이학박사학위논문

형질전환 생쥐를 이용한 학습과 기억의 분자적 기전에 관한 연구

Studies on molecular mechanisms of learning and memory using cell-type specific SHP-2^{D61G} expressing mice, *Mib2* KO mice and *Cd38* KO mice

2017 년 6 월 7 일

서울대학교 대학원

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ABSTRACT

Studies on molecular mechanisms of learning and memory using cell-type specific SHP-2^{D61G} expressing mice, *Mib2* KO mice and *Cd38* KO mice

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The process of learning and memory requires various molecular signaling pathways and molecules to coordinate in complex network of brain cells. There have been many studies searching molecules and molecular pathways critical for learning and memory but there are still lot to be revealed yet. Transgenic animal model is an excellent tool for studying the role of a specific gene product. I used different transgenic animals in each chapter of this study to examine the molecular mechanisms of learning and memory.

In the first part of the study, cell-type specific effect of mutant SHP-2 protein expression in mouse hippocampus on learning and memory was analyzed to study which neuron type is responsible for the

cognitive impairment in Noonan syndrome patients. SHP-2 is a protein tyrosine kinase which positively regulates RAS-MAPK signaling pathway. SHP-2^{D61G} mutation was reported in human Noonan syndrome patient and SHP-2^{D61G} expressing mice showed Noonan syndrome-like physical features during development and had mild cognitive impairment, also reported in human patients. Even though SHP-2^{D61G} was expressed throughout the body, data from preceding researches detected abnormalities in only a subset of cells. However, neither the identity of these cells nor the cell-type specific effect of SHP-2^{D61G} has not been studied. Here I reveal that excitatory neurons, but not inhibitory interneurons, are responsible for the cognitive impairment by examining the effect of selective expression of mutant SHP-2 protein in excitatory or inhibitory interneurons in the mouse hippocampal CA1 area. Also, I present that SHP-2^{D61G} only hyperactivates MAPK signaling when expressed in excitatory neurons.

In the second part of the study, I used a mind bomb-2 deletion mutant mice to address how Notch signaling is regulated during learning and memory. *Mib* and its paralogue *Mib2* were identified as notch signaling regulators during zebrafish development. The mouse homologue *Mib1* also regulates Notch signaling during embryonic development and in adult brain neurons. Disruption of Notch signaling by *Mib1* deletion resulted deficits in synaptic plasticity, learning and memory. Mouse *Mib2* is mainly expressed in adult tissue, rather than in embryonic tissue, suggesting that *Mib2* might regulated non-developmental Notch signaling such as learning and memory. However, whether *Mib2* is also important in learning and memory has never been tested. In this chapter I report that *Mib2* deletion resulted in hippocampus-dependent learning and memory deficits in mouse. Also, *Mib2* knockout (KO) mouse showed impaired synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD). Unexpectedly, when the Notch signaling activation in the hippocampi of these mice was tested, the cleaved Notch1 level, which indicates Notch signaling activation, was normal in *Mib2* KO hippocampi. However, when cleaved Notch1 level was lower in *Mib2* KO hippocampi than WT hippocampi after mild foot shock. These data indicate that Mib2 is

required for hippocampus-dependent learning and memory as a neuronal activity-dependent Notch signaling activator.

In the final chapter, hippocampus-dependent learning and memory of *Cd38* KO mice was tested. CD38 regulates Ca^{2+} release from internal Ca^{2+} storage and regulates oxytocin (OXT) secretion from OXT neuron axon terminals. *Cd38* is related to autism-spectrum disorder (ASD) in both human patients and mouse model. The social behavior of *Cd38* KO mice was tested in a previous study while the learning and memory of these mice remains untested. As ASD patients often show cognitive impairment, *Cd38* KO mice are likely to have learning and memory impairment. As expected, *Cd38* KO mice had impaired hippocampus-dependent memory. I also found that these mice not only had impaired social recognition memory (which was shown in previous studies), but also impaired object recognition memory. Interestingly, synaptic plasticity such as LTP and LTD was intact in CD38 null hippocampi. Also, the pERK1/2 level, which is known to be elevated by OXT treatment, was comparable between *Cd38* KO and their wild type (WT) littermates. However, CD38 null hippocampi showed greater increment of pERK1/2 level after OXT treatment, indicating that there might be some compensation mechanism to maintain MAPK activation level without OXT. Although the behavioral data clearly indicate that *Cd38* KO mice have impaired learning and memory, further investigation is required to identify the underlying molecular mechanism.

I have identified the importance of three different molecules in learning and memory in this study. Even though they may not share the same intracellular signaling processes, SHP-2, Mib2, and CD38 were all indispensable and disruption of any one of these caused cognitive impairment in mice. These findings not only fill the empty pieces or the molecular puzzle of learning and memory, but may also contribute to developing treatments to various cognitive impairment patients including Noonan syndrome, ASD and Notch related diseases such as Down syndrome.

Keywords: Learning and memory, hippocampus, Ptpn11, *Mib2*, *Cd38*

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CHAPTER I

INTRODUCTION

BACKGROUND

Transgenic mice in learning and memory study

From the first successful attempt to generate transgene expressing mice by Rudolf Jaenisch in 1974 (Jaenisch and Mintz, 1974), transgenic animal generation technique has greatly advanced. Now it is possible to specifically manipulate the expression of a gene of interest with multiple options. The transgene-expressing mice (Tg mice) are generated via introducing gene of interest to a fertilized egg. When generating Tg mice, gene of interest may be inserted wherever in the chromosomes if there are no side effects such as destruction of already existing gene structure from the insertion. The knock-in (KI) mice differs from Tg mice that the targeting cassette is specifically inserted into the desire location on mouse chromosome. In some cases, gene of interest is positively manipulated such as Alzheimer's disease model mice expressing excessive amount of APP (Hsiao et al., 1996; Masuda et al., 2016; Saito et al., 2014) or the famous 'smart mice' with more copies of NR2B (Tang et al., 1999). KI mice also provides negative modulation of gene function by replacing the original gene with the loss-of-function mutant gene as in the case of CaMKIIa autophosphorylation deficient mice (Giese et al., 1998).Another common purpose of Tg or KI mice is to introduce genes those are not present in mouse genome. Depending on the expressed gene, these mice can be used for various experiments from studying the function of a gene to analyzing the systematic network of neurons in the brain. Especially, Cre recombinase expressing mice lines are powerful, multi-purposed tools. Cre recombinase is originated from P1 bacteriophage and recognizes specific DNA sequence which is not present in mouse genome and recombinates two recognition sites. A gene of interest can be inserted, deleted or flipped according to the orientation of the Cre recognition sites. With gene construct with proper Cre recognition site orientation and by expressing Cre recombinase in a desired cell population, researchers can turn on or off a gene of interest in Cre expressing cells without generating new transgenic animals every time. In Chapter II of this study, I used two Cre expressing mice lines to express a mutant gene construct in two different cell populations.

The KO mice lines are another powerful tool for studying gene function *in vivo*. They differ from Tg mice that when generating KO mice lines, targeting construct should be exactly inserted in a specific site of a chromosome. KO mice are generated through homologous recombination between targeting construct and the gene of interest. Usually, there are exons on the chromosomes those are critical for the gene expression. Deletion of these exons (or exon) through inserting the targeting construct disrupts gene expression, thus the animal does not express the gene of interest anymore. With the simplest idea that if there are any abnormalities in the KO mice, those are related to the function of the deleted gene, KO mice are widely used to investigate the role of various genes *in vivo*. Behavioral, physiological phenotypes of KO mice are widely studied in the field of learning and memory. Many important questions to understand the molecular mechanisms of synaptic plasticity, learning and memory were solved with the aid of KO mice. For example, the function of CA1 and CA3 in the hippocampus were investigated with region-specific *Grin1* KO mice (Nakazawa et al., 2002; Tsien et al., 1996b) and the role of different AMPAR subunits, which mediate synaptic transmission, was studied with *Gria2, Gria3* KO mice (Meng et al., 2003). Some may serve as disease models such as *Fmr1* KO mice to study fragile X mental retardation (Bakker et al., 1994), *Mecp2* KO mice as Rett syndrome model (Guy et al., 2001) to understand the pathology of cognitive impairment and find new treatments for the patients. In Chapter III of this study, I used *Mib2* KO mice to study the role of Mib2 in memory formation. In Chapter IV, the ASD model mice, *Cd38* KO mice were tested for their memory and synaptic plasticity to study the cognitive impairment showed in ASD patients.

The hippocampus

It is crucial to remember and learn from the events happened in your life. Our memory not only serves us the basic survival information such as where to find food and how to avoid danger, but also stands as the foundation of who we are, as no twins are two identical men. From ancient time, people wondered how and where the memory is stored. They once believed the memory lies in our souls or our heart, but now we know that the nervous system should take the credit.

The brain may appear as a single organ, but it has many subregions performing various tasks from regulating homeostasis of our system to sensory sensation and memory storage. Combined works of neurobiologists, psychiatrists and neurosurgeons led to the identification of the functions of each region. Among those works, the case study of a human patient, H. M., provided the key concept of the storage of episodic memory in the human brain (Scoville and Milner, 1957). H. M. suffered from epilepsy and doctors performed lobectomy to cure his symptom. This included the removal of hippocampi of his brain and after that, H. M. showed anterograde amnesia symptom. The amnesia was restricted to episodic memory, while intrinsic memory such as motor skill learning was intact. Although later brain imaging study revealed that lobectomy had affected more various regions than expected (Corkin et al., 1997), the case study of H. M. drew great attention to the hippocampus as the key of episodic memory storage.

Along with the case study of H. M., tremendous researches were performed to dissect the memory formation process in the hippocampus for last 60 years. And now we understand that the hippocampus is critical for initial storage and retrieval of episodic memory, spatial memory and recognition memory. The discovery of LTP with the hippocampus was another landmark in learning and memory study (Bliss and Gardnerm.Ar, 1973; Bliss and Lomo, 1973). LTP is strongly suggested to be one of the cellular mechanisms of learning and memory and extensively studied in wide-range of synaptic connections, including the SC-CA1 synapses in the hippocampus. Various key molecules in the hippopcampus for memory formation and synaptic plasticity were identified including ion channels (Huganir and Nicoll, 2013; Paoletti et al., 2013), transcription factors(Alberini, 2009; Chen et al., 2012; Kim et al., 2014; Lakhina et al., 2015) and protein kinases (Jalil et al., 2015; Kandel, 2012; Park et al., 2014; Ryu and Lee, 2016; Sacktor, 2008). These findings give crude view of the cellular, molecular event in the brain undergoing memory formation and there are still gaps to fill in. For more detailed information of the mechanism of learning and memory, I investigated the role of SHP-2, Mib2 and CD38 in the hippocampus using transgenic mice models in this study.

PURPOSE OF THIS STUDY

1. Cell type-specific expression of mutant SHP-2 and its effect on learning and memory

The brain is composed of various cell types, including neurons and glial cells. Each cell type has its unique function and can be defined by their morphology, electrophysiological properties and molecular markers. Sometimes, different types of cell residing in the same brain area lead to opposing behavioral output when activated (Oka et al., 2015). Therefore, we should approach the brain by dissecting the actions of specific cell types rather than the whole brain or brain area.

Glutamate releasing excitatory neurons and γ-Aminobutyric acid (GABA) releasing inhibitory interneurons are two major types of neurons in the brain. Previous result that SHP-2^{D61G} caused increased mEPSC frequency, but not mIPSC frequency indicates that cognitive impairment following SHP-2^{D61G} might be due to the malfunction of excitatory neurons. To address this question, cell type specific SHP-2^{D61G} expression in the mouse brain should be performed. This approach can be accomplished by using Cre dependent gene expressing rAAV system and Cre recombinase expressing transgenic mouse lines.

SHP-2 is innately expressed in both excitatory and inhibitory neurons. Thus, if there are any difference in SHP-2^{D61G} expression, the molecular signaling pathway SHP-2 is involved might be different in each cell type. Studying this difference will give better understanding of cognitive impairment in Noonan syndrome patients and furthermore, the molecular mechanism of learning and memory.

The first goal of this study is to identify the neuronal cell type responsible for the cognitive impairment of Noonan syndrome patients. The second goal of this study is to identify the molecular partners of SHP-2 that makes the difference between excitatory and inhibitory neurons.

2. Regulation of Notch signaling by Mind bomb-2 in hippocampal synaptic plasticity, learning and memory

While the Notch signaling is already known to be important in synaptic plasticity, learning and memory, there are much to be learned on the precise mechanism of how the Notch signaling is regulated in such events. Since zebrafish Mib and Mib2 have redundant function, their orthologue Mib1 and Mib2 are likely to share the molecular target, Notch1. However, they seem to function in different circumstances. While Mib1 null mice were embryonic lethal and Mib1 cKO mice show several developmental Notch mutant phenotypes, Mib2 KO mice were grossly normal (Koo et al., 2007). Combined with the differential expression pattern of Mib1 and Mib2, Mib2 might act as a non-developmental Notch1 regulator.

Given that Mib1 cKO mice showed impaired synaptic plasticity, learning and memory, Mib1 might act as a general Notch1 regulator throughout the lifetime of an animal. If the Mib2 KO mice also show synaptic plasticity or learning and memory deficit and differently regulated with Mib1 under such condition, study on Mib2 regulation would provide better understanding of the Notch 1 signaling in learning and memory.

The first goal of this study is to confirm if Mib2 KO mice also show deficits in hippocampal synaptic plasticity, learning and memory. The second goal of this study is to confirm whether Mib2 has distinguished role in Notch1 signaling pathway or has redundant role with Mib1.

3. Hippocampus-dependent learning and memory in *Cd38* knockout mice

Although it is known that OXT-neurons of the hypothalamus send their projections to the hippocampus wherein OXT receptors are highly expressed and OXT regulates hippocampal plasticity, it remains largely unknown whether *Cd38* deletion affects hippocampal synaptic plasticity and hippocampus-dependent behaviors including learning and memory. As it is common with ASD patients to have learning and memory disability, studying *Cd38* KO mice, which shows ASD-like phenotype, will provide better insight for understanding ASD patients. This study will demonstrate the hippocampal synaptic plasticity and hippocampus-dependent learning and memory of *Cd38* KO mice for the very first time.

The first goal of this study is to confirm of *Cd38* KO mice has impaired hippocampal synaptic plasticity, learning and memory. The second goal of this study is to confirm if any, these impairments could be rescued by OXT treatment.

CHAPTER II

Cell type-specific expression of mutant SHP-2 and its effect on learning and memory

INTRODUCTION

Noonan syndrome, recognized by Jacqueline Noonan (Noonan and Ehmke, 1963), is a developmental disorder, caused by autosomal mutations and have a prevalence of 1 in 1000 to 1 in 2500 (Tartaglia et al., 2001). The key features of Noonan syndrome are cardiac abnormality, chest deformity, short stature, cryptorchidism, distinctive facial appearance and cognitive impairment (Collins and Turner, 1973; Romano et al., 2010; Turner, 2014). The morphological features, especially the cardiomyopathy of Noonan syndrome has been the major focus of previous studies (Calcagni et al., 2016; Colquitt and Noonan, 2014; Ko et al., 2008; Lepri et al., 2011; Rojnueangnit et al., 2015; Rusu et al., 2014; Yagasaki et al., 2015). However, little has known about the molecular mechanism underlying impaired cognitive ability of patients.

The genetic cause of Noonan syndrome is heterogeneous within the mutations in the genes regulating RAS-MAPK signaling pathway, including PTPN11 (Tartaglia et al., 2001), SOS1 (Roberts et al., 2007), KRAS (Schubbert et al., 2006), RAF1 (Pandit et al., 2007) and NRAS (Cirstea et al., 2010). Among these genes, PTPN11 mutation is known to be the major case of Noonan syndrome patients (Tartaglia et al., 2002; Turner, 2014). PTPN11 encodes src homology 2 (SH2) domain-containing protein tyrosine phosphatase SHP-2, which positively regulates RAS-MAPK signaling pathway (Costa-Mattioli, 2014; Neel et al., 2003). SHP-2 is expressed in dividing brain cells during embryonic development, but only expressed in neurons and reactive astrocytes in adult brain (Servidei et al., 1998). While not all Noonan syndrome associated mutation cause cognitive impairment, PTPN11 mutant patients are well known to show impaired learning and memory (Pierpont et al., 2009).

In previous study, researchers generated a gain-of-function mutant SHP-2; SHP-2^{D61G} to investigate the molecular mechanism underlying Noonan syndrome (Araki et al., 2004). These mice showed characteristic Noonan syndrome-like phenotypes such as craniofacial abnormalities, short stature, decreased viability, cardiac dysfunction and increased ERK signaling. Another group of researchers used these mice to

investigate the cognitive function and synaptic plasticity of Noonan syndrome patients (Lee et al., 2014). In line with clinical reports (Alfieri et al., 2011; Pierpont et al., 2013; van der Burgt et al., 1999), SHP-$2^{+/D61G}$ mice showed impaired spatial learning and memory (Lee et al., 2014). Also, hippocampal slices of these mice showed impaired long-term potentiation (LTP), which is thought to be an underlying mechanism of memory (Nicoll, 2017). They confirmed that SHP-2^{D61G} hyperactivates Erk by expressing rAAV (rAAV-PTPN11D61G) in naïve mouse hippocampus. Just as SHP-$2^{+/D61G}$ mice, rAAV-PTPN11D61G injected mice showed impaired spatial learning and memory with LTP deficit, indicating that cognitive impairment in Noonan syndrome patients is not due to developmental abnormality. The MEK inhibiting drug SL327 and RAS inhibiting drug Lovastatin reversed the effect of SHP-2D61G expression, suggesting that these impairments in cognitive function and synaptic plasticity was due to hyperactivated RAS-MAPK signaling pathway. When they tested the electrophysiological features of SHP-2^{D61G} expressing neurons, surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) was increased and AMPA/ N-Methyl-D-aspartic acid (NMDA) current ratio and miniature excitatory post synaptic current (mEPSC) frequency was increased, while mEPSC amplitude was intact. However, miniature inhibitory post synaptic current (mIPSC) frequency and amplitude was comparable between two groups, implicating that the Noonan syndrome-like phenotype was mainly due to the disruption of RAS-MAPK pathway in excitatory neurons.

Even though this study implicated that SHP-2^{D61G} might have different effect on different type of neurons, confirming the hypothesis is not possible since SHP-2^{D61G} was express in regardless of cell type. For better understanding, experiment expressing SHP-2^{D61G} exclusively in excitatory neurons or inhibitory neurons should be performed. Given that SHP-2 is naturally expressed in both excitatory and inhibitory neurons (Kusakari et al., 2015; Servidei et al., 1998), it is possible that molecular partners interacting with SHP-2 protein are different in each type of neurons. Using cell-type specific Cre recombinase expressing mouse lines, I have successfully expressed SHP-2^{D61G} exclusively in excitatory neurons or inhibitory interneurons. When the spatial learning and memory

of these mice was analyzed, SHP-2^{D61G} only caused cognitive impairment when expressed in excitatory neurons. Also, I have found that SHP-2^{D61G} expressed excitatory neurons, but not SHP-2^{D61G} expressed interneurons had upregulated ERK. These results show that even though SHP-2 is naturally expressed in both excitatory neurons and interneurons, SHP-2^{D61G} selectively disrupts RAS-MAPK signaling pathway in excitatory neurons, leading to learning and memory disability.

EXPERIMENTAL PROCEDURES

Viral vector construct

SHP-2^{D61G}-HA was amplified by polymerase chain reaction (PCR) by using the following primers: 5'-cccgctagcgccaccatgacatcgcggagatgg-3' and 5'-atggcgcgcctcaagc gtaatctggaacatcgtatgggtatctgaaacttttctgctgttg-3'. Sequence for HA tag is underlined. The PCR product was digested with Nhe I and Asc I and ligated into the pAAV-EF1a::DIO-EYFP-WPRE plasmid.

rAAV packaging

7 x 10^{12} HEK 293T cells were plated on 150 mm culture dish (Thermo 157150) with 15 ml D10 culture medium (DMEM (Thermo SH30243.01) + 10% FBS (Thermo SH30919.03)) in a 37 ℃, 5% CO2 incubator for 24 hr. 13 μg of p5E18-RxC1 plasmid and 26 μg of pAd-ΔF6 plasmid with 13 μg of pAAV-EF1a::DIO-EYFP-WPRE or pAAV-EF1a::DIO-Ptpn11D61G-HA-WPRE plasmid was transfected to HEK 293T cells by CaPO4 transfection method. Cells were washed with DMEM 6~8 hr after transfection and the culture medium was replaced with 20 ml of new D10 medium. After 72 hr culture medium in the culture dishes was harvested for rAAV purification. Harvested culture medium, 6 ml of 15% iodixanol (Opti-Prep; Axis-shield 1045) solution (1 M NaCl, 1 mM MgCl2, 2.5 mM KCl and 25% Opti-prep in PBS), 5 ml of 25% iodixanol solution (1 mM MgCl2, 2.5 mM KCl, 0.2% phenol red and 42% Opti-prep in PBS), 5 ml of 40% iodixanol solution (1 mM MgCl2, 2.5 mM KCl and 67% Opti-prep in PBS), 4 ml of 60% iodixanol solution (1 mM MgCl2, 2.5 mM KCl, 0.2% phenol red in Opti-prep) was stacked in order (from top to bottom) in an ultracentrifuge tube (Beckman 324214). Tubes were centrifuged at 69000 rpm, 18 ℃, 1 hr using Beckman UltimaTm L-100K ultracentrifuge and a 70Ti rotor. Approximately 4 ml of 40% iodixanol solution was harvested from the centrifuged column with a 5ml syringe (KOVAX ND.SY1030-005). Harvested solution was mixed with 11 ml of PBS and filtered with Amicon ultra-15 filter tube (Millipore UFC910024) and

then the filter was washed with 15 ml PBS for two times. Leftover solution was harvested and viral particles in the solution were quantified via q-RT PCR

Stereotaxic viral injection

7~8 weeks old male CaMKIIa-Cre mice or VGAT-IRES-Cre mice were anesthetized with 10 μl/g ketamine / xylazine solution and mounted on a stereotaxic frame. Hippocampal CA1 region was targeted by following coordinates: AP: -1.8 mm, ML: ±1.0 mm, DV: -1.7 mm/ AP: -2.5 mm, ML: ±2 mm, DV: -1.8 mm). 1 μl of 2 x 10^12 vg/ml AAV solution was injected into each point. All mice had at least 3 weeks of recovery time after further experiments. Experimenters were blinded to the type of injecting viral vectors.

Morris water maze test

Mice were handled for 3 minutes at the same time for 7 consecutive days before performing the test. When handling was over, mice were put into a gray opaque cylinder shaped tank (140 cm diameter, 100 cm height) placed in a room with multiple spatial cues including a water tap, and a computer desk where the experimenter sits. The tank was divided into 4 virtual quadrants and a 10 cm diameter-platform was placed at the center of a quadrant (TQ). Other 3 quadrants were named by their position from TQ. The tank was filled with water (20~22 °C) until the water level was 1 cm higher than the platform and white paint was added. Before the first training trial of each mouse on training day 1, mice were placed on the platform for 30 seconds. On training days, mice were released at the edge of the maze facing the inner wall of the tank and trained to reach the platform for 60 seconds. Releasing point was randomly chosen at each trial. When the mice failed to reach the platform, they were guided to or placed on the platform for 10 seconds and were rescued from the maze. When the mice successfully reached the platform and stayed on the platform more than 1 second, mice were rescued from the maze after 10 seconds. Mice were trained with 4 trials per one training day and the trial interval between trial 1 and 2 or trial 3 and 4 was 1 minute and between trial 2 and 3 was 30 ~ 45

minutes. Every mouse received 4 training trials per day for 5 consecutive days. Probe tests were performed in the same condition with training trials except the absence of the platform and the mice were tracked for 1 minute with a tracking program (EthoVision 3.1; Nodulus). Probe test 1 was performed on training day 3 after all training trials were completed. Probe test 2 was performed 24 hours after the training trials of training day 5. Experimenters were blinded to the type of injected viral vectors.

Immunohistochemical analysis

After all behavioral experiments, mice were anesthetized with isoflurane (Hana medical) and decapitated. Brain were fixed in 4% PFA (Sigma-Aldrich P6148) in PBS solution for 24 hr and then transferred into 30% sucrose (Sigma-Aldrich S5391) solution for 48 hr. The brain samples were than froze at -80°C until sectioning. 40 μm brain slices were acquired via cryostat and stored in 50% glycerol in PBS solution at -20°C. Brain slices were washed in PBS for 5 min duration 3 times and transferred into blocking solution (4% goat serum (Rockland D104-00-0050), 0.2% Triton-X 100 (Sigma-Aldrich T8787) in PBS) for 1 hr at room temperature. Slices were then incubated with primary antibody (anti-HA rat IgG 1: 50, Roche 11867423001) in blocking solution for 48 hr at 4-10°C cold room, washed with PBS for 5 min duration 3 times, and incubated with secondary antibody (1: 250 anti-rat IgG Alexa 568 conjugated, Invitrogen A-11077). Slices were washed in PBS for 5 min duration 3 times, and mounted between a slide glass and a coverslip with Vectashield with DAPI (Vector laboratories H-1200). For p-Erk IHC, all procedure was identical with above but hippocampi separated from mouse brain was used and 20 mM Na_3VO_4 and 100 mM NaF were added in all solutions. Primary antibodies were: anti-pErk rabbit IgG 1:400 (Cell signaling technology 4370s), anti-HA rat IgG 1:50. Secondary antibodies were: anti-rat IgG Alexa 488 conjugated (Invitrogen A21208), anti-rabbit IgG Alexa 555 conjugated (Invitrogen A21428).

Confocal microscopy

Slice images were acquired using Zeiss LSM-700 confocal microscope and ZEN image analysis software. Experimenters were blinded to the type of injected viral vectors during imaging.

Statistics

For water maze data, we used two-way ANOVAs to analyze to examine if there is significant effect of injected virus (EYFP vs. D61G), followed by Bonferroni posttest for the comparison of target quadrant occupancy. Proximity measures between two groups were analyzed by the unpaired two-tailed t-test. Learning curves were analyzed by using repeated measure two-way ANOVAs. For IHC data, MAPK activation score was analyzed by the unpaired two-tailed t-test for the effect of injected virus. Data distribution was assumed to be normal but this was not formally tested. All the data are represented as mean ± s.e.m. In all cases, statistical significance was indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RESULTS

Cell-type specific Cre-recombinase expressing mouse lines

Glutamate releasing excitatory neurons and γ-Aminobutyric acid (GABA) releasing inhibitory interneurons are two major types of neurons in the brain. Previous result that SHP-2^{D61G} caused increased mEPSC frequency, but not mIPSC frequency indicates that cognitive impairment following SHP-2^{D61G} might be due to the malfunction of excitatory neurons. To address this question, cell type specific SHP-2^{D61G} expression in the mouse brain should be performed. This approach can be accomplished by using Cre dependent gene expressing rAAV system and Cre recombinase expressing transgenic mouse lines. Ca2+/calmodulin-dependent protein kinase II alpha (CaMKIIa)-Cre mice express Cre recombinase under CaMKIIa promoter and represent forebrain excitatory neurons (Tsien et al., 1996a). Vesicular GABA transporter (VGAT)-IRES-Cre mice have Cre recombinase coding sequence inserted after *Slc32a1* gene, which encodes VGAT, with internal ribosome entry sequence (IRES) positive cells and well represent GABAergic inhibitory interneurons (Vong et al., 2011). These two mouse lines were used with Cre-dependent rAAV gene expression system to selectively express SHP-2^{D61G} with cell-type specificity.

Packaging of a Cre-dependent SHP-2^{D61G} and enhanced yellow fluorescent protein (EYFP) expressing rAAV

Infecting the brain of these Cre-line mice with rAAV carrying Cre-dependent gene expression cassette results in successful gene expression in selective cell type. There are several Cre recombinase dependent gene expressing cassette (Cre-On system). One of the most popular construct has a stop cassette floxed by lox sequence after 5' untranslated sequence (UTR) and coding sequence(CDS). Another popular construct is the double floxed inverted open reading frame (DIO) construct. In this construct, the open reading frame (ORF) of the gene is oriented in the opposite direction from other regulatory elements such as promoter. However, in the presence of Cre recombinase, the double floxed sequence (by loxP and lox2272) 'flips' the ORF, enabling proper gene expression. Since the DIO construct has tighter regulation in Cre-negative cells, we decided to use the DIO construct, rather than floxed stop cassette construct.

The hemagglutinin (HA) tagged mutant PTPN11 construct was obtained from the author of 2014 Nat. Neurosci. paper (Lee et al., 2014). The SHP-2D61G coding sequence was subcloned into pAAV-Ef1a::DIO-EYFP-WPRE-hGHpA, a rAAV vector containing EYFP in DIO orientation, distributed from the optogenetics resource center operated by Karl Deisseroth lab (**Fig. 1A**).

Using the rAAV vectors above, rAAV will be purified from mammalian cell culture system. To avoid the rAAV from replicating inside infected mouse brain tissue, three independent plasmids, each containing viral capsid, viral enzymes and gene expression cassette, will be transfected into 293T cells. Among these three plasmids, only gene expression cassette contains AAV inverted terminal repeat (ITR), which acts as an entry sequence into viral capsid. These packaged virus particles were purified with iodixanol discontinuous gradient ultracentrifugation.

Viral gene expression in hippocampal CA1 neurons

The packaged virus particles were streotaxically infused into dorsal hippocampal CA1 neurons of CaMKIIa-Cre mice and VGAT-IRES-Cre mice. Immunohistochemical staining of the AAV-EF1a::DIO-PTPN11^{D61G} infused CaMKIIa-Cre mice (CaMKIIa-Cre:SHP-2^{D61G}) and VGAT-IRES-Cre mice (VGAT-IRES-Cre:SHP-2^{D61G}) brain slices with HA-antibody showed characteristic HA signal expression of excitatory neurons and inhibitory neurons (**Fig. 1B, C**).

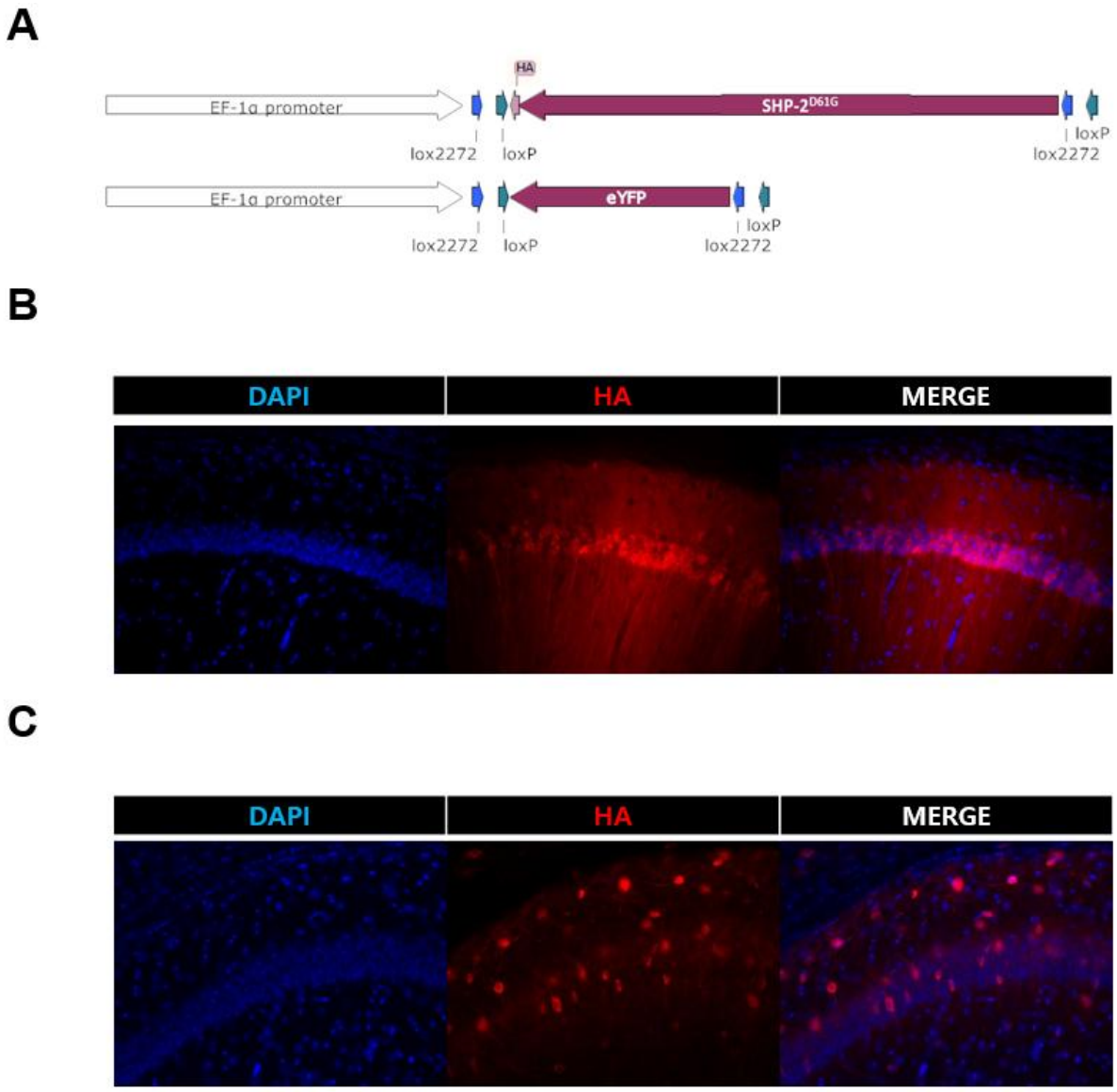


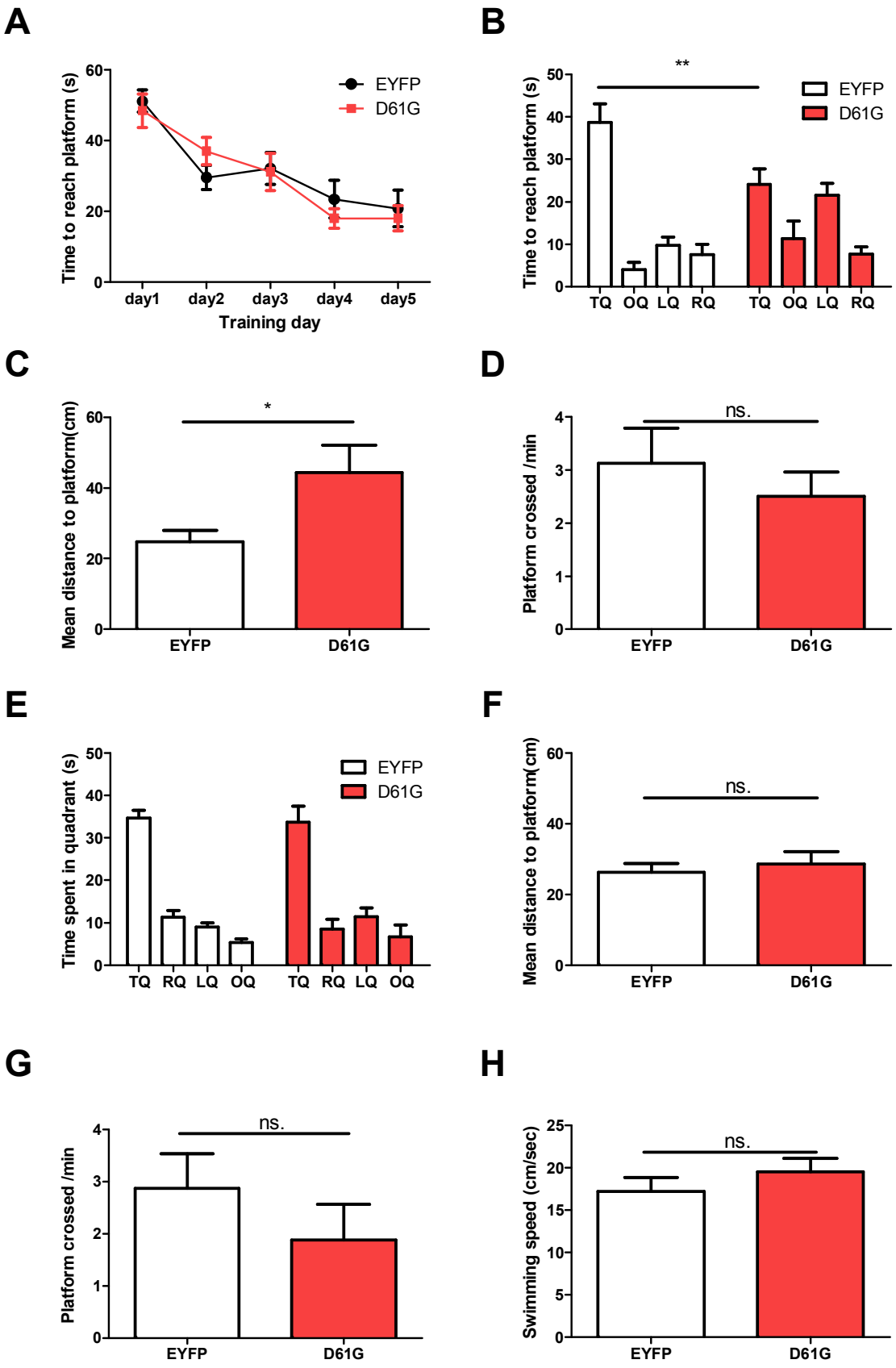
Figure 1. Immunohistochemical analysis of cell type-specific SHP-2^{D61G} expression in Cre mice

(**A**) Two AAV constructs encoding Cre-dependent double floxed inversed open reading frame (DIO) HA-tagged SHP-2^{D61G}. (**B**) HA staining imaging of CaMKIIa-Cre:SHP-2^{D61G} mice hippocampus. (**C**) HA staining imaging of VGAT-IRES-Cre:SHP-2^{D61G}.

SHP-2^{D61G} expression in hippocampal excitatory neurons impair spatial memory of mice.

To test if SHP-2^{D61G} expression in excitatory neurons causes cognitive impairment, I performed MWM test. First I tested the effect of SHP-2^{D61G} in excitatory neurons (**Fig. 2**). During the 5-day training session, CaMKIIa-Cre:SHP-2^{D61G} mice and CaMKIIa-Cre:EYFP mice showed no significant difference in time to reach the hidden platform, which indicates the learning capability of the mice was unaffected by SHP-2^{D61G} expression. (EYFP, n = 8; SHP-2^{D61G}, n = 8; two-way repeated measure ANOVA, effect of virus, $F_{1, 56} = 0.047$, $p = 0.832$, **Fig. 2A**). However, when I tested the spatial memory of these mice after 3 days of training (1st probe test, **Fig. 2B-D**), there was significant effect of SHP-2^{D61G} expression. While CaMKIIa-Cre:EYFP control group showed good memory of platform's original location with significant preference to target quadrant (TQ), CaMKIIa-Cre:SHP-2^{D61G} group showed poor spatial memory of platform's original location with no significant preference to TQ (EYFP, n = 8; SHP-2D61G, n = 8; two-way repeated measure ANOVA, interaction between virus and quadrant, $F_{3, 42} = 5.902$, p = 0.002, Bonferroni posttest, target quadrant comparison, $^{**}p < 0.01$, **Fig. 2B**). CaMKIIa-Cre:SHP-2^{D61G} also searched further from the platform location (EYFP, n = 8; SHP-2D61G, n = 8; unpaired t-test, $^{*}p < 0.05$, **Fig. 2C**). CaMKIIa-Cre:EYFP mice had a tendency to swim across platform's original location more frequently during 1st probe test but not significantly (EYFP, n=8; SHP-2^{D61G}, n=8; unpaired t-test, $p = 0.454$, **Fig. 2D**). However, after 5 days of training session, both group formed good spatial memory of the platform and showed no significant difference in the 2nd probe test, which was performed 24 hours after the last training session (EYFP, n=8; SHP-2^{D61G}, n=8; 2nd probe test, two-way repeated measure ANOVA interaction between virus and quadrant, $F_{3,42} = 0.420$, $p = 0.740$, **Fig. 2E**, mean distance to platform during 2nd probe test, unpaired t-test, $p = 0.705$, **Fig. 2F**, platform crossing during 2nd probe test, unpaired t-test, $p = 0.316$, **Fig. 2G**). The swimming speed was unaffected by SHP-2^{D61G} expression (EYFP, n=8; SHP-2^{D61G}, n=8; unpaired t-

test, $p = 0.640$, **Fig. 2H**), indicating that my result was not generated by locomotive defects caused by SHP-2^{D61G} expression.


A
Time to reach platform (s)
60
40
20
0
day1
day2
day3
day4
day5
Training day
EYFP
D61G
B
Time to reach platform (s)
50
40
30
20
10
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EYFP
D61G
TQ OQ LQ RQ TQ OQ LQ RQ
C
Mean distance to platform(cm)
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40
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*
EYFP
D61G
D
Platform crossed /min
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2
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ns.
EYFP
D61G
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Time spent in quadrant (s)
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EYFP
D61G
TQ RQ LQ OQ TQ RQ LQ OQ
F
Mean distance to platform(cm)
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ns.
EYFP
D61G
G
Platform crossed /min
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3
2
1
0
ns.
EYFP
D61G
H
Swimming speed (cm/sec)
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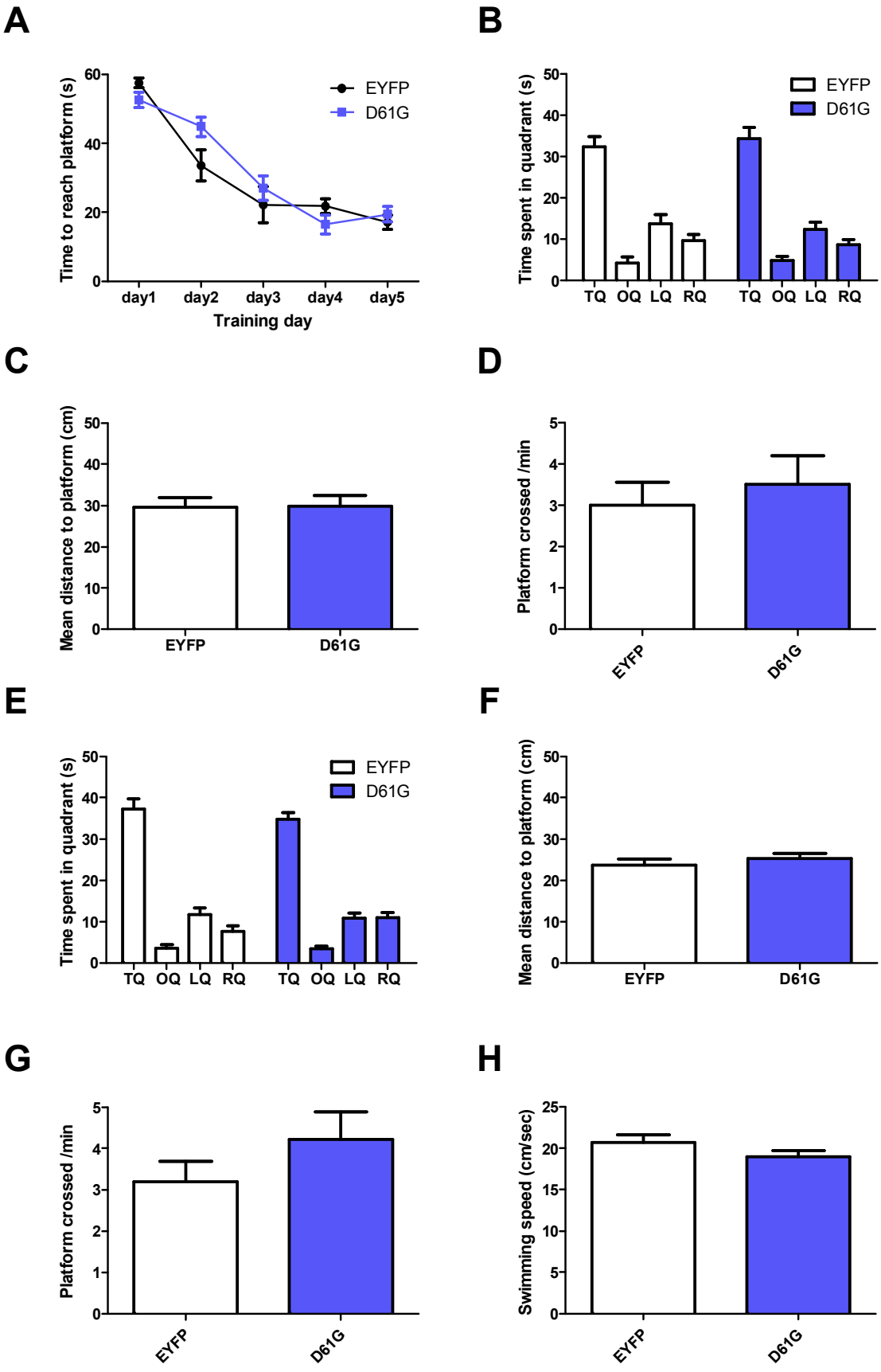
20
15
10
5
0
ns.
EYFP
D61G

Figure 2. SHP-2^{D61G} overexpression in excitatory neurons impairs learning and memory

(**A, B**) SHP-2^{D61G} overexpression in excitatory neurons impairs spatial memory. (**A**) CaMKIIa-Cre:SHP-2^{D61G} and CaMKIIa-Cre:EYFP mice performed comparably during training trials in the Morris water maze task. (**B**) CaMKIIa-Cre:SHP-2^{D61G} mice spent less time in the target quadrant than CaMKIIa-Cre:EYFP control mice in the 1st probe test. (TQ: target quadrant, RQ: right quadrant, LQ: left quadrant, OQ: opposite quadrant.) (**C**) When searching for the platform, CaMKIIa-Cre:SHP-2^{D61G} mice searched farther from the platform's location compared to CaMKIIa-Cre:EYFP control group. (**D**) During the 1st probe test, CaMKIIa-Cre:EYFP group and CaMKIIa-Cre:SHP-2^{D61G} group swam above the platform's original location comparably. (**E-G**) During 2nd probe test after 5 days of training, CaMKIIa-Cre:SHP-2^{D61G} and CaMKIIa-Cre:EYFP mice showed good spatial memory of platform location. (**H**) CaMKIIa-Cre:SHP-2^{D61G} and CaMKIIa-Cre:EYFP mice showed comparable swimming speed.

SHP-2^{D61G} expression in hippocampal inhibitory neurons have no effect on spatial memory of mice

If the cognitive impairments in Noonan syndrome patients and Noonan syndrome model mice was solely due to excitatory neurons, SHP-2^{D61G} expression in inhibitory interneurons should have no effect on cognitive function of mice. To test this hypothesis, I performed MWM with VGAT-IRES-Cre:SHP-2^{D61G} and VGAT-IRES-Cre:EYFP mice as with CaMKIIa-Cre:SHP-2^{D61G} and CaMKIIa-Cre:EYFP mice (**Fig. 3**.). VGAT-IRES-Cre:SHP-2D61G and VGAT-IRES-Cre:EYFP mice showed no difference in any aspects during the 5-day training session (EYFP, n = 11; D61G, n = 16; learning curve, two-way repeated measure ANOVA, effect of virus, $F_{1, 100} = 0.362$, $p = 0.553$, **Fig. 3A**) or 1st probe test after 3-days of training (EYFP, n = 11; D61G, n = 16; 1st probe test, two-way repeated measure ANOVA, interaction between virus and quadrant, $F_{3, 75} = 0.257$, $p = 0.856$, **Fig 3B**, mean distance to platform during the 1st probe test, unpaired t-test, $p = 0.956$, **Fig 3C**, platform crossing during the 1st probe test, unpaired t-test, $p = 0.607$, **Fig 3D**). After 5 days of training session, VGAT-IRES-Cre:EYFP and VGAT-IRES-Cre:SHP-2^{D61G} mice showed good spatial memory during the 2nd probe test (EYFP, n = 11; D61G, n = 16; 2nd probe test, two-way repeated measure ANOVA, interaction between virus and quadrant, $F_{3,75} = 1.144$, $p = 0.337$, **Fig 3E**, mean distance to platform during the 2nd probe test, unpaired t-test, $p = 0.435$, **Fig. 3F**, platform crossed during the 2nd probe test, unpaired t-test, $p = 0.316$, **Fig. 3G**). As in the excitatory specific SHP-2^{D61G} expression experiment, SHP-2^{D61G} expression in inhibitory interneurons did not alter swimming speed of the mice (EYFP, n = 11; D61G, n = 16; unpaired t-test, $p = 0.150$, **Fig. 3H**).


A
Time to reach platform (s)
60
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day1
day2
day3
day4
day5
Training day
EYFP
D61G
B
Time spent in quadrant (s)
50
40
30
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10
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TQ OQ LQ RQ TQ OQ LQ RQ
EYFP
D61G
C
Mean distance to platform (cm)
50
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30
20
10
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EYFP
D61G
D
Platform crossed /min
5
4
3
2
1
0
EYFP
D61G
E
Time spent in quadrant (s)
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TQ OQ LQ RQ TQ OQ LQ RQ
EYFP
D61G
F
Mean distance to platform (cm)
50
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EYFP
D61G
G
Platform crossed /min
5
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3
2
1
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EYFP
D61G
H
Swimming speed (cm/sec)
25
20
15
10
5
0
EYFP
D61G

Figure 3. SHP-2^{D61G} overexpression in inhibitory neurons has no effect on learning and memory

(**A, B**) SHP-2^{D61G} overexpression in inhibitory neurons have no effect on spatial memory. (**A**) VGAT-IRES-Cre:SHP-2^{D61G} and VGAT-IRES-Cre:EYFP mice performed comparably during training trials in the Morris water maze task. (**B**) VGAT-IRES-Cre:SHP-2^{D61G} mice spent similar time in the target quadrant with VGAT-IRES-Cre:EYFP control mice in the 1st probe test. (**C**) During the 1st VGAT-IRES-Cre:SHP-2^{D61G} mice and VGAT-IRES-Cre:EYFP mice was searching for the platform in similar proximity from the platform's original location. (**D**) During the 1st probe test, VGAT-IRES-Cre:EYFP group and VGAT-IRES-Cre:SHP-2^{D61G} group swam above the platform's original location comparably. (**E-G**) During 2nd probe test after 5 days of training, VGAT-IRES-Cre:SHP-2^{D61G} and VGAT-IRES-Cre:EYFP mice showed good spatial memory of platform location. (**H**) VGAT-IRES-Cre:SHP-2^{D61G} and VGAT-IRES-Cre:EYFP mice showed comparable swimming speed.

Measuring RAS-MAPK activation through immunohistochemical assay of phosphorylated ERK1/2

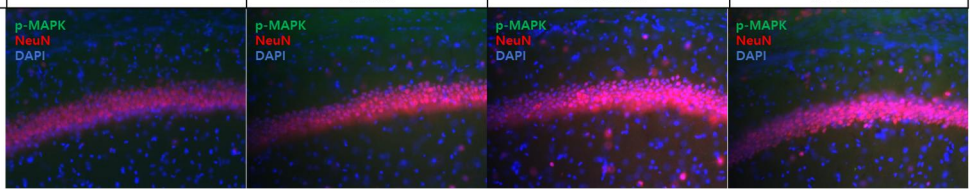
From the behavioral studies I concluded that excitatory neurons are responsible for the cognitive impairment phenotype of SHP-2^{D61G} genotype. In the preceding studies (Araki et al., 2004; Lee et al., 2014), SHP-2^{D61G} increased pERK1/2 level, which is a marker for Ras-MAPK pathway activation (Her et al., 1993), and reversing this increment by MAPK inhibitor SL-327 was able to rescue the cognitive impairment in mice. Assuming that elevated RAS-MAPK pathway is the molecular event underlying cognitive impairment, there can be two possible explanations for cell-type specific effect of SHP-2^{D61G} expression. The first possibility is SHP-2^{D61G} upregulating RAS-MAPK pathway in both excitatory and inhibitory neurons, but cognitive impairment occurs only when RAS-MAPK is upregulated in excitatory neurons. The second possibility is that even though SHP-2^{D61G} is equally expressed in both cell-types, it only upregulates RAS-MAPK pathway when expressed in excitatory neurons. Because inhibitory interneurons have far smaller cell population compared to excitatory neurons in the hippocampus, western blot analysis result of pERK1/2 might not properly reflect the RAS-MAPK pathway hyperactivation in inhibitory interneurons. Therefore, I planned to perform immunohistochemical (IHC) analysis of pERK1/2 with SHP-2^{D61G} and EYFP expressed mice brains.

The phosphate groups of phosphorylated proteins are easily lost after tissue preparation due to phosphatase activity remaining in the tissue. To avoid this issue, many laboratory protocols use phosphatase inhibitors to preserve the phosphorylated signals of proteins of interest. To preserve the phosphorylated state of proteins in brain tissue I added two phosphatase inhibitors, sodium fluoride (NaF) and sodium orthovanadate (Na_3VO_4), to all of the solutions used in IHC experiment. NaF is a well-known Ser/Thr phosphatase inhibitor (Shenolikar and Nairn, 1991) and Na_3VO_4 is a Tyr and alkaline phosphatase inhibitor (Gordon, 1991). I first tried to find the adequate concentration of these two chemicals in the solution and performed a pERK1/2 IHC with 30um-sectioned naïve mouse brain slice, adding 0 mM to 20mM of NaF and 0 mM to 100 mM of Na_3VO_4 (**Fig.** 4A). However, I could

not detect any pERK1/2 signal in the hippocampus with any of the phosphatase inhibitor concentration, while the control neuronal nuclei (NeuN) signal indicated the IHC experiment was performed well. However, when I looked at the brain slices with lower magnification, I found that superficial layers of the cortex had higher background signal of Alexa Fluor 488 signal, the secondary antibody used for pERK1/2 primary antibody (**Fig. 4B**), and had neurons stained with pERK1/2 (**Fig. 4C**). I assumed that this result indicates that phosphatase inhibitors was unable to penetrate deep enough through the brain tissue, thus giving phosphorylated signal only in superficial area. To test this hypothesis, I sliced half of the brain between two cerebral hemispheres to see if this could be enough to expose hippocampus to phosphatase inhibitors added to 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) fixative (**Fig. 4D**, left panels). In this condition, cortex area had neurons with good pERK1/2 positive signal, while hippocampal area still did not show any pERK1/2 positive neurons. As the final attempt, I separated hippocampi from the brain in ice-chilled PBS and then fixed them in fixative with phosphatase inhibitors. When these samples were sliced and immunostained, pERK1/2 positive neurons were reliably detected from CA1, CA2, CA3 and DG areas (CA1 area, **Fig. 4E**, CA2, CA3 and DG data not shown).

A

NaF	-	1mM	2mM	20mM
Na_3VO_4	-	2mM	20mM	100mM



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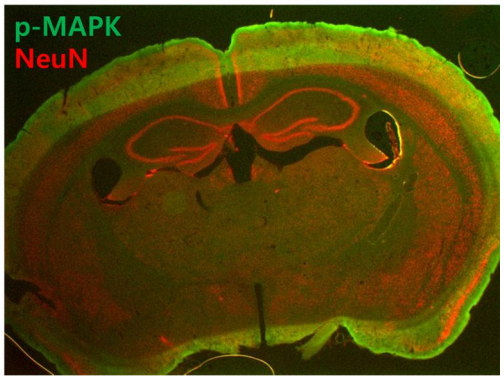
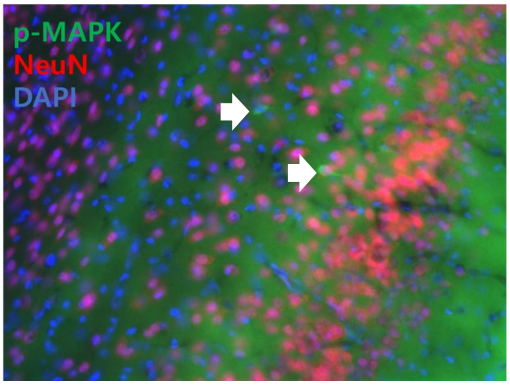


C



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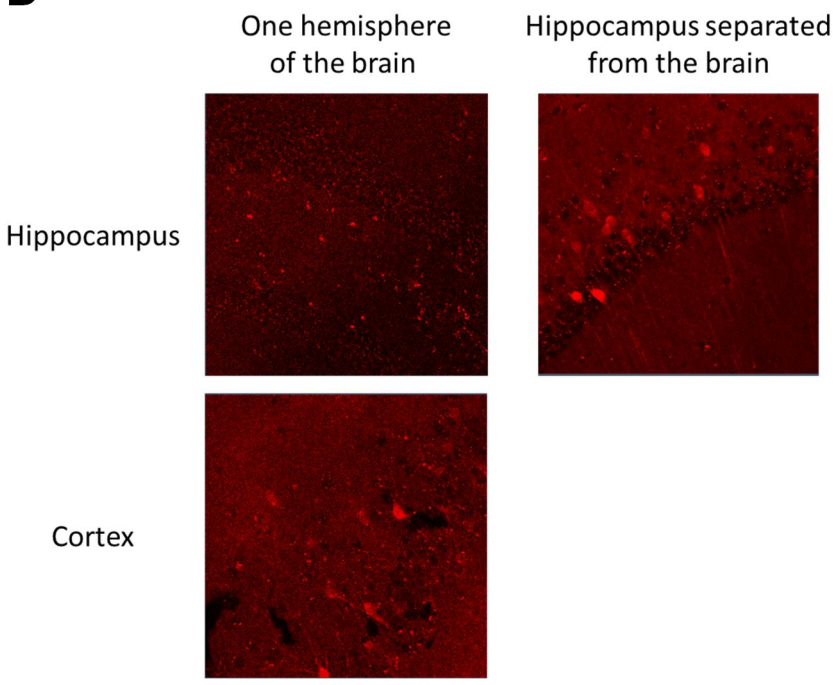


Figure 4. Fixing separated hippocampus in 4 % PFA in PBS with 20 mM NaF and 100 mM Na_3VO_4 yields pERK1/2 signal in the hippocampal CA1 region

(**A**) When whole brain was fixed in 4 % PFA in PBS, no pERK1/2 signal was detected in CA1 area regardless of phosphatase inhibitor concentration. (**B**, **C**) Fixing brain tissue in 4 % PFA in PBS with 20 mM NaF and 100 mM Na_3VO_4 resulted in higher Alexa 488 Fluor background signal with pERK1/2 positive neurons in superficial area. (**D**) Fixing separated hippocampi, but not half-sliced brain tissues gave pERK1/2 signal in the hippocampal area.

SHP-2^{D61G} upregulates pERK1/2 in excitatory neurons but not in inhibitory interneurons

With the pERK1/2 IHC protocol tested in the previous section, I performed pERK1/2 IHC with SHP-2^{D61G} and EYFP virus expressed CaMKIIa-Cre and VGAT-IRES-Cre mice. Since the excitatory neurons and inhibitory interneurons greatly differ in their number in hippocampal CA1 region direct comparison of pERK1/2 positive cell number will be unfair. Therefore, rather directly comparing number of neurons with activated RAS-MAPK signaling pathway, I calculated MAPK activation score (MA) as follow:

pERK1/2 activation in EYFP expressed neurons,

$$\text{MA} = \frac{pERK1/2(+)\,\text{and}\,EYFP(+)}{EYFP(+)}$$

pERK1/2 activation in SHP-2^{D61G} expressed neurons (HA tag on SHP-2^{D61G}),

$$\text{MA} = \frac{pERK1/2(+)\,\text{and}\,HA(+)}{HA(+)}$$

thereby comparing effect of infected virus on RAS-MAPK signaling activation. I found that CaMKIIa-Cre:SHP-2^{D61G} had higher MA compared to CaMKIIa-Cre:EYFP group, while VGAT-IRES-Cre:SHP-2^{D61G} had comparable MA with VGAT-IRES-Cre:EYFP while showing greatly smaller MA compared to CaMKII-Cre mice group (CaMKIIa-Cre:EYFP n = 14; CaMKIIa-Cre:SHP-2^{D61G} n = 16; One-way ANOVA, $F_{3, 39} = 16.29$, ***$p < 0.0001$, Bonferroni's multiple comparison test, CaMKIIa-Cre:SHP-2^{D61G} vs. CaMKIIa-Cre:EYFP, **$p < 0.01$, **Fig. 5C**). When the total number of pERK1/2 positive cells on the counted area was compared between group, there was no significant difference (VGAT-IRES-Cre:EYFP n = 7; VGAT-IRES-Cre:SHP-2D61G n = 5; One-way ANOVA, $F_{3, 37} = 1.624$, $p = 0.200$, **Fig. 5D**) and the number of viral construct expressing cells was also comparable within each cell-type (CaMKIIa-Cre:EYFP n = 14; CaMKIIa-Cre:SHP-2^{D61G} n = 16; unpaired t-test, $p = 0.188$, VGAT-IRES-Cre:EYFP n = 7; VGAT-IRES-Cre:SHP-2D61G n = 5;

unpaired t-test, p = 0.824, **Fig. 5E**), indicating that the difference in MA was not due to any unintended bias during image analysis.

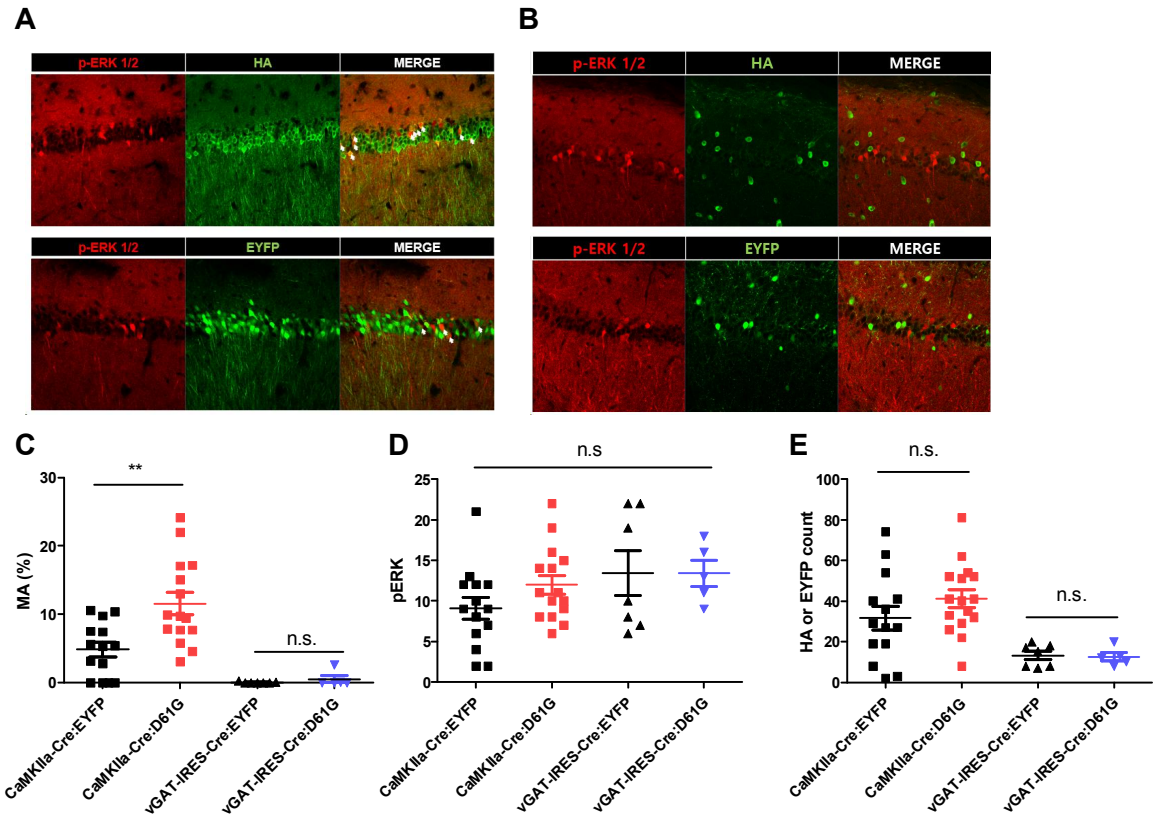


Figure 5 SHP-2^{D61G} hyperactivated MAPK pathway only in excitatory neurons

(**A**) CaMKIIa-Cre:SHP-2^{D61G} (up) and CaMKIIa-Cre:EYFP (down) mouse hippocampal CA1 region immunostained with pERK1/2 and HA antibody. (**B**) VGAT-IRES-Cre:SHP-2^{D61G} (up) and VGAT-IRES-Cre:EYFP (down) mouse hippocampal CA1 region immunostained with pERK1/2 and HA antibody. (**C**) SHP-2^{D61G} upregulated RAS-MAPK signaling pathway in excitatory neurons but not in inhibitory neurons. (**D**) The total number of pERK1/2 positive neurons among counted cells were not significantly different between groups. (**E**) The number of cells expressing viral constructs was comparable within CaMKIIa-Cre mice and VGAT-IRES-Cre mice.

Discussion

In this part of the study, I have demonstrated that the gain-of-function mutant SHP-2, SHP-2^{D61G} expression in excitatory neurons, but not inhibitory neurons in the hippocampal CA1 region induces cognitive impairment in mice. When SHP-2^{D61G} was expressed in CaMKIIa-Cre mice, CaMKIIa-Cre:SHP-2^{D61G} failed to locate the hidden platform's location during the first probe test while CaMKIIa-Cre:EYFP mice spent significantly more time in TQ. In contrast, VGAT-IRES-Cre:SHP-2^{D61G} and VGAT-IRES-Cre:EYFP mice showed comparable performance during the first probe test with significant time spent in TQ. However, this didn't mean the total failure of spatial memory formation in CaMKIIa-Cre:SHP-2^{D61G} mice, since they showed comparable learning curve with CaMKIIa-Cre:EYFP mice and they performed as well as other mice during the second probe test. This result was a good replication of human clinical data showing mild cognitive impairment in Noonan syndrome patients with SHP-2^{D61G} mutation (Alfieri et al., 2011; Pierpont et al., 2013; van der Burgt et al., 1999), suggesting these mice to be a good model for studying Noonan syndrome pathology.

Then what causes the cognitive impairment in CaMKIIa-Cre:SHP-2^{D61G} mice? In previous studies, SHP-2^{D61G} has been known to hyperactivate RAS-MAPK pathway and MAPK inhibitor SL327 was able to rescue cognitive impairment (Lee et al., 2014). From given information, it will be reasonable to suspect RAS-MAPK hyperactivation to be the cause. Then, we can think about two possible situations. First, SHP-2^{D61G} may hyperactivate RAS-MAPK in both excitatory neurons and inhibitory neurons, while upregulated RAS-MAPK only accompanies behavioral phenotype. The second possibility is that by some reason, SHP-2^{D61G} may only hyperactivate RAS-MAPK in excitatory neurons, thus CaMKIIa-Cre:SHP-2^{D61G} mice only show cognitive impairment. While looking for which of the two hypothesis fits the molecular event underlying cell-type specific cognitive impairment due to SHP-2^{D61G} expression, I found that SHP-2^{D61G} only hyperactivates RAS-MAPK pathway in excitatory neurons. This was rather surprising because SHP-2 is intrinsically expressed in both excitatory and inhibitory neurons. For a single gene product which is naturally expressed in both

cell types to have different outcome, we may assume that the interacting molecular partners are different in each cell type. If further study could reveal the molecular partners interacting with SHP-2 in each cell types, they should be good targets for the treatment of NS patients (with SHP-2^{D61G} mutation, at least). It is because while MAPK inhibitor successfully rescued the cognitive impairment in whole-body SHP-2^{D61G} expressing mice, it should also inhibit normal level of MAPK activity in SHP-2^{D61G} insensitive cells such as inhibitory neurons and may cause unwanted side effects.

Although this study has focused only on the NS, the conclusion of this study may be applied to other RAS-MAPK signaling associated disorders (RASopathies). Neurofibromatosis type 1 (NF1), associated with the loss-of-function mutations in RAS-MAPK negative regulator *Nf1* gene, is another developmental disorder including cognitive impairment and share many aspects with NS shown in this study. Studies with NF1 model mice reported memory impairment (Costa et al., 2002; Cui et al., 2008; Li et al., 2005; Silva et al., 1997) and reducing RAS-MAPK activity was able to rescue NF1 phenotype (Costa et al., 2002; Li et al., 2005). While my work demonstrates abnormal RAS-MAPK activity in excitatory neurons, NF1 model mice exhibited enhanced activity of GABA-releasing interneurons (Costa et al., 2002; Cui et al., 2008; Shilyansky et al., 2010). These reports may implicate that the other RASopaties may also have cell-type specificity. This idea is supported by the fact that may RAS signaling regulators show distinct expression patterns in different cell types (Ryu and Lee, 2016). Therefore, the next round of RASopathy study should be identifying the cell-type specificity of each disorder and generating target specific treatment for human patients.

This study demonstrated that previously reported cognitive impairment in NS model mice was due to SHP-2^{D61G} expression in excitatory neurons. In correlation with the behavioral experiment, I also found that there were more pERK1/2 positive neurons in SHP-2^{D61G} expressed excitatory neurons. Though this study confirmed that excitatory neurons are responsible for the learning and memory impairment in NS model mice, further studies such as electrophysiological study and identifying the molecular mechanism separating the response in excitatory neurons and inhibitory neurons still remains.

CHAPTER III

Mind bomb-2 regulates hippocampus-dependent memory formation and synaptic plasticity

INTRODUCTION

The Notch signaling pathway is an intracellular signal transduction mechanism, which is highly conserved throughout multicellular eukaryotic species (Gazave et al., 2009). To date, five Notch ligands and four Notch receptors have been identified in mammalian cells. As these are all type 1 transmembrane proteins, Notch signaling can occur only between physically adjacent cells (Sorkin and Von Zastrow, 2002). During the development of invertebrate and vertebrate species, Notch signaling plays a critical role in regulating cell fate and integrating other developmental cues (Artavanis-Tsakonas et al., 1999; Baron, 2003; Greenwald, 1998; Mumm and Kopan, 2000). During the development of the mammalian central nervous system (CNS), Notch signaling maintains the progenitor cell population and regulates its maturation (Yoon and Gaiano, 2005). Besides the well-known developmental role of Notch signaling, recent studies report that Notch signaling in the CNS is also involved in synaptic plasticity, learning, and memory (Ge et al., 2004; Grant et al., 1992; Sargin et al., 2013; Wang et al., 2004; Yoon and Gaiano, 2005). These studies show that LTP and LTD were both impaired in hippocampal slices from transgenic mice with reduced Notch level, , suggesting that Notch signaling is involved in modification of CA 1 synapses during stimulation (Wang et al., 2004). Not only Notch 1 itself but also its ligand Jagged 1 were found at the synapse and activated by neuronal activity. When Notch 1 was conditionally deleted in postnatal hippocampus, LTP and LTD were impaired, leading to learning and short-term memory deficits (Alberi et al., 2011).

The *mib* gene was originally found in zebrafish; it encodes the protein Mind bomb (Mib), which mediates Notch signaling by ubiquitinating the Notch ligand Delta and promoting its endocytosis (Itoh et al., 2003). Kong and his colleagues identified the mouse homologue of *mib,* and created knock-out (KO) transgenic mice lines for Mind bomb-1 (*Mib1*) and its paralogue Mind bomb-2 (*Mib2*) (Koo et al., 2005a; Koo et al., 2007). The E3 ubiquitin ligase activity of both Mib1 and Mib2 is mediated by the C-terminal Really Interesting New Gene (RING) domain, and Mib1 and Mib2 interact with *Xenopus* Delta (XD) via their N-terminal region (Itoh et al., 2003). When expression

levels are compared in mice, Mib2 is highly expressed in adult tissues and is less abundant in embryos; however, Mib1 is highly expressed in both adult and embryotic tissues (Koo et al., 2005b). Even though zebrafish Mib and Mib2, which are orthologous to mouse Mib1 and Mib2, have redundant roles in zebrafish development (Zhang et al., 2007), the different expression patterns of Mib1 and Mib2 suggest that mouse Mib1 and Mib2 may have different roles.

Even though the role of Notch signaling in development and synaptic plasticity is already known (Alberi et al., 2011), the role of Mib2 in learning and memory is still unclear. In the previous study of my colleagues, that conditional knockout of *Mib1* in mature neurons of the mouse forebrain results in impaired synaptic plasticity, learning, and memory was reported (Yoon et al., 2012). To assess whether Mib2 has a different function compared with Mib1, me and my colleagues used *Mib2* KO mice and examined their learning and memory functions. We found impaired hippocampus-dependent long-term memory, such as contextual fear memory, in the *Mib*2 KO mice compared with their WT littermates. Similarly, acute hippocampal slices prepared from *Mib2* KO mice exhibited impairments in various forms of LTP. When we measured protein levels in the hippocampus of these mice, we found that the level of cleaved Notch1 was lower in *Mib2* KO mice than in their WT littermates after mild foot shock. These results suggest that Mib2-mediated Notch signaling is essential for regulating synaptic plasticity and memory formation in the hippocampus.

EXPERIMENTAL PROCEDURES

Mice

We used 8–15-week-old male *Mib2* KO and wild type (WT) littermates on the C57BL/6N genetic background for behavioral experiments. Mice were kept on a 12-h light: dark cycle, and behavioral experiments were performed during the light phase. Food and water were provided *ad libitum.* The Animal Care and Use Committee of Seoul National University approved the animal protocols used in this study.

Behavioral tests

For all behavioral tasks, 8-15 weeks old male mice were used. Before performing the task, mice were placed on a shelf for at least 40 minutes for accommodation.

Morris water maze task

The basic procedure of MWM test was performed as described in Chapter II, except that the probe test was only performed after 5 days of training and the platform was moved to another quadrant (which was previously OQ) for reversal learning on day 7 and day 8.

Contextual fear conditioning

Prior to contextual fear conditioning, we handled each mouse for 3 minutes on 4 consecutive days. For contextual fear conditioning, the mice were placed in a chamber for 3 minutes. After 148 seconds, they were presented with an unexpected foot shock for 2 seconds (0.4 mA). Mice were returned to the chamber for testing their fear memories after 24 hours. Freezing (immobile posture except for respiration) level was measured automatically by a computer program (FreezeFrame; Coulbourn).

Elevated plus maze task

During the test, mice were placed at the center of the plus maze, and their movement was tracked for 5 minutes by a tracking program (EthoVision 3.1; Nodulus) under fluorescent light. The maze was made of white Plexiglas, and its arms were 150-cm long. The two opposing closed arms had additional walls with a height of 20 cm.

Open field task

Mice were put in a square opaque box (40 × 40 × 40 cm). The tasks were performed under dim light, and mice were tracked with a tracking program (EthoVision 3.1; Nodulus) for 10 minutes.

Electrophysiology

Field excitatory postsynaptic potential recordings were performed as described previously (Choi et al., 2014). Using a manual tissue chopper, transverse hippocampal slices (400-μm thick) were prepared from 4–5-week-old mice for measuring the *N*-methyl-D-aspartate receptor (NMDAR) mediated long-term depression (LTD) or from 8–12-week-old mice (male and female) for other protocols. Animals were deeply anesthetized with isoflurane (Hana Medical), decapitated, and their brains were removed and sectioned. Hippocampal slices were maintained in an interface chamber at 28°C oxygenated with 95% O_2 and 5% CO_2 and perfused (1–1.5 ml/min) with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgSO_4$). Slices were incubated in the interface chamber for at least 2 hours. After this recovery period, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from the Schaffer collaterals (SC) of the CA1 region using a glass pipette electrode filled with ACSF (1 MΩ). The SC were stimulated every 30 seconds using concentric bipolar electrodes (MCE-100; Kopf Instruments) placed at the CA1 region. Field potentials were amplified, low-pass filtered (GeneClamp 500; Axon Instruments), and then digitized (NI PCI-6221; National Instruments) for measurement.

Data were monitored, analyzed online, and reanalyzed offline using the WinLTP program (WinLTP Ltd., winltp.com, The University of Bristol, UK). For measuring long-term potentiation (LTP) and LTD, stimulation intensity was adjusted to produce a fEPSP slope that is approximately 40% of the slice's maximum slope. Two successive responses elicited twice per minute at this test stimulation intensity were averaged, and then expressed relative to the normalized baseline. After a stable baseline was recorded, we induced LTP by high-frequency stimulation (HFS; 100 Hz stimulation, 1 second, test intensity), LTD by low-frequency stimulation (LFS; 900 stimuli, 1 Hz, test intensity), and LTP by theta burst stimulation (TBS; 3 × TBS, 1 second each, test intensity). Data were monitored, analyzed online, and reanalyzed offline using the WinLTP program.

Western blot

For western blot analysis, hippocampi of 8-12 weeks old mice were used. Mice were deeply anesthetized with isoflurane and decapitated; then, their hippocampi were removed and frozen immediately in liquid nitrogen and stored at -80°C until lysis with a lysis buffer (1.6% SDS, 10 mM TrisCl; pH 6.8). Protease inhibitor cocktail (Roche 11873580001) and protein phosphatase inhibitor cocktail (Roche 04906845001) were also added to the lysis buffer. Hippocampal tissue samples were lysed in 400 □l lysis buffer with the QIAGEN TissueLyser LT (50 Hz, 3 min). For analysis of cleaved Notch1, mice were given a mild foot shock (0.5 mA, 2 sec) using the same protocol as for contextual fear conditioning. Fifteen micrograms of the protein samples were run by electrophoresis, and the blotted membranes were immunolabeled with antibodies against phospho-GluRepsilon2 (pGluN2B, Sigma-Aldrich, M2442), GluRepsilon2 (GluN2B, NeuroMab, 75-097), Actin (Sigma-Aldrich, A2066), Tubulin (NeuroMab 75-330), cleaved Notch1 (CST, 2421) and Mib2 (Abnova H00142678-M01A). Mib1 antibody was gift from Dr. P. Gallagher

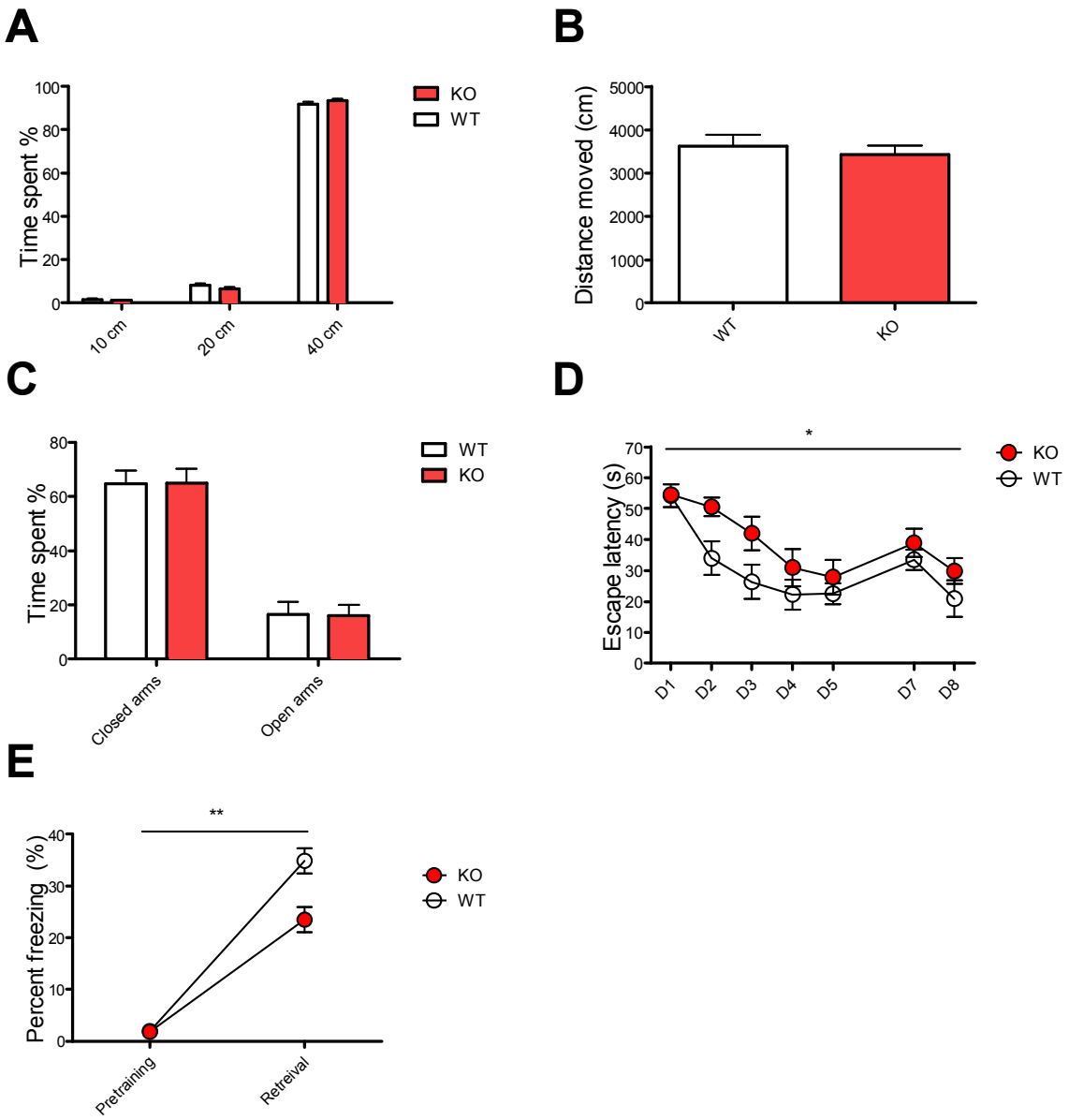
RESULTS

Hippocampus-dependent memory is abnormal in *Mib2* KO mice

Most of the learning and memory test requires animal's movement in the test apparatus such as fear conditioning chamber and MWM. To configure if a newly generated transgenic animal can perform these task without physical and psychiatric disability, general locomotion and anxiety should be measured. The open field test (OFT) measures the movement of a mouse while it explores a novel, square box (**Fig. 6A, B**). The more anxious the mouse is, the less the mouse explores in the center of the box. General locomotion of the mouse can also be measured by counting total distance moved and movement speed. During OFT test, *Mib2* KO mice did not show any noticeable difference from their WT littermates in general anxiety (WT n = 8; *Mib2* KO n = 11; two-way repeated measure ANOVA interaction between genotype and area, $F_{2, 34} = 1.082$, $p = 0.350$, **Fig. 6A**) and locomotion (WT n = 8; *Mib2* KO n = 11; unpaired t-test, p = 0.562, **Fig. 6B**). Elevated plus maze (EPM) is another test that can measure the anxiety of the mouse (**Fig. 6C**). EPM apparatus has 4 arms, with 2 open arms and 2 closed arms elevated above the ground. The more anxious the mouse is the more time it spends in the closed arms. The anxiety of the mouse can be measured by counting time spent in open arm and closed arm and *Mib2* showed comparable anxiety with their WT littermates (WT n = 7; *Mib2* KO n = 8; two-way repeated measure ANOVA interaction between genotype and maze arm, $F_{1, 13} = 0.002$, $p = 0.966$, **Fig. 6C**).

Since the Mib1 cKO mouse showed impaired hippocampus-dependent learning and memory, two hippocampus-dependent learning and memory task, MWM and contextual fear conditioning (CFC) was used to test whether deletion of Mib2 gene impairs hippocampal function. During the Morris water maze (Vorhees and Williams, 2006) task, mice have to learn and remember the location of a hidden platform beneath the water surface using spatial cues in the room. Mice were trained to find a

platform in a fixed location from the first 5 days of training and at the seventh and eighth day, the platform was moved into the OQ to test the plasticity of spatial memory of the mice. In the training trials, the latency to reach the hidden platform was significantly longer in the KOs than in their WT littermates (WT n = 9; *Mib2* n = 9; two-way repeated measure ANOVA effect of genotype, $F_{1, 96}$ = 7.191, * p = 0.012, **Fig. 6D**). These results suggest that deletion of Mib2 impairs spatial learning in mice. CFC is one of the wide-used experiment to test hippocampal function of an animal (Fanselow, 2000). In this test, unexpected electrical foot shock is given while the mouse is exploring a chamber. When the animal is placed in the same chamber 24hr later, it will freeze because of the memory of the foot shock in the very context. Higher freezing level represents stronger memory retrieval. If *Mib2* KO mice have impaired hippocampus-dependent memory formation or retrieval, it will show lower freezing level compared to its WT littermate. Consistent with the result of the Morris water maze, *Mib2* KO mice exhibited significantly less freezing behavior than WT mice (WT n = 8; *Mib2* KO n = 11; two-way repeated measure ANOVA interaction between conditioning and genotype, $F_{1, 17}$ = 14.39, **p < 0.01, **Fig. 6E**), demonstrating that Mib2 is required for proper hippocampal function during learning and memory.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60
40
20
0
10 cm
20 cm
40 cm
KO
WT
B
Distance moved (cm)
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4000
3000
2000
1000
0
WT
KO
C
Time spent %
80
60
40
20
0
Closed arms
Open arms
WT
KO
D
Escape latency (s)
70
60
50
40
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D1
D2
D3
D4
D5
D7
D8
KO
WT
E
Percent freezing (%)
40
30
20
10
0
**
Pretraining
Retreival
KO
WT

Figure 6. *Mib2* KO mice show normal anxiety and locomotion, but show impaired hippocampus-dependent learning and memory

(**A, B**) *Mib2* KO and their WT littermates showed comparable anxiety and locomotion during OFT. (**C**) *Mib2* KO and their WT littermates showed similar anxiety level in EPM test. (**D**) *Mib2* KO required longer time to reach the hidden platform and escape from water during the training sessions of MWM test. (**E**) *Mib2* KO mice showed less freezing level during the fear memory retrieval test, 24 hr after mild foot shock.

Basal synaptic transmission and LTD is normal in *Mib2* KO mice

To identify the mechanism responsible for the memory deficits caused by Mib2 deletion, my colleagues Somi Kim and Hye-Ryeon Lee examined the electrophysiological properties of *Mib2* KO mice with extracellular field recordings at the SC–CA1 synapses in acute hippocampal slices. The input-output relationship (WT, n = 8; KO, n = 5; two-way repeated measure ANOVA interaction between stimulus intensity and genotype, $F_{9, 126} = 0.115$, $p = 0.999$, **Fig. 7A**) and paired-pulse ratio (WT, n = 9; KO, n = 8; two-way repeated measure ANOVA interaction between stimulus interval and genotype, $F_{5, 75} = 0.390$, $p = 0.855$, **Fig. 7B**) were indistinguishable, demonstrating that the basal synaptic transmission is intact in *Mib2* KO mice. Since spatial memory of *Mib2* KO mice was impaired in the NMDAR-dependent (Hayashi et al., 2014) water maze task, we examined the NMDAR-dependent LTD at the SC–CA1 pathway by delivering LFS (900 pulses at 1 Hz) using acute hippocampal slices. We found no significant difference in the level of LTD between genotypes (WT n = 6; *Mib2* KO n = 9; unpaired t-test of last 5min, $p = 0.878$, **Fig. 7C**), indicating that the genetic deletion of Mib2 does not affect NMDAR-LTD.

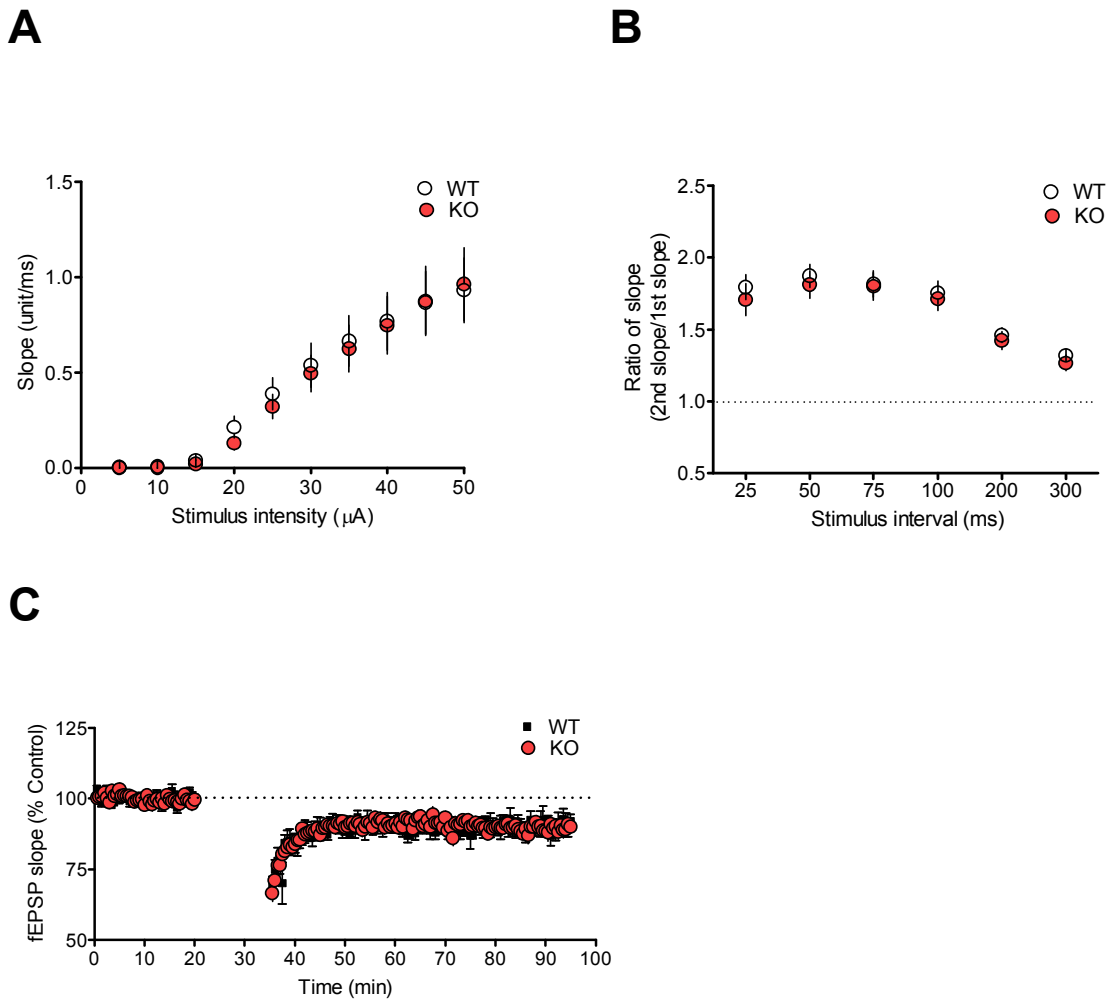


Figure 7. *Mib2* KO mice hippocampi show normal synaptic transmission and long-term depression synaptic plasticity

(**A**) Input-output relationship at the SC-CA1 synapses was not different between *Mib2* KO and WT littermates. (**B**) Paired pulse ratio was normal in *Mib2* KO mice. (**C**) NMDAR-dependent LTD was similar in WT and *Mib2* KO mice.

Mib2 KO mice have stimulation protocol dependent deficits in E-LTP and L-LTP

LTP at the SC-CA1 synapses plays an important role in spatial learning and memory. Mutant mice with impaired LTP often exhibit deficits in the hippocampus-dependent learning and memory (Grant et al., 1992). Consistent with the results of behavioral tasks, which investigated the hippocampus-dependent memory, *Mib2* KO mice had an abnormal TBS-induced early-phase LTP (E-LTP) (WT n = 12; *Mib2* KO n = 9; unpaired t-test of last 5 min, $*p < 0.05$, **Fig. 8A**). In contrast, the potentiation level of HFS (100 Hz) induced E-LTP was normal in *Mib2* KO mice compared to WT controls in the last 5 minutes (WT n = 7; *Mib2* KO n = 8; unpaired t-test of last 5 min, $p = 0.900$, **Fig. 8B**).

Interestingly, this protocol-dependent deficit of synaptic plasticity in *Mib2* KO mice reversed during the late-phase LTP (L-LTP), which is a *de novo* protein synthesis dependent form of synaptic plasticity (Bliss and Collingridge, 2013; Karpova et al., 2006; Sanhueza and Lisman, 2013). When we induced L-LTP in the hippocampal slices by delivering four pulses of high frequency tetanus with 5 minutes intervals, L-LTP was significantly decreased in *Mib2* KO mice compared to their WT littermates (WT n = 3; *Mib2* KO n = 4; unpaired t-test of last 5 min, $*p < 0.05$, **Fig. 8C**). However, when we induced L-LTP by delivering TBS three times with 10 minutes intervals, the potentiation level during the last 5 minutes was similar in the *Mib2* KO and WT littermates (WT n = 11; *Mib2* KO n = 9; unpaired t-test of last 5 min, $p = 0.250$, **Fig. 8D**). These results suggest that Mib2 selectively regulates E-LTP and protein synthesis-dependent L-LTP at hippocampal SC-CA1 synapses.

These protocol-dependent deficits of synaptic plasticity in *Mib2* KO mice may be explained by the difference between the two stimulation protocols. TBS and HFS both induce LTP, but they resemble two separate prominent rhythms in the brain: theta rhythm of 5 to 7 Hz and high frequency gamma rhythm of 50 to 100 Hz. While TBS is known to more accurately replicate the stimulus pattern of the naturally occurring rhythmic activity in hippocampus in vivo (Lynch, 2004), increasing evidences

show that they do not share intracellular mechanisms when inducing LTP. TBS-induced LTP requires ERK MAPK activity (Siarey et al., 2006)while HFS-induced LTP does not (Selcher et al., 2003). A more recent study compared the two protocols and showed that although they share mechanisms like actin polymerization, TBS-induced LTP involves calpain-1 activation and suprachiasmatic nucleus circadian oscillatory protein degradation, while HFS-induced LTP does not. Instead, HFS requires adenosine A2 receptors and PKA (Zhu et al., 2015). TBS and HFS may represent different physiological conditions of learning and some cases have been reported where transgenic mice show LTP deficits in only one protocol (Costa and Grybko, 2005). Therefore, the protocol-dependent LTP and L-LTP deficit in *Mib2* KO mice suggests that Mib2 is important in selective synaptic plasticity pathways.

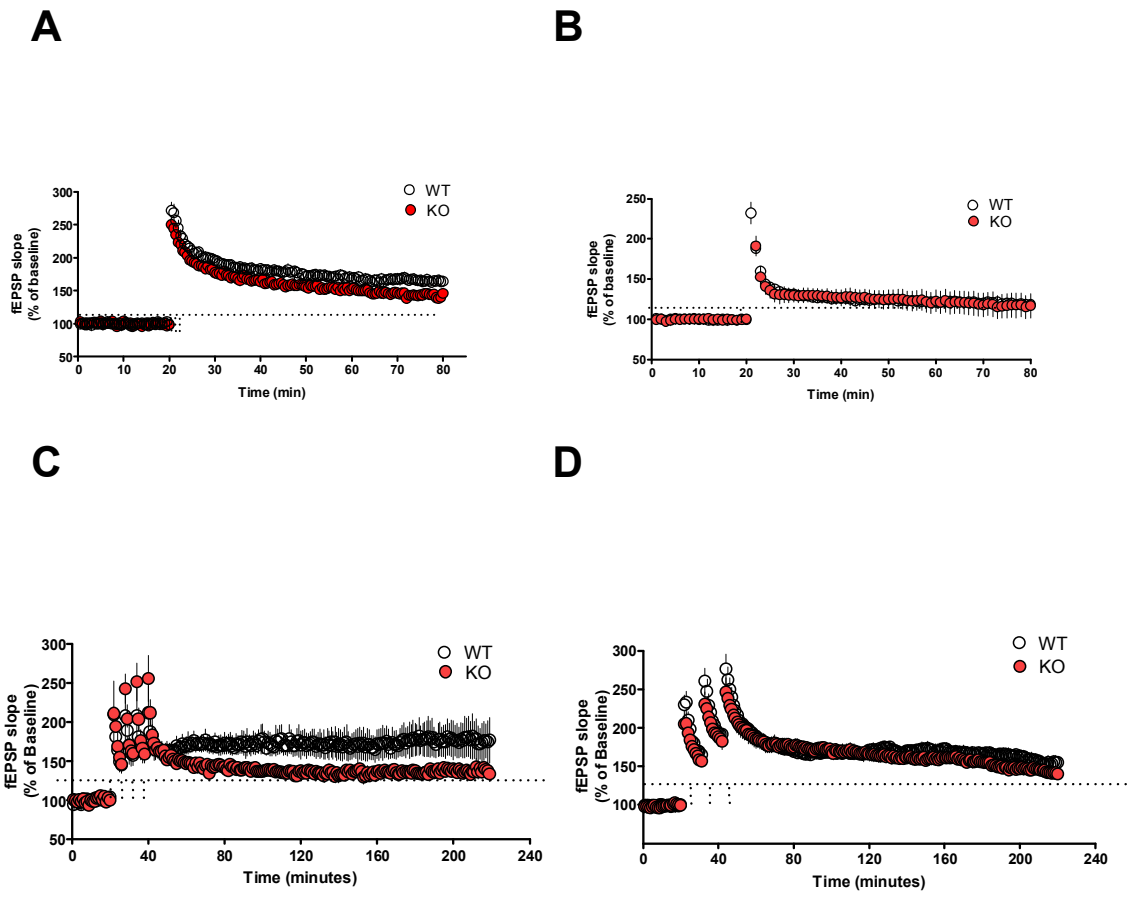
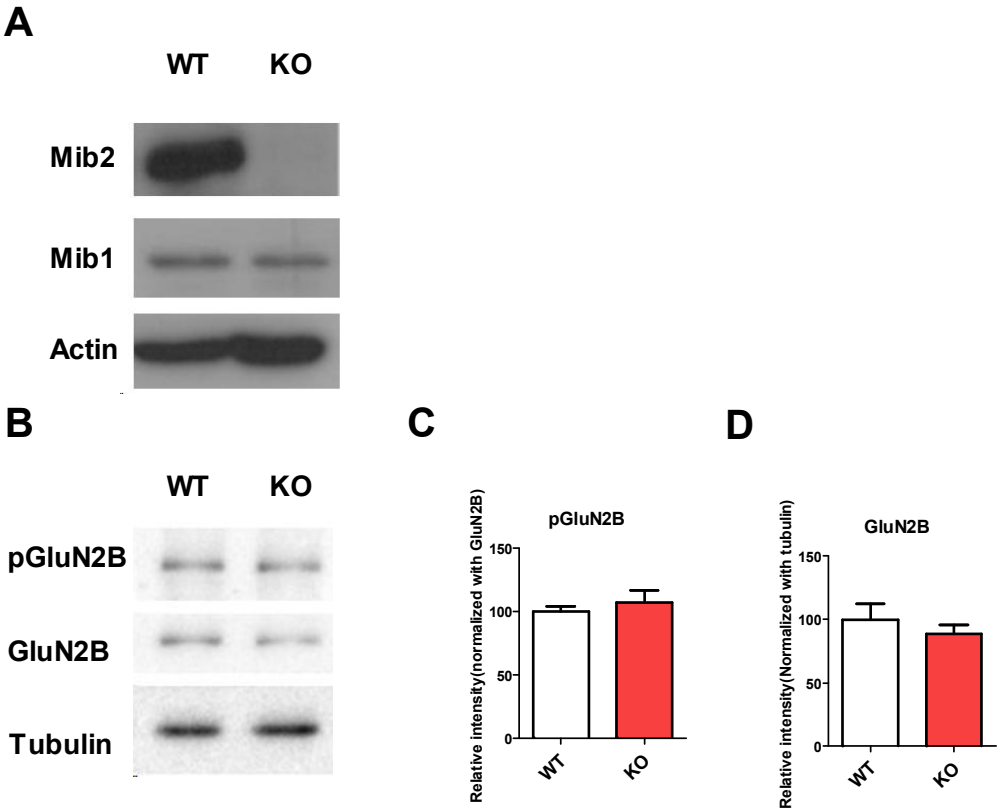


Figure 8. E-LTP and L-LTP are selectively impaired in *Mib2* KO mouse depending on the induction protocol

(**A**) TBS-induced E-LTP was impaired in *Mib2* KO. (**B**) HFS-induced E-LTP at the SC-CA1 synapses was not different in WT and *Mib2* KO mice. (**C**) The potentiation level of HFS-induced L-LTP was different during the last 5 minutes in WT and *Mib2* KO mice. (**D**) The potentiation level of TBS-induced L-LTP was not different during the last 5 minutes in WT and *Mib2* KO.

Mib2 KO mice have a normal GluN2B level, but a diminished Notch signaling

What is the molecular mechanism that caused memory impairments and synaptic plasticity deficits in *Mib2* KO mice? To answer this question, I prepared hippocampal lysates from *Mib2* KO and WT littermates. In the hippocampi of *Mib2* KO mice, compared to the hippocampi of their WT littermates, Mib2, but not Mib1 was specifically deleted (**Fig. 9A**). A previous study reported that Mib2 ubiquitinates the GluN2B subunit of the NMDAR receptor (Jurd et al., 2008). However, I found no difference in the levels of pGluN2B (WT n = 5; *Mib2* KO n = 4; unpaired t-test, $p = 0.490$, **Fig. 9B, C**) and GluN2B (WT n = 5; *Mib2* KO n = 4; unpaired t-test, $p = 0.494$, **Fig. 9B, D**) between *Mib2* KO and WT littermates. Next, I measured the level of cleaved Notch1 in the hippocampi of *Mib2* KO and WT mice without foot shock and 1 hour after mild foot (**Fig. 9E**). While there was no difference in intracellular cleaved Notch1 level between *Mib2* KO and their WT littermates without foot shock (WT n = 2; *Mib2* KO n = 3; unpaired t-test, $p = 0.778$, **Fig. 9E, F**), *Mib2* KO mice had a reduced level of hippocampal cleaved Notch1 after mild foot shock compared with their WT littermates shock (WT n = 5; *Mib2* KO n = 4; unpaired t-test, $*p < 0.05$, **Fig. 9E, G**). These results show that the deficits in *Mib2* KO mice might be a result of impaired activity-dependent Notch signaling.


A
WT
KO
Mib2
Mib1
Actin
B
WT
KO
pGluN2B
GluN2B
Tubulin
C
pGluN2B
Relative intensity(normalized with GluN2B)
150
100
50
0
WT
KO
D
GluN2B
Relative intensity(Normalized with tubulin)
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100
50
0
WT
KO
E
WT
KO
WT
SM
KO
Footshock
-
-
+
+
Cleaved-
Notch 1
Tubulin
F
NICD1
Relative intensity(Normalized with tubulin)
150
100
50
0
WT
KO
G
Cleaved Notch1
Relative intensity(Normalized with tubulin)
*
150
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50
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WT
KO

Figure 9. *Mib2* KO mice have a normal GluN2B level, but a reduced Notch signaling

(**A**) Protein expression of Mib2, but not Mib1, was downregulated in *Mib2* KO mouse hippocampi. (**B-D**) pGluN2B and GluN2B levels were comparable in *Mib2* KO and WT littermates. (**C**) pGluN2B levels normalized to the total GluN2B levels. (**D**) GluN2B levels normalized with tubulin. (**E-G**) Cleaved Notch1 level in the hippocampus was comparable in WT and *Mib2* KO littermates in the home cage condition. However, *Mib2* KO mice had a lower level of cleaved Notch1 than their WT littermates after a mild foot shock. SM: size marker.

Discussion

In the early developmental stages of *Drosophila* and zebrafish, Mib2 mediates Notch signaling by ubiquitinating the Notch ligand Delta (Koo et al., 2005b). Despite its abundant expression, the function of Mib2 in the adult mouse brain was previously unknown. In this study, we report that Mib2 plays an important role in hippocampus-dependent memory formation and synaptic plasticity by regulating Notch signaling.

Previous studies suggested that Notch signaling is involved in learning and memory formation (Costa et al., 2003; Ge et al., 2004; Sargin et al., 2013), synaptic plasticity such as LTP and LTD (Wang et al., 2004), and neuronal activity-dependent immediate early gene expression (Alberi et al., 2011). Notch1 null heterozygous knockout and conditional knockout mice exhibit normal locomotion, but impaired spatial memory (Alberi et al., 2011; Costa et al., 2003). Similarly, this study showed impaired spatial learning of *Mib2* KO mice in the Morris water maze test.

Are these defects in the hippocampus-dependent behavioral tasks and synaptic plasticity of *Mib2* KO mice caused by impaired Notch signaling in the hippocampus? The basal level of cleaved Notch1 was similar in WT and *Mib2* KO littermates, suggesting that Mib2 does not mediate basal Notch signaling. However, *Mib2* KO mice had a lower level of cleaved Notch1 compared to WT mice 1 hour after a mild foot shock. Considering that hippocampal neuronal activity and synaptic plasticity are critical for contextual fear memory formation (Ressler et al., 2002), Mib2 might mediate Notch signaling in the adult hippocampus during hippocampus-dependent learning and neuronal activity.

The input-output curve and paired pulse ratio confirmed that the basal transmission is not impaired in *Mib2* KO mice. However, *Mib2* KO mice clearly showed deficits in two different forms of LTP in a stimulation protocol dependent manner. As Notch is a transcription co-activator, and Mib2 ubiquitinates a Notch ligand (Koo et al., 2005b), the deficit in L-LTP in *Mib2* KO mice suggests that

Mib2 might regulate L-LTP via transcription regulation. Additionally, the deficient E-LTP in *Mib2* KO mice suggests that potentiation of glutamate receptor responses might be disrupted in synapses occurring in these mice (Kelleher et al., 2004). Therefore, Mib2 might be important in the formation and maintenance of synaptic plasticity.

Mib2 has been reported to ubiquitinate the GluN2B subunit of the NMDAR receptor in the postsynaptic site (Jurd et al., 2008). Therefore, we investigated if deficits observed in Mib2 mice during behavioral experiments and electrophysiological recordings were due to the dysregulation of GluN2B caused by the absence of Mib2. However, we found similar levels of pGluN2B and GluN2B in *Mib2* KO and WT mouse hippocampi. In this study, we used whole hippocampal lysates. If Mib2 ubiquitinates GluN2B subunits only at the postsynaptic, but not at the extrasynaptic site, the misregulation of postsynaptic GluN2B ubiquitination could be obscured by the non-affected extrasynaptic GluN2B population. In another report (Koo et al., 2005b), Mib2 was shown to regulate the Notch signaling pathway by ubiquitinating the ligand protein Delta. Although Notch signaling is mainly known to regulate development (Williams et al., 1995), recent studies showed that it is also activated by neuronal activity and regulates synaptic plasticity (Alberi et al., 2011). Because Notch signaling in neuronal cells regulates activity-dependent synaptic plasticity (de Bivort et al., 2009; Wang et al., 2004), the impaired memory and synaptic plasticity in *Mib2* KO mice might be due to a decreased Notch signaling. More importantly, previous report from my colleagues showed that conditional knock-out (cKO) of Mib1, a Mib2 paralogue, in mature neurons of the mouse forebrain results in impaired Notch signaling (Yoon et al., 2012). To test if Mib2 is also involved in Notch signaling in the adult mouse brain, I measured the level of cleaved Notch1 in the hippocampi of *Mib2* KO and WT mice. Unlike the forebrain of Mib1 cKO mice (Yoon et al., 2012), *Mib2* KO and WT mouse hippocampi had a comparable level of cleaved Notch1. However, when cleaved Notch1 levels were measured 1 hour after mild foot shock, *Mib2* KO mouse hippocampi contained a lower level of cleaved Notch1 than WT hippocampi. Mib2 is highly expressed in adult brain tissues, but shows a lower expression level in embryonic brain tissues; however, Mib1 is constantly expressed at a high

level (Koo et al., 2005b). Therefore, my data indicate that Mib1 is involved in basal Notch signaling in the adult brain, while Mib2 is involved in neural activity-dependent Notch signaling. However, since *Mib2* KO mice are conventional KO mice line, the possibility that normal levels of basal Notch signaling was maintained by a compensatory mechanism in Mib2 mice still remains.

The lower level of cleaved Notch1 after fear conditioning and the impaired synaptic plasticity in *Mib2* KO mice implicates that Mib2 might mediate Notch signaling during activity-dependent synaptic plasticity in the hippocampus. My study suggests that the absence of Mib2 leads to decreased hippocampal Notch signaling during learning, and thus causes impaired hippocampus-dependent learning, memory formation, and synaptic plasticity in *Mib2* KO mice.

CHAPTER IV

Impaired hippocampus-dependent learning and memory in *Cd38* knockout mice

INTRODUCTION

CD38 is a type II glycoprotein that catalyzes ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) at the plasma membrane and nuclear membrane. These catalytic substrates act as a signal to mobilize Ca^{2+} from intracellular stores (Ceni et al., 2003; Deshpande et al., 2005; Lee, 2001; Orciani et al., 2008). Although CD38 was first described as a lymphoid differentiation marker (Kung et al., 1979), it is also expressed in the brain tissue (Ceni et al., 2003). Internal Ca^{2+} release is critical for neuronal functions including neurotransmitter release and synaptic plasticity (Rose and Konnerth, 2001). In the oxytocin (OXT) secreting neurons of the hypothalamus, CD38 mediates OXT release at the neuronal terminals via intracellular Ca^{2+} mobilization (Higashida et al., 2012; Jin et al., 2007).

In *Cd38* KO mice, decreased OXT and Ca^{2+} release in response to depolarization was detected. Also, these mice showed autism spectrum disorder (ASD)-like behavioral phenotypes such as impaired offspring nurturing behavior and social recognition. These phenotypes were reversed via OXT treatment, issuing that impaired OXT release was causing the ASD-like behaviors (Higashida et al., 2012; Jin et al., 2007). In line with this report, *Cd38* mutations in human patients are also known to be associated with ASD (Ceroni et al., 2014; Munesue et al., 2010). From the human case reports (Vivanti et al., 2013), we can assume that mice with ASD-like phenotype may also have learning disabilities. However, previous studies with *Cd38* KO mice only focused on ASD-like social behaviors. OXT receptors are highly expressed in the hippocampus, and innervated by OXT neurons from the hypothalamus (Meyer-Lindenberg et al., 2011; Owen et al., 2013; Tribollet et al., 1991). Since OXT is well known to regulate hippocampal synaptic plasticity (Lin et al., 2012; Tomizawa et al., 2003), *Cd38* mutant mice is very likely to have hippocampal synaptic plasticity, learning and memory deficit.

Although it is known that OXT-neurons of the hypothalamus send their projections to the hippocampus wherein OXT receptors are highly expressed and OXT regulates hippocampal

plasticity, it remains largely unknown whether *Cd38* deletion affects hippocampal synaptic plasticity and hippocampus-dependent behaviors including learning and memory. As it is common with ASD patients to have learning and memory disability, studying *Cd38* KO mice, which shows ASD-like phenotype, will provide better insight for understanding ASD patients. This study will demonstrate the hippocampal synaptic plasticity and hippocampus-dependent learning and memory of *Cd38* KO mice for the very first time.

EXPERIMENTAL PROCEDURES

Mice

Eight to fifteen week old male CD38 KO and wild-type (WT) littermates with a C57BL/6J genetic background were used for the behavioral experiments. The Animal Care and Use Committee of Seoul National University approved the animal protocols.

Behavioral tests

For all behavioral tasks, 8-15 weeks old male mice were used. Before performing the task, mice were placed on a shelf for at least 30 minutes for accommodation.

Morris water maze

MWM test was performed as described in Chapter II.

Contextual fear conditioning test

CFC test was performed as described in Chapter III.

Three-chamber test

Stranger mice were handled for 3 minutes and then habituated in a wired cup placed in the 3-chamber apparatus for 5-10 minutes for 4 consecutive days. When the handling was over, the test mice were habituated to the 3-chamber apparatus for 10 minutes with the doors opened. When the habituation was completed, the test mouse was guided to the center chamber and the doors were closed. A wired cup with stranger 1 mouse and empty cup (for sociability test) were introduced into the other two chambers, and then opened the doors. The movement of the test mouse was tracked for 10 minutes with a tracking program (EthoVision 3.1; Nodulus). When the sociability test

session was over, the test mouse was guided to the center chamber and the doors were closed. Another stranger mouse (stranger 2) was introduced into the empty cup (for social recognition test). The doors were opened and the movement of the subject mouse was tracked for another 10 minutes with the same tracking program. For each set of experiment, the orientation of two wired cups containing stranger 1 or stranger 2 (or empty) was counterbalanced.

Novel object recognition (NOR) test

Mice were handled for 3 minutes for 4 consecutive days before performing NOR test. The task was performed in an open field box, which was made of opaque acryl (40 × 40 × 40 cm). Object A was a light bulb and object B was a glass vial and there were no differences in preference between objects. Each object height was 10 cm, hard for mice to climb to object during task. On the day of sample phase, mice were placed to the open field box with two identical objects for 15 minutes. On retention phase test, which was performed 24 hours after sample phase, mice were placed to the arena again for 10 minutes with two different objects (one was the same object presented at the sample phase, the other was a novel object newly presented at the retention phase). The time spent for the mice exploring each object was recorded. The discrimination index was calculated as the difference (AT-BT) in time spent by each mouse exploring the novel object compared with the familiar object divided by the total time spent exploring both of two objects (AT+BT). Preference was derived from the exploration time to a novel object divided by the total time spent exploring both of two objects.

Elevated plus maze test

EPM test was performed as described in Chapter III.

Open field test

OFT test was performed as described in Chapter III.

Electrophysiology

For extracellular field recordings, transverse hippocampal slices (400 μm thick) were prepared from deeply anesthetized (Isoflurane) 4~5 weeks old mice for NMDAR-LTD or 8~12 weeks old mice for other experiments using manual tissue chopper and incubated in an interface chamber for at least 2 hours to recover After the recovery period, the slices were placed in a recording chamber at 25℃, perfused (1~1.5 ml/min) with oxygenated artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, and 2 mM $MgSO_4$, 290 Osm), and extracellular field EPSPs (fEPSPs) were recorded from the CA1 area using a glass electrode filled with ACSF (1 MΩ). The SC pathway was stimulated every 30 seconds using concentric bipolar electrodes (MCE-100; Kopf Instruments). Field potentials were amplified, low-pass filtered (GeneClamp 500; Axon Instruments), and then digitized (NI PCI-6221; National Instruments) for measurement. Data were monitored, analyzed online and reanalyzed offline using WinLTP program. . . For LTP and LTD experiments, after a stable baseline was recorded, high frequency stimulation (100 Hz, 1 second for HFS-LTP), four trains of high frequency stimulation (4 x 100 Hz, 1 second each, 5 min inter-train interval for HFS-L-LTP), low frequency stimulation (1 Hz, 900 stimuli for LFS-LTD), or theta burst stimulation (3 x TBS, 1 second each for TBS-LTP) was delivered. For L-LTP rescue experiment, Oxytocin (0.2 μM, Tocris) was perfused for 40 min per slice during baseline (20 min) and stimulation (20 min)

Western blot

Hippocampal slices from 7 to 8 weeks old mice were prepared as described in electrophysiology section. Slices were incubated in a submerge chamber for 2 hours. For oxytocin treatment group, oxytocin was added to the chamber for 0.2 uM final concentration. Slices were incubated for

additional 40minuet in ACSF or ACSF with 0.2 uM oxytocin and then collected in a 1.5ml e-tube. Slices were frozen in liquid nitrogen and stored in -80°C. Slices were lysed in 200ul radio-immunoprecipitation assay buffer (RIPA buffer: 50 mM Tris-Cl pH 7.4, 2 mM EDTA, 0.1% SDS, 0.5% sodium dioxycholate, 150 mM NaCl, 1% NP-40, 1 mM DTT) with protease inhibitor cocktail (Roche 11873580001) and protein phosphatase inhibitor cocktail (Roche 04906845001) with TissueLyser LT (QUIAGEN) using 50Hz, 3minute protocol. 5ug protein sample was loaded for each well in acrylamide gel and blotted with pErk1/2 antibody (1:3000, Cell Signaling Technology CST 9101). Blotted membrane were stripped in stripping buffer (Thermo Scientific, NCI1059KR) for 45 minutes and then blotted with Erk1/2 antibody (1:5000, Cell Signaling Technology CST 9102). Signal intensity of each lane was measured with Image Lab 3.0 program (Bio-Rad).

Statistics

For water maze data, we used one-way ANOVAs to analyze quadrant occupancy (% time spent in quadrant). When two groups were compared, unpaired or paired two-tailed t-test was used. LTP data were analyzed by using unpaired two-tailed t-test on the average of the last 5 min of recordings. All the data are represented as mean ± SEM.

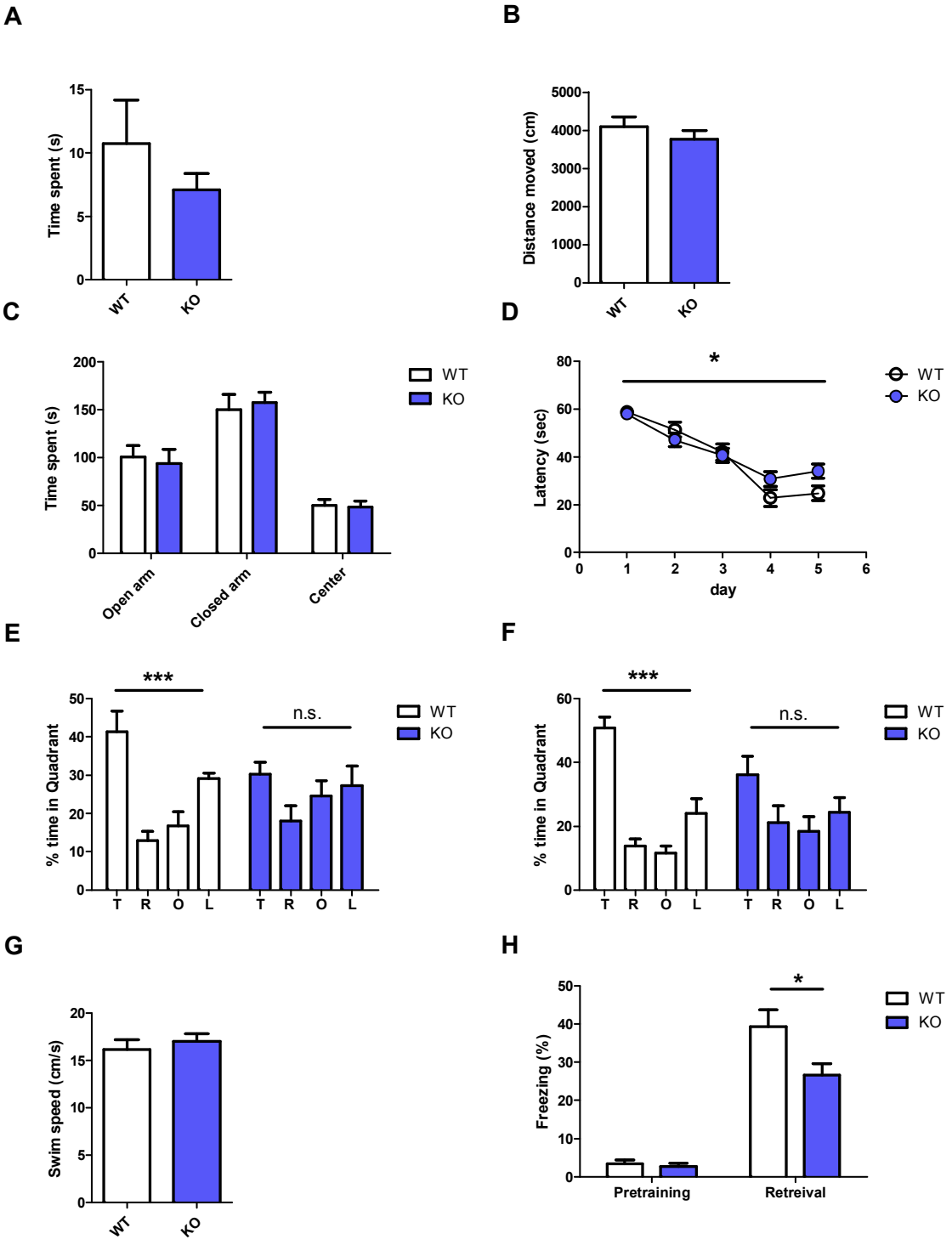
RESULTS

CD38 deletion impairs hippocampus-dependent learning and memory

Before testing whether *Cd38* KO mice show cognitive impairment as seen in ASD patients, I wanted to confirm that these mice do not have any abnormality in anxiety or locomotion. My colleague Hye-Ryeon Lee performed OFT and EPM to check these aspects. *Cd38* KO mice showed no significant difference from their WT littermates in anxiety (WT n = 7; *Cd38* KO n = 20; unpaired t-test, $p = 0.272$, **Fig. 10A**) and locomotion (WT n = 7; *Cd38* KO n = 10; unpaired t-test, $p = 0.366$, **Fig. 10B**) during OFT. They also spent similar time in open arms of EPM (WT n = 12, *Cd38* KO n = 9, two-way repeated measure ANOVA interaction between maze arms and genotype, $F_{2, 38} = 0.112$, $p = 0.888$, **Fig. 10C**), indicating that basal anxiety and locomotion was unaffected by *Cd38* deletion.

Next, hippocampus-dependent learning and memory was tested with CFC and MWM test. My colleague Yong-Seok Lee studied whether spatial memory was impaired in *Cd38* KO mice with MWM test. *Cd38* KO took more time to locate the hidden platform and escape from the water during the 5-day training session (WT n = 9; *Cd38* KO n = 13; two-way repeated measure ANOVA, interaction between time and genotype, $F_{4, 344} = 2.53$, $^{*}p < 0.05$, **Fig. 10D**). Also, *Cd38* KO mice failed to remember the hidden platform's location during the probe test session after 3 days (WT n = 9; one-way ANOVA, F = 13.23, $^{***}p < 0.001$, *Cd38* KO n = 13; one-way ANOVA, F = 2.323, $p = 0.087$, **Fig. 10E**) and even after 5 days (WT n = 9; one-way ANOVA, F = 30.43, $^{***}p < 0.001$, *Cd38* KO n = 13; one-way ANOVA, F = 1.637, $p = 0.193$, **Fig. 10F**) of training. As it was confirmed in OFT test, *Cd38* KO mice did not show any difference in swimming speed compared to their WT littermates (WT n = 9; *Cd38* KO n = 13; unpaired t-test, $p = 0.528$, **Fig. 10G**), indicating that the impaired performance in MWM test was solely due to impaired cognitive function. CFC was performed with aid from my colleague Eun-Hye Jang. When the contextual fear memory was tested, I found that *Cd38* deletion caused decreased freezing level during the retrieval test 24 hr after conditioning (WT n = 10; *Cd38* KO n = 9; unpaired t-test, $^{*}p < 0.05$, **Fig. 10H**).

Even though there was no difference in locomotion and anxiety, *Cd38* KO mice exhibit impaired spatial learning and memory in MWM test and impaired contextual fear memory in CFC test. These data strongly suggest that CD38 is critical in hippocampal function during hippocampus-dependent learning and memory process.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Distance moved (cm)
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Figure 10. *Cd38* KO mice show normal locomotion and general anxiety, but impaired hippopcampus-dependent learning and memory

(**A**) *Cd38* KO and their WT littermate spent comparable time in the center 10 x 10 cm area during OFT test. (**B**) *Cd38* KO mice showed comparable locomotion during OFT test. (**C**) Time spent in each area of EPM apparatus did not differ between *Cd38* KO and their WT littermates. (**D**) *Cd38* KO mice required more time to escape from the water than their WT littermates during the 4^{th} and 5^{th} day of training session. (**E, F**) *Cd38* KO failed to locate the platform's location while their WT littermates showed strong preference to TQ area during the 1^{st} and 2^{nd} probe test after 3 days and 5 days of training. (**G**) *Cd38* deletion did not affect the swimming speed of mice during MWM test. (**H**) The freezing level of *Cd38* KO mice was lower than their WT littermates when tested 24 hr after mild foot shock was given.

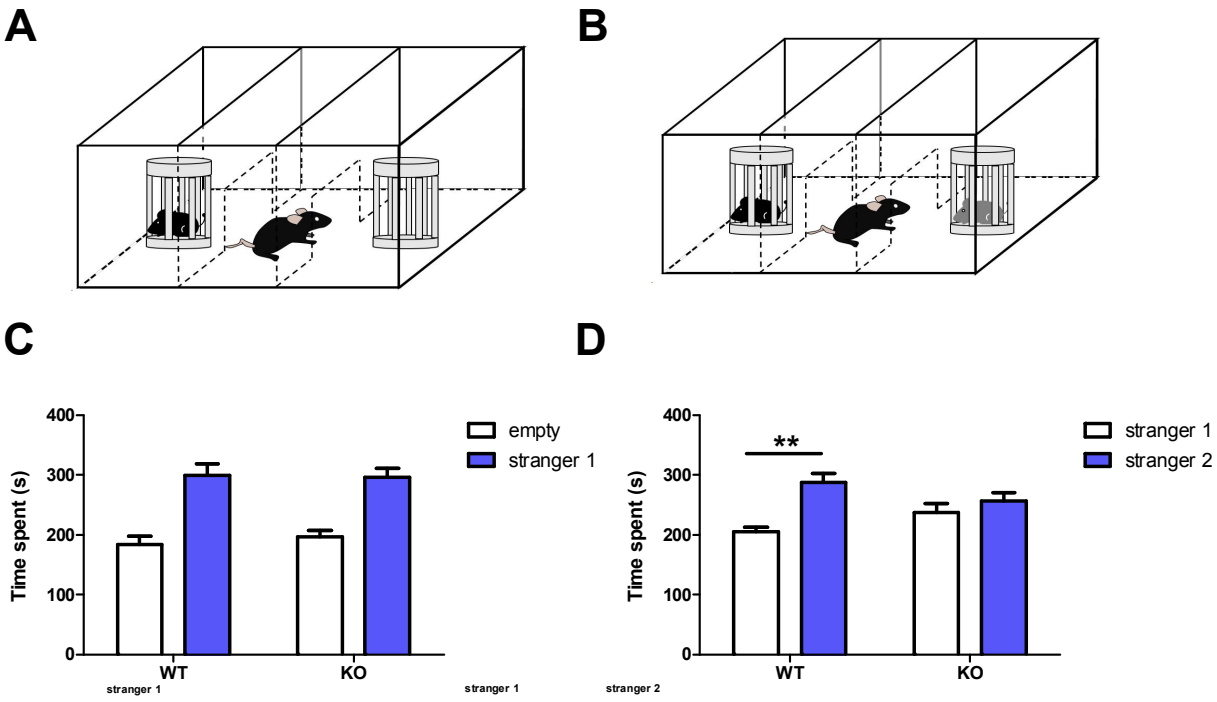
Not only social recognition memory, but also nonsocial recognition memory is impaired in *Cd38* KO mice

Previous reports show the social behavior such as social memory and nurturing of their offspring is impaired in *Cd38* KO mice (Akther et al., 2013; Jin et al., 2007). Because OXT mediated signaling is known to be important in the social behaviors (Ferguson et al., 2000; Lee et al., 2008; Takayanagi et al., 2005), the authors reasoned that the impaired OXT release from OXT neurons in *Cd38* KO mice caused the social memory impairment. However, the social memory deficit of *Cd38* KO mice could be due to the general recognition memory impairment, rather than social-specific phenotype. To test this hypothesis, social and nonsocial recognition memory of *Cd38* KO mice was tested with three-chamber test and novel object recognition test, respectively. These tests were performed by my colleague, Juyoun Yoo. First, the sociability of mice was tested by introducing a stranger mouse (stranger 1) in a wire cage in one of the chambers as described in **Fig. 11A**. In this test, *Cd38* KO mice did not show any noticeable difference from their WT littermates (WT n = 9; paired t-test, $^{**}p < 0.01$, *Cd38* KO n = 9; paired t-test, $^{**}p < 0.01$, **Fig. 11C**), showing normal level of preference to a stranger mouse than an empty cage. Right after measuring the sociability, social memory of these mice was tested. Along with the stranger mouse presented in the sociability test (which is now a 'familiar' mouse), another stranger mouse (stranger 2) was presented in the opposite chamber (**Fig. 11B**). Innately, mice show more interest to a novel mouse or object and spend more time exploring it. While their WT littermates showed more interest to the stranger 2 mouse, *Cd38* KO mice did not show any preference to the stranger 2 mouse (WT n = 9; paired t-test, $^{**}p < 0.01$, *Cd38* KO n = 9; paired t-test, $p = 0.522$, **Fig. 11D**).

Next one of the nonsocial recognition memory, novel object recognition (NOR) memory was tested. In this test, mice are presented with two identical objects during the training session. Then, we change one of the objects to a novel object, and see if the mice show more interest to the new object (**Fig. 11**

E). As I suspected, *Cd38* KO mice showed lower preference to the novel object compared to their WT littermates (WT n = 7; *Cd38* KO n = 10; unpaired t-test, $*p < 0.05$, **Fig. 11 F**).

I confirmed that *Cd38* KO mice have normal social preference, but show impaired social memory as reported in preceding studies (Akther et al., 2013; Jin et al., 2007). Further, we have shown that recognition memory deficit of *Cd38* KO mice is not only restricted to social aspect, but novel object recognition memory is also impaired in this mice. These result may suggest that CD38 is not just involved in OXT supply to hippocampus during recognition memory formation but rather its other function such as intracellular Ca^{2+} mobilization might be important, as other reports suggest that OXT reduction is not related to NOR impairment (Zhang et al., 2016) or even excessive OXT treatment impairs NOR (Zhang et al., 2015).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Time spent (s)
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Discrimination index (%)
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Figure 11. Social and nonsocial recognition memory is impaired in *Cd38* KO mice

(**A-B**) Experimental design for 3-chamber tests measuring sociability (**A**) and social recognition memory (**B**). (**C**) Exploration time of *Cd38* KO mice and their WT littermates for the empty cup and the cup with the stranger mouse was comparable. (**D**) Exploration time for the cup with the familiar mouse and the cup with the stranger mouse was significantly different in WT mice but not in *Cd38* KO mice. (**E**) Experimental design for novel object recognition memory test. (F) *Cd38* KO mice showed less preference to the novel object than their WT littermates.

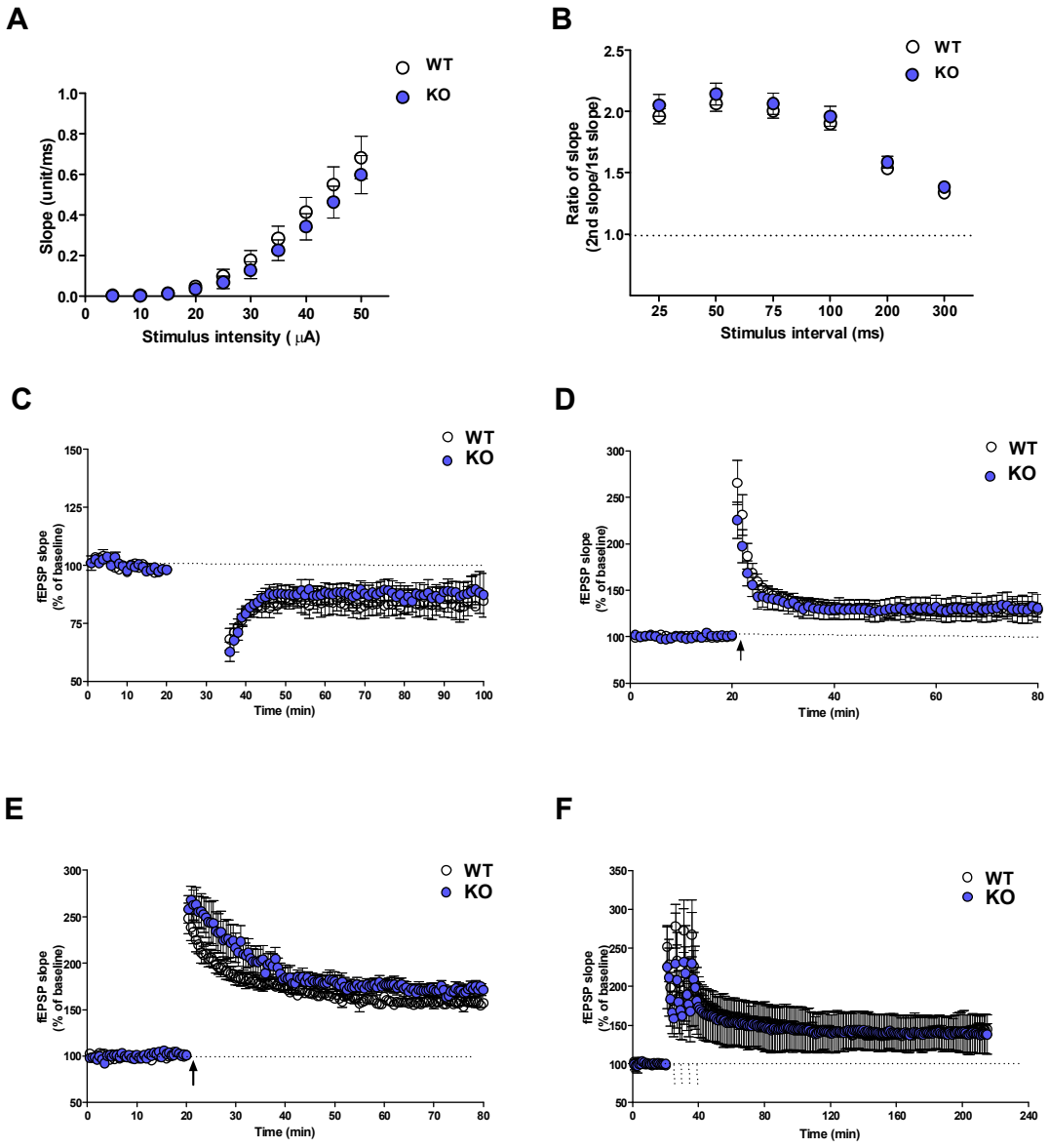
Cd38 KO mice have normal LTD and E-LTP in SC-CA1 synapses

To identify the mechanism underlying hippocampus-dependent memory, electrophysiological properties at the SC-CA1 synapses of acute hippocampal slices from *Cd38* KO and WT mice were tested. Extracellular field recording was performed by my colleagues, Somi Kim and Hye-Ryeon Lee. Before testing synaptic plasticity, whether basal synaptic transmission is affected by *Cd38* deletion was tested (**Fig. 12A, B**). Input-output relationship and paired-pulse facilitation (PPF) ratios were indistinguishable between WT and *Cd38* KO mice (Input-output, WT n = 18; *Cd38* KO, n = 12; Repeated measure two-way ANOVA, effect of genotype, $F_{1,252} = 0.4931$, $p = 0.4883$, **Fig. 11A**; PPF, WT n = 14; *Cd38* KO n = 10; Repeated measure two-way ANOVA, effect of genotype, $F_{1,110} = 0.5807$, $p = 0.4541$, **Fig. 11B**), demonstrating that the genetic deletion of CD38 does not affect basal synaptic transmission.

Next, NMDAR-dependent LTD was tested by delivering low frequency stimulation (900 pulses at 1Hz). There was a report that the catalytic product of Cd38, cADPR, is critical for the formation of hippocampal LTD as it mediates Ca^{2+} release from ryanodine-sensitive intracellular storage of presynaptic neurons (Reyes-Harde et al., 1999). Even though the presynaptic neurotransmitter release was unchanged in the SC-CA1 synapses of *Cd38* KO mice, *Cd38* deletion may have disrupted LTD formation. However, we found that NMDAR-LTD was not significantly different between *Cd38* KO and WT mice (average of fEPSP slopes for last 5 min; WT n = 6; *Cd38* KO n = 8; unpaired t-test; $p = 0.552$, **Fig. 11C**).

LTP is another form of synaptic plasticity and learning and memory impairment is generally accompanied with LTP deficits (Lee, 2014; Lee and Silva, 2009). Therefore, my colleagues tested whether *Cd38* KO mice show LTP impairment in SC-CA1 synapses of their acute hippocampal slices. *Cd38* KO mice showed comparable level of early-phase LTP (E-LTP) induced by a single pulse of high frequency (100 Hz) stimulation to WT controls (average of fEPSP slopes for last 5

min; WT n = 6 ; *Cd38* KO n = 6; unpaired t-test; p = 0.9689; **Fig. 11D**). Consistently, theta-burst stimulation (TBS)-induced E-LTP was not different between genotypes (average of fEPSP slopes for last 5 min; WT n = 7; *Cd38* KO n = 6; unpaired t-test; p = 0.0808**Fig. 11E**). Late-phase LTP (L-LTP) is a form of synaptic plasticity that is dependent on *de novo* protein synthesis and is considered a mechanism for long-lasting memory (Kandel et al., 2014). We induced L-LTP in the hippocampal slices by delivering four pulses of high frequency tetanus in 5 min intervals and found that *Cd38* KO mice showed similar level of L-LTP compared to WT littermates (average for last 10 min; WT n = 10; *Cd38* KO, n = 9; unpaired t-test, p = 0.653, **Fig. 11F**). This result shows that the genetic deletion of CD38 does not affect LTP in hippocampal SC-CA1.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Time (min)
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Time (min)

Figure 12. SC-CA1 synaptic plasticity is unaffected by *Cd38* deletion in acute hippocampal slices

(**A**) Input-output relationship was unchanged in *Cd38* KO mice. (**B**) Paired-pulse ratio was unchanged in *Cd38* KO mice. (**C**) LFS-LTD was induced to similar level in hippocampal slices of both *Cd38* KO and their WT littermates. (**D**) HFS induced E-LTP was unchanged in *Cd38* KO mice. (**E**) TBS induced E-LTP was unchanged in *Cd38* KO mice. (**F**) L-LTP in SC-CA1 synapses was unaffected by *Cd38* deletion.

Cd38 KO mice hippocampal slices show higher response do OXT treatment

What would be the underlying molecular mechanism of learning and memory impairment caused by *Cd38* deletion? It has been demonstrated that social behavior deficits can be successfully rescued via OXT administration (Akther et al., 2013; Jin et al., 2007). Because OXT was known to positively regulate MAPK cascade (Lee et al., 2015), I measured pERK1/2 level in the hippocampal slices of *Cd38* KO and their WT littermates through western blot analysis. Even though OXT signaling is absent in *Cd38* KO mice hippocampus, pERK1/2 level was comparable between *Cd38* KO and WT mice hippocampal slices (pErk1; WT n = 9, *Cd38* KO n = 7; unpaired t-test between ACSF condition, $p = 0.987$, pErk2; WT n = 9, *Cd38* KO n = 7; unpaired t-test between ACSF condition, $p = 0.955$, **Fig. 12**). However, when 0.2 uM of OXT was treated to the hippocampal slices, slices from *Cd38* KO mice showed more sensitive response to OXT treatment, showing elevated pErk1/2 level (pErk1; WT n = 9, *Cd38* KO n = 7; two-way repeated measure ANOVA, interaction between genotype and OXT, $F_{1, 14} = 13.10$, $^{**}p < 0.01$, **Fig. 12A, B**, two-way repeated measure ANOVA, interaction between genotype and OXT, $F_{1, 14}$, $^{*}p < 0.05$, **Fig. 12A, C**).

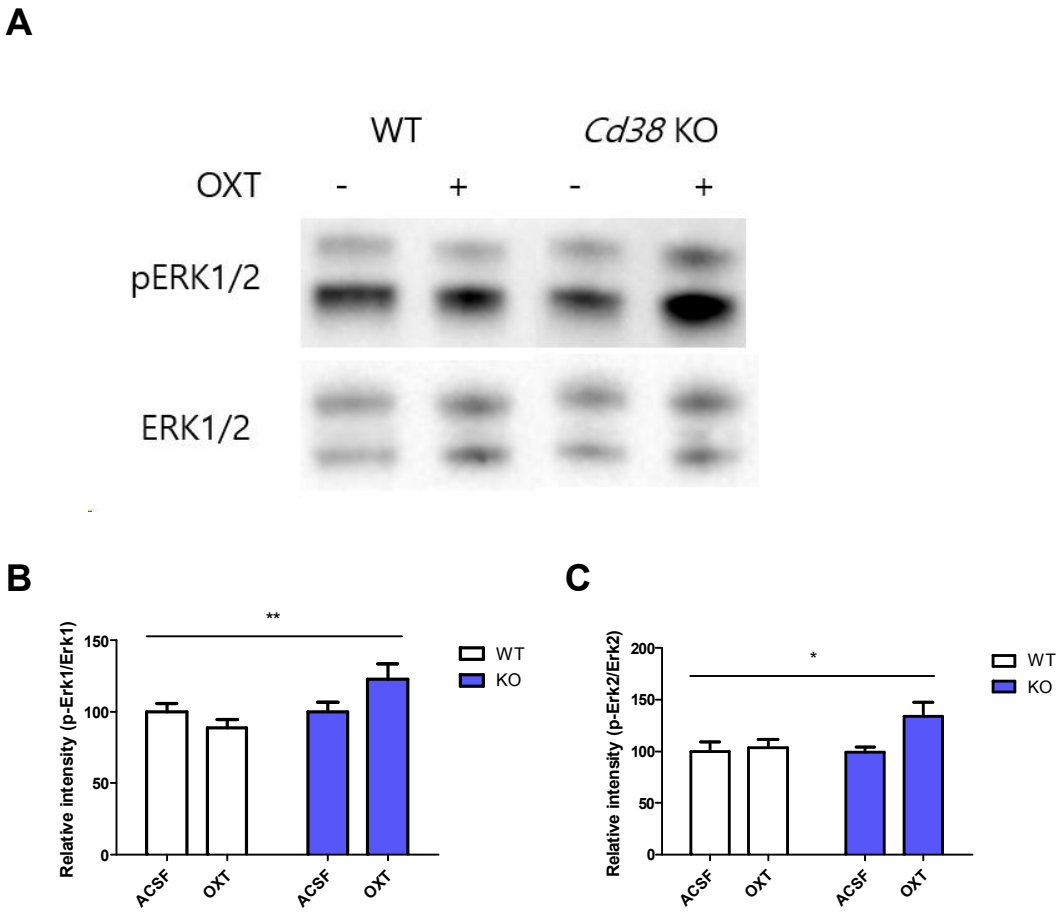

Figure 13. *Cd38* KO mice hippocampal slices are hypersensitive to OXT

(**A**) Representative blot image of pERK1/2 and total ERK1/2 with *Cd38* KO and WT mice hippocampal slices. (**B**) After OXT treatment, *Cd38* KO mice hippocampal slices showed upregulated pERK1 level. (**C**) After OXT treatment, *Cd38* KO mice hippocampal slices showed upregulated pERK2 level.

DISCUSSION

Cd38 mutation is associated with ASD in human patients (Ceroni et al., 2014; Munesue et al., 2010) and *Cd38* KO mice also show impaired social behavior (Higashida et al., 2012; Jin et al., 2007), one of the key phenotype of ASD patients. It is also true that ASD is closely associated with cognitive impairment in humans (Vivanti et al., 2013; Williams et al., 2006). In this study, I have found that *Cd38* KO mice show hippocampus-dependent cognitive impairments as in human ASD patients.

In MWM test, *Cd38* KO mice required more time to escape to hidden platform during the training session. Also, they failed to specify TQ, where the hidden platform was located during the training session, in the two probe test performed after 3 days and 5 days of training. It is noticeable that the time to reach platform of *Cd38* KO mice reached plateau during the fourth and fifth training day as their WT littermates, indicating that Cd38 deletion have not just delayed learning, but rather reduced the learning capacity of the mice. Meanwhile, there was a report that *Cd38* KO mice did not show any difference between their WT littermates during MWM (Young and Kirkland, 2008). However, this report is only measuring the mean distance to the platform of the mice during the training sessions, which is hardly used as an indicator of spatial learning instead of time to escape reach platform. And even with this indicator, there were slight tendency of increased distance in *Cd38* KO mice group. Also, they did not show any probe test data, which can be used as a more rigorous criterion for spatial memory (Crawley, 2007), while I have clearly demonstrated spatial memory impairment in *Cd38* KO mice group. While I could not specify whether memory storage or retrieval was specifically impaired in *Cd38* KO mice, they showed lower freezing level compared to their WT littermates. Next, I tested whether previously reported recognition memory impairment in *Cd38* KO mice was specific in social aspects or rather recognition memory is generally impaired in these mice. The social memory impairment was clearly replicated in the 3-chamber test settings in our lab and further, I found that the

novel object recognition memory, another form of hippocampus-dependent memory, was also impaired in *Cd38* KO mice.

Data from this study clearly and consistently show hippocampus-dependent memory impairment in *Cd38* KO mice. While not all of the hippocampus-dependent memory is impaired in *Cd38* KO mice (Jin et al., 2007), they show a wide-spectrum of cognitive impairment including social memory as in ASD patients (Williams et al., 2006). Then what is the cellular and molecular mechanism underlying these cognitive impairments? We tested the electrophysiological properties of *Cd38* KO mice hippocampal SC-CA1 synapses with field electrophysiology to find the cellular mechanism of cognitive impairment in this mice. From the basal synaptic transmission to synaptic plasticity, hippocampal slices from *Cd38* KO mice were indistinguishable from hippocampal slices from WT littermates. This was a very unexpected result for following reasons. First, *Cd38* KO mice demonstrated clear hippocampus-dependent learning and memory deficit during the behavioral experiments and learning and memory impairment is usually followed by LTP deficits (Lee, 2014; Lee and Silva, 2009). Second, OXT has been known to enhance L-LTP (Lin et al., 2012; Tomizawa et al., 2003). As hippocampi of *Cd38* KO mice should lack of OXT signal input due to OXT release impairment of OXT neurons, I expected the LTP to be impaired in these mice.

If the synaptic plasticity impairment in SC-CA1 synapses was not the cause of cognitive impairment in these mice, what would be possible explanation for this phenomenon? First, as I haven't tested synaptic properties in other synaptic connections in the hippocampus, I cannot rule out the possibility of malfunctioning synaptic connections other than SC-CA1 synapses. Second, even though OXT treatment was able to rescue social behaviors, CD38 may have other roles in hippocampus-dependent learning and memory such as intracellular Ca^{2+} mobilization. Indeed, there have been report that OXT treatment can enhance social behavior while impairing novel object recognition memory (Zhang et al., 2015). According to this study, NOR memory impairment in *Cd38* KO mice cannot be explained with OXT secretion deficiency in these mice. Finally, as *Cd38* KO mice line used in this study is a

conventional KO line, some compensation mechanism to maintain biological homeostasis (yet still insufficient to gain normal learning and memory) might have shaded the underlying cellular and molecular mechanism to be detected. This was shown in my pERK1/2 analysis in the hippocampal slices of *Cd38* KO and WT mice. As OXT is known to upregulate pERK1/2 (Lee et al., 2015), OXT depletion in hippocampal slices in *Cd38* KO mice should reduce pERK1/2 by some extent. However, I found that *Cd38* KO and WT mice hippocampal slices show comparable pERK1/2 level without OXT treatment. Meanwhile, pERK1/2 level in *Cd38* KO mice hippocampal slices was hypersensitive to OXT treatment compared to WT hippocampal slices, indicating that there were some compensational mechanisms to maintain pERK1/2 level without OXT in *Cd38* KO mice hippocampal slices.

While the underlying mechanism remains unraveled, clear hippocampus-dependent learning and memory deficit was detected in *Cd38* KO mice was detected in this study. These data indicate that *Cd38* KO mice can be a good model for studying learning and memory deficit in ASD patients. Further study to reveal and treat the molecular events responsible for cognitive impairments in these mice may aid finding the cure to treat cognitive impairments in ASD patients.

CONCLUSION

In this study, I investigated the molecular event during the learning and memory process with two Cre expressing mice lines and two KO mice.

In Chapter II, I demonstrated that the cognitive impairment due to SHP-2^{D61G} expression was caused by excitatory neurons in the hippocampus. During the MWM test, SHP-2^{D61G} caused learning and memory deficit when expressed in excitatory neurons but not in inhibitory neurons. Also, I found that pERK1/2 was only upregulated in the hippocampi of SHP-2^{D61G} expressing excitatory neurons, implicating that SHP-2^{D61G} have different depending on expressed cell-types. This may because different cell-types have different molecular partners interacting with SHP-2, but further study must be done to evaluate this hypothesis.

In Chapter III, Notch signaling regulation in hippocampus-dependent learning and memory was studied. As *Mib1* KO mice, *Mib2* KO mice showed deficits in hippocampus-dependent learning and memory tasks and the SC-CA1 synapses of these mice showed impaired synaptic plasticity. However, even though the behavioral output was similar, underlying molecular event was different. While *Mib1* KO mice showed lower basal NICD1 level in their hippocampi, *Mib2* KO mice hippocampi showed comparable basal level of NICD1 with the hippocampi of their WT littermates. However, *Mib2* KO mice failed to produce sufficient NICD1 level after mild foot shock was given to the animal, indicating that Mib2 is important in neural activity-dependent Notch signaling regulation.

In Chapter IV, whether *Cd38* KO mice show impaired hippocampus-dependent learning and memory impairment as well as social behavior deficit was tested. the behavioral test results clearly demonstrated that CD38 is critical for proper hippocampal function during hippocampus-dependent learning and memory tests such as CFC, MWM and NOR tests. However, I failed to identify any abnormalities in electrophysiological properties at SC-CA1 synapses of these mice. When I looked at the pERK1/2 level in the hippocampal slices of *Cd38* KO and WT mice, they had comparable level of

pERK1/2 without OXT treatment. However, after OXT treatment, *Cd38* KO mice hippocampi showed greater elevation of pERK1/2. These results may implicate that there were some compensatory mechanisms undergone in *Cd38* KO mice hippocampi to compensate OXT deprivation, veiling electrophysiological and molecular function of CD38 from being revealed in this study.

To fully understand the mechanism of learning and memory in the brain, large scale analysis such as omics study or neural circuit level study and detailed analysis such as cell-type specific analysis or single-gene/molecule target study should be paralleled. This study focused on the detailed mechanism of cognition in mice by analyzing cell-type specificity of cognitive impairment in NS model mice (Chapter II), specific gene regulating Notch signaling pathway during learning (Chapter III) and a single gene related to ASD (Chapter IV). This was the first report of cell-type dependency of cognitive impairment in NS and the role of Mib2 and CD38 in learning and memory. Further study may address more detailed information such as interacting partners of SHP-2 in each cell type, upstream regulators of Mib2 during learning or the molecular mechanism underlying memory impairment of *Cd38* KO mice.

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국문 초록

우리 뇌에서 이루어지는 학습과 기억은 복잡한 네트워크 구조를 이루고 있는 뇌세포들간의 상호작용과 다양한 분자적 신호전달 체계가 정교하게 맞아떨어져야 하는 과정을 통해 이루어 진다. 수많은 연구자들이 학습과 기억에 관여하는 다양한 분자들과 그 신호전달 체계를 연구하였고 많은 성과를 이루었지만, 아직 우리가 밝혀야 할 부분이 많이 남아있는 것이 현실이다. 유전자 산물의 발현을 인위 적으로 조절한 형질전환 동물 모델은 특정한 유전자 산물이 학습과 기억의 형성과정에서 어떠한 역할을 하는지 연구하는데 있어 아주 좋은 도구로 사용되고 있다. 나는 이 연구의 각 장마다 다른 형질전환 동물을 사용하여 학습과 기억의 분자적 메커니즘을 연구해 보았다.

첫 번째 장에서는 누난 증후군 환자들에서 보고된 인지능력 감소가 뇌를 구성하는 세포들 중 어떠한 세포들 때문에 나타나는지를 밝혀보고자 한다. SHP-2^{D61G} 단백질을 발현시키는 돌연변이는 누난 증후군을 일으키는 것으로 알려져 있다. 다른 연구진들이 선행연구로써 이 돌연변이를 선천적으로 지닌 형질 변환 생쥐를 만들어 학계에 보고한 바 있는데, 이 쥐를 이용한 연구들에서 SHP-2^{D61G} 를 발현하는 생쥐들은 누난 증후군 환자들에서 나타나는 발달장애와 인지 장애를 보인다는 것이 보고되었다. 이 연구들에서 주목할만한 점은 생쥐가 지닌 모든 세포에 SHP-2^{D61G} 단백질이 발현되었음에도 모든 세포에서 이상현상이 나타나는 것이 아닌, 일부 세포들에서만 이상현상이 나타나는 것이었다. 하지만, 이 세포들의 정체가 무엇인지, 또 왜 일부 세포들에서만 이상이 나타나는지는 지금까지 보고된 바가 없었다. 나는 이 연구에서 우리 뇌를 구성하는 신경세포들 중, 흥분성신경세포가 SHP-2^{D61G} 단백질 발현에 의한 인지능력 감퇴를 유발한다는 것을 밝혔다. 또한, SHP-2^{D61G} 단백질을 생쥐 해마

CA1 을 구성하고 있는 흥분성신경세포와 억제성신경세포에 발현시켰을 경우, 흥분성신경세포에서만 MAPK 신호전달체계가 과활성 되는 것을 발견하였다.

두 번째 장에서는 mind bomb-2 유전자 결손 생쥐를 이용해 Notch 신호전달 체계가 학습과 기억에서 어떻게 조절되는지를 알아보았다. *Mib* 과 *Mib2* 유전자에서 발현되는 산물은 제브라 피쉬의 초기 발생단계에서 Notch 신호전달 체계를 관장하는 것으로 알려졌다. 생쥐에서도 *Mib1* 과 *Mib2* 유전자가 발현되는데, Mib1 은 발생과정에서 Notch 신호전달 체계를 조절하며, *Mib* 결손 생쥐는 학습과 기억 능력이 정상 생쥐에 비해 떨어지는 것이 우리 실험실의 기존 연구를 통해 밝혀진 바 있다. 한 가지 특이한 사항은 제브라 피쉬에서는 Mib 과 Mib2 가 Notch 신호전달 체계를 조절하는데 있어 중복적인 역할을 수행하는 것으로 알려졌는데, 생쥐에서는 그렇지 않을 가능성을 보였다는 점이다. 생쥐의 발생단계에서 Mib1 과 Mib2 가 발현되는 패턴을 살펴보면 Mib1 은 발생 초기부터 성체가 될 때까지 높은 발현 정도를 보이는 반면, Mib2 는 발생 초기에 거의 발현되지 않다가 성체가 되면서 발현이 높아진다는 점이다. 이렇게 상이한 발현 패턴을 지닌다는 것은 두 단백질의 역할이 다를 수 있음을 시사한다고 볼 수 있다. 따라서 나는 *Mib2* 결손이 *Mib1* 의 결손과 마찬가지로 생쥐의 학습과 기억에 어떠한 영향을 끼치는 지를 보고자 하였다. 이 연구에서 나는 *Mib2* 결손 생쥐가 해마 의존적 학습과 기억에 장애를 보이며, 이 생쥐의 해마에서 나타나는 시냅스 가소성 또한 일반 생쥐에 미치지 못한다는 것을 확인할 수 있었다. *Mib2* 결손 생쥐의 해마에서 Notch 신호전달 체계를 살펴보았을 때, 평소에는 일반 생쥐와 별 차이를 보이지 않는 것을 확인할 수 있었다. 하지만, 약한 전기자극을 쥐의 발에 주는 실험을 진행 하여 학습이 일어나게 한 후에 는 *Mib2* 결손 생쥐에서 Notch 신호전달 체계가 덜 활성화 되어 있는 것을 확인할 수 있었다. 이를 통해 Mib2 가

생쥐 해마에서 신경세포의 활성에 따라 Notch 신호전달 체계를 조절하는 역할을 함을 알 수 있었다.

마지막 세 번째 장에서는 *Cd38* 결손 생쥐의 해마 의존적 학습과 기억을 살펴보았다. CD38 은 세포 내 Ca^{2+} 저장소에서 Ca^{2+} 의 분비를 조절하며, 옥시토신 호르몬이 옥시토신 생성 신경세포의 세포말단에서 분비되는 과정에 필요한 것으로 알려져 있다. 또한, *Cd38* 유전자의 돌연변이는 자폐 증상에 관련이 되어 있는 것으로 사람과 쥐에서 보고된 바 있다. 그러나 자폐증 환자들에게서 학습과 기억 능력 저하가 빈번하게 보고됨에도 불구하고 CD38 이 학습과 기억에 관련되어 있는지는 알려진 바가 없다. *Cd38* 결손 생쥐는 해마 의존적 학습과 기억 능력에 장애를 보였으며, 기존 연구에서 알려진 사회적 인식기억뿐만 아니라 새로운 객체를 인식하는 데에도 장애를 보이는 것을 발견하였다. 다만, 예기치 못하게 SC-CA1 으로 이어지는 시냅스에서 시냅스 가소성을 조사해 보았을 때, *Cd38* 결손 생쥐의 해마 절편은 정상 생쥐 해마 절편과 아무런 차이를 보이지 않았다. 또한, *Cd38* 결손 생쥐의 해마 절편은 정상 생쥐의 해마 절편과 비슷한 수준의 pERK1/2 를 지니고 있었는데, 옥시토신 호르몬을 처리해 주면, *Cd38* 결손 생쥐의 해마절편에서 pERK1/2 가 더 크게 증가하는 것을 볼 수 있었다. 이를 통해 *Cd38* 결손 생쥐의 뇌에서 Cd38 의 부재를 보완해 주기 위한 변화가 일어나 있음을 짐작해 볼 수 있었다.

나는 본 연구에서 세 가지 다른 분자가 학습과 기억에 중요함을 밝혔다. 비록 SHP-2, Mib2, CD38 세 단백질이 세포내 신호전달체계를 직접적으로 공유하는 것은 아니지만, 어느 한나라도 결손 되거나 기능에 이상이 있을 시에 생쥐의 학습과 기억 능력에 직접적인 영향을 주는 것을 확인할 수 있었다. 이 연구를 통한 발견들은 학습과 기억의 분자적 메커니즘이라는 큰 퍼즐의 빈 자리를 메꾸는 동시에 누난

증후군, 자폐스펙트럼 증후군과 다운 증후군 등 각종 인지 장애를 동반한 질병 치료 개발의 초석이 될 것이다.

핵심어: 학습과 기억, 해마, PTPN11, *Mib2*, *Cd38*

학번: 2012-20303