium influx most likely through NMDA receptor activation. Finally, psoralidin induced structural modifications of excitatory synapses by increasing density of synapses marked by PSD95 and VGLUT1 antibodies using confocal microscopy, which was completely abolished by NMDA

receptor antagonist and MAPK inhibitor. Taken together, these results suggest that psoralidin regulates the activation of NMDA receptor and subsequent intracellular downstream signaling molecules to modify the expression of synaptic plasticity-related proteins and synapse structure, which presumably affects the function of synapse and neuronal activity.

The influx of calcium through NMDA receptor activates intracellular signaling pathway involved in LTP, including Ras-ERK and PI3K-Akt pathways [57-61]. Since psoralidin activated Erk1/2 through NMDA receptor and calcium influx, but not Akt, we speculate that psoralidin may act as a modulator of NMDA receptor activity, rather than neurotropic factors such as BDNF or Reelin which strongly activates both Akt and Erk1/2 [35-37]. However, psoralidin might has a different property from NMDA which has shown the activation of Akt which can be masked by basal neuronal activities [59, 62]. Also, psoralidin might have a preferential action on either synaptic or extrasynaptic NMDA receptor, depending on the affinity to specific type of NMDA receptor subunits [63]. For future utilization of psoralidin, additional studies such as electrophysiological analysis and receptor binding assay need to be done.

In summary, we investigated the effect of psoralidin in synaptic plasticity-related gene transcription and synaptic structural change via modulation of NMDA receptor activity. Since psoralidin has been widely studied for its anti-inflammatory, anti-osteoporosis and anti-cancer, the specificity of psoralidin in the central nervous system described in this study is a very important issue for utilization of psoralidin in any clinical field. Furthermore, not all NMDA receptor modulator are convulsants [64] and several *in vivo* studies of psoralidin haven't described any seizure in animals [65, 66], which conveys that discovering the potential role of psoralidin might lead to the development of a new therapeutic in neurological disorders as well as a specific agent for mechanism study.

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