UNIVERSITY OF CALIFORNIA SAN DIEGO

# Signaling Mechanism of Synapse Development and Function

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Xiaojia Wang

Committee in charge:

Professor Yimin Zou, Chair
Professor Yishi Jin
Professor Gentry Patrick

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The thesis of Xiaojia Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

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# PREFACE

This research project was accomplished by collaborating with Dr. Yue Ban, the postdoc in Dr. Yimin Zou's lab. Prickle2$^{E8Q}$ mice were generated by Dr. Ting Yu.

In the experiment of using AAV delivery of guide RNA in the brain of Cre-dependent Cas9 mice to knock out Prickle2, I performed the majority of the perfusion, cryosectioning and synapse quantification.

In the experiment testing the interaction between Prickle1 and Prickle2 in HEK293T cells, I did the initial test.

In the experiment testing colocalization between Prickle1, Prickle2, and PSD-95, Dr. Yue Ban did the colocalization experiment and I analyzed the data.

All the other experiments, including examining the level of synaptic proteins in the crude synaptosome of WT and Prickel2$^{E8Q/E8Q}$ mice, quantifying synapse density in WT, Prickel2$^{E8Q/+}$ and Prickel2$^{E8Q/E8Q}$ mice, and testing the impact of Prickle2$^{E8Q}$ mutation on the interaction between Prickle2 and Vangl2, were accomplished by collaborating with Dr. Yue Ban.

ACKNOWLEDGEMENTS

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I would also like to thank my committee members, Professor Yishi Jin and Professor Gentry Patrick, who kindly accepted the request to be my committee members. Thank Dr. Patrick for being my co-advisor.

I would like to thank Dr. Yue Ban for her guidance and collaboration on the experiments and her advice on my thesis. Yue and I worked closely on the role of Prickle1 and Prickle2 in synapse development and function that every experiment presented in this thesis had symbolized our joint contribution.

I would like to thank Dr. Ting Yu for her technical support which generated Prickle2$^{E8Q}$ mice with CRISPR gene editing, enabling us to start our experiments.

I would like to thank our former lab manager YeoRang Lee and technician Clayton Baker for assisting the virus injection and cryosectioning and thank Akumbir S Grewal for managing mouse colonies. I would like to thank undergraduate students Elaine Chu, Sogoli Sadraeinouri, and Can Liu for their help in genotyping mice colonies.

At last, I would like to express my gratitude to my parents for their unconditional support in the past two years.

Part of the data in this thesis is currently being prepared for submission for publication. Yimin Zou; Yue Ban; Xiaojia Wang. Dr. Yimin Zou is the principal researcher/author on the paper. The author of this thesis and Dr. Yue Ban are the co-authors.

ABSTRACT OF THE THESIS

Signaling Mechanism of Synapse Development and Function

by

Xiaojia Wang

Master of Science in Biology

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Professor Yimin Zou, Chair

The signaling mechanisms of regulating excitatory synapses are the foundation for understanding neural circuit functions and disorders but have not been fully understood. Previously our lab has found an opposing role of core planar cell polarity (PCP) components, Celsr3 and Vangl2, in regulating excitatory synapse formation. Celsr3 is required for excitatory synapse formation, while Vangl2 inhibits excitatory synapse formation. Here, we took a further step to focus on another PCP component called Prickle. We found that the deletion of Prickle homolog 2 (Prickle2/Pk2) in developing and adult mice's dorsal hippocampus and medial prefrontal cortex (mPFC) reduced the density of excitatory synapses. Moreover, Prickle2 p.E8Q mutation (Prickle2$^{E8Q}$), which was found in human patients diagnosed with Autism Spectrum Disorder (ASD), reduced the density of excitatory synapses in the stratum radiatum of mice

CA1 region. The reduced synapse density may be due to the decreased level of NMDA receptors, AMPA receptors, and MAGUK family proteins that are essential for synapse development. We also found that Prickle2$^{E8Q}$ protein was less stable than WT Prickle2, which may be due to the increased interaction between Prickle2 and Vangl2 that enhanced the proteasomal degradation of Prickle2. In addition, in HEK293T cells, Prickle2 interacted with Prickle homolog 1 (Prickle1/Pk1), which contributes to ASD and epilepsy. In mice's stratum radiatum of CA1 region and layer I-III of mPFC, Prickle2 and Prickle1 colocalized with each other while both colocalized with postsynaptic density 95 (PSD-95). These data together indicated Prickle2 plays an essential role in regulating synapse formation and function.

# Chapter 1. Introduction

While the signaling mechanisms regulating the formation of excitatory synapses are the foundation of understanding brain functions underlying neurodevelopmental disorders, they remain elusive. The Wnt signaling pathway is an evolutionarily conserved pathway that plays an essential role in developmental processes including cell fate determination, polarity, migration, neutral patterning and organogenesis (Komiya and Habas, 2008). In addition, recent studies indicate Wnts also play a role in axon remodeling, synaptic assembly and plasticity (Budnik and Salinas, 2011). The Wnt signaling pathway is characterized into the canonical pathway that requires β-catenin, and the non-canonical pathway that is independent of β-catenin, and the non-canonical pathway is further divided into the planar cell polarity (PCP) pathway and the $Ca^{2+}$ pathway (Komiya and Habas, 2008). The PCP proteins are best known for their functions on patterning epithelial cells and migrating cells, and recent studies show PCP components also plays a role in regulating axon guidance and synapse formation (Shafer et al., 2011; Onishi et al., 2013; Thakar et al., 2017).

PCP components were first discovered in Drosophila epithelial cells (Wang and Nathans, 2007). There are six core PCP components asymmetrically distributed at the proximal or distal faces and forming protein complexes at the junctions that connect neighboring epithelial cells (Wang and Nathans, 2007). While Prickle and Vangl are more abundantly accumulated at the proximal face, Frizzle, Dishevelled, and Diego are more abundantly expressed in the distal face (Wang and Nathans, 2007). And Celsr, also called flamingo, is localized at both faces (Wang and Nathans, 2007). WT Drosophila wing hairs are oriented from proximal to distal direction, but mutations in core PCP components randomize the alignment, indicating the importance of PCP components in patterning epithelial cells (Wang and Nathans, 2007).

PCP components are asymmetrically enriched in the presynaptic and postsynaptic compartments of excitatory synapses similarly to the Drosophila wing epithelial cells (Thakar et al., 2017). Vangl2 is enriched in the postsynaptic density, Dishevelled1 and the hyperphosphorylated form of Frizzled3 are enriched in the synaptic membrane fraction, while Celsr3 is expressed in both fractions (Thakar et al., 2017). In addition, Vangl2 and Celsr3 play opposing roles in regulating excitatory synapse formation. Celsr3 is required for synapse formation, and Vangl2 inhibits excitatory synapse formation (Thakar et al., 2017). This study suggests PCP components play different roles in regulating synapse formation and function.

Prickle is another core PCP component that is highly conserved in many species. Four Prickle homologs, Prickle-like protein 1 (Prickle1/Pk1), Prickle-like protein 2 (Prickle2/Pk2), Prickle-like protein 3 (Prickle3/PK3), and Prickle-like protein 4 (Prickle4/PK4) were found in the mouse genome (The UniProt Consortium, 2019). Among the four homologs, Prickle1 and Prickle2 are abundantly expressed in the mice brain (Katoh, M and Katoh, M., 2003). Disruptions of Prickle1 and Prickle2 contribute to neurodevelopmental disorders Autism spectrum disorder (ASD) and epilepsy (Tao et al., 2011). Moreover, Prickle2 loss of function mutation can induce deficits in hippocampal-dependent learning in mouse model, suggesting Prickle2 may play an essential role on the hippocampal synaptic function (Sowers et al., 2013).

Whether Prickle2 plays a role in regulating synapse formation and function remains reclusive, but investigation of that may help develop the diagnosis and treatment of ASD and epilepsy. Therefore, in this study, we tried to investigate the role of Prickle2 in synapse formation and function. Considering the regulatory role of core PCP components Vangl2 and Celsr3 in excitatory synapse formation, we hypothesized that Prickle2 also plays an important role in excitatory synapse formation and function.

## Chapter 2. Prickle2 is Required for Synapse Formation and Maintenance

Prickle2 mutation has been found relative to ASD and epilepsy in human patients. ASD is a neurodevelopmental disorder that is estimated to link to 400-1000 genes (Krishnan et al. 2016; Sowers et al., 2013). Epilepsy is a central nervous disorder that is usually caused by hyperactivation of the brain or a part of the brain and patients with epilepsy show various severity of symptoms (Stafstrom and Carmant, 2015). Prickle2 disruption in mice hippocampus contributes to ASD-like behaviors including reduced social interaction, deficits in hippocampal-related learning, and behavioral inflexibility (Sowers et al., 2013). Moreover, mice with disrupted Prickle2 shows reduced PSD size and decreased miniature excitatory post synaptic current frequency, suggesting the behavioral deficits may due to synaptic abnormalities (Sowers et al., 2013). However, the direct relationship between synapse development and Prickle2 has not been fully discovered yet.

Here, we knocked out Prickle2 using AAV delivery of sgRNA in the brain of Cre-dependent Cas9 knock-in mice. We knocked out Prickle2 in developing mice at postnatal 7 and also knocked out Prickle2 in adult mice at 8 weeks, and we focused on the density change of synapse in the dorsal hippocampus (more specifically, between the Schaffer collaterals and the CA1 region of hippocampus spanning the stratum radiatum) and the layer I-III of medial prefrontal cortex. We used two guide RNA of Prickle2 to target the exon 2 (sgPk2-1) and exon 4 (sgPk2-2). The synapse numbers of Prickle2 knocked-out mice and of the control mice injected with control sgRNA were compared to check if Prickle2 is required for synapse formation and maintenance.

## 2.1 Methods

*Animals*

All animal experiments performed here were approved by the University of California San Diego Institutional Animal Care and Use committee. AAV sgRNA creation and virus injection of the Cre-dependent Cas9 mice used to knock out Prickle2 were mainly done by Dr. Yue Ban.

*Subcellular fractionation*

To obtain the total protein and crude synaptosome fraction (P2) from mouse cortex and hippocampus, subcellular fractionation was performed on mice from postnatal 3 to 5-month-old. Mice cortex and hippocampus were homogenized to 10% (weight/volume) in ice-cold 0.32M sucrose buffer containing 1mM MgCl2, 0.5mM CaCl2, 1mM NaHCO3, and protease inhibitors using 16 strokes with a glass Dounce. The homogenates (H) were spun at 710g for 30 minutes at 4°C to pellet out nuclei and large debris (P1). The supernatant (S1) was further centrifuged at 13800g for 10 min at 4°C to get the crude synaptosome.

*Western blot*

Protein samples were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After incubating with 5% nonfat milk in tris-buffered saline (TBST) for 1 hour, membranes were incubated with primary antibodies (Mouse anti-Prickle2 from MilliporeSigma, Cat# MABN1529; 1:2000) overnight. Membranes were then washed with TBST for 10 minutes three times after primary antibody incubation, and then incubated with secondary antibody (Donkey anti-Mouse IgG from Jackson ImmunoResearch, Cat#715-035-151,

1:10000 in 5% nonfat milk) for one hour, followed with three washes with TBST for 10 minutes. Membranes were incubated with SuperSignal West Pico PLUS Chemiluminescent substrate for 3 minutes and signals were detected in the darkroom.

*Perfusion and cryosectioning*

After virus injection, mice were perfused with 1x phosphate-buffered saline (PBS) first to remove the blood and switched to 4% paraformaldehyde (PFA) in 1x PBS when the color of the liver turned to light yellow. 15 ml 1X PBS and 4% PFA were used for mice at postnatal 7 and postnatal 14. 20 ml 1X PBS and 4% PFA were used for adult animals. After perfusion, mice brains were dissected out and fixed in 4% PFA overnight, then dehydrated with 30% sucrose at 4°C until the brains sank to the bottom of 15 ml tubes. The brains were then embedded with 30% sucrose mixed with optimal cutting temperature (OCT) compound (1:1). If the tissue would not be used immediately, they were wrapped with aluminum foil and stored in -80 °C. Next, the frozen tissues were sectioned with cryostat. Each slice was 40 μm thick and stored at -20 °C fridge in anti-freeze solution (30% ethylaneglycol + 30% glycerol in 1x PBS) until use.

*Immunohistochemistry*

Mouse brains were collected from Prickle2$^{E8Q/E8Q}$, Prickle2$^{E8Q/+}$and WT littermates. Mice were perfused, and their brains were sectioned with cryostat. Each brain slice was 40 μm thick. Mice brain slices were washed with 1xPBS for 5 minutes twice at room temperature to remove OCT or anti-freeze solution. Slices were then incubated with 1% sodium dodecyl sulfate (SDS) in 1xPBS for 5 minutes. After SDS treatment, slices were washed with 1xPBS for 10 minutes twice to remove SDS, and then incubated with the blocking buffer (1% BSA, 5% Donkey serum,

0.1% Tritonx100 in 1x PBS) at room temperature for 1 hour. Next, the slices were incubated with primary antibody (Goat anti-PSD-95 from Abcam, Cat# ab12093, 1:1000; Guinea pig anti-Bassoon from Synaptic Systems, Cat# 141 004, 1:500) diluted in AB dilution buffer (0.1% triton in 1xPBS with 0.25% BSA and 1% Donkey serum) at 4 °C overnight. On the next day, brain slices were washed with 0.1% triton in 1xPBS for 10 minutes three times and then incubated with secondary antibodies (Donkey anti-Goat IgG Alexa Fluor 568 from ThermoFisher Scientific, Cat# A-11057, 1:300; Donkey anti Guinea pig Alexa Fluor 488 from Jackson Immunoresarch, Cat# 706-545-148, 1:300) diluted in AB dilution buffer for 2 hours at room temperature. After secondary antibody incubation, slices were washed with PBST (0.1% triton in 1xPBS) for 10 minutes and incubated with DAPI for 5 minutes. Then slices were washed with PBST twice, and then mounted on plain glass slides with Fluoromount Southern Biotech for imaging. Images were analyzed with ImageJ, and the number of synapses was quantified with the Synapse counter downloaded from GitHub.

## 2.2 Results

*Prickle2 is constantly expressed in mouse hippocampus and cortex through the early developmental stages to adulthood*

Before testing the role of Prickle2 in regulating synapse formation, we checked the expression level of Prickle2 in the hippocampus and cortex of mice at different ages. WT mice from postnatal 3 to 5-month-old were selected. Mice's cortex and hippocampus were extracted and fractionated to get protein homogenate and crude synaptosome. The Prickle2 expression level was examined with western blot (Figure 1). As the result, Prickle2 expression was detected in both developing and adult mice hippocampus and cortex, suggesting Prickle2 is constantly expressed in mice's homogenate and crude synaptosome through the early developmental stages to adulthood (Figure 1).

*Prickle2 is required for synapse formation and maintenance*

To check the role of Prickle2 in regulating synapse development, we first knocked out Prickle2 using AAV delivery of sgRNA in the brain of Cre-dependent Cas9 knock-in mice. We focused on the density change of excitatory synapses in the mouse dorsal hippocampus and medial prefrontal cortex. Cas9 was widely expressed in the mouse dorsal hippocampus and medial prefrontal cortex, shown in green (Figure 2A).

To test if Prickle2 is required for excitatory synapse formation, Prickle2 was knocked out at postnatal 7, and the density of synapses was quantified at postnatal 21. In Prickle2 knocked-out mice, the number of presynaptic puncta, postsynaptic puncta, and colocalized puncta were all decreased in both the dorsal hippocampus and medial prefrontal cortex, suggesting Prickle2 is required for excitatory synapse formation (Figure 2B, 2C)

To test if Prickle2 is required for synapse maintenance, Prickle2 was knocked out at 8 weeks and the synapse number was quantified at 11.5 weeks (Figure 3A). The result showed a reduced number of presynaptic puncta, postsynaptic puncta, and colocalized puncta in Prickle2-deleted mice, suggesting Prickle2 is also required for excitatory synapse maintenance (Figure 3B).

**Prickle2 Expression Profile**

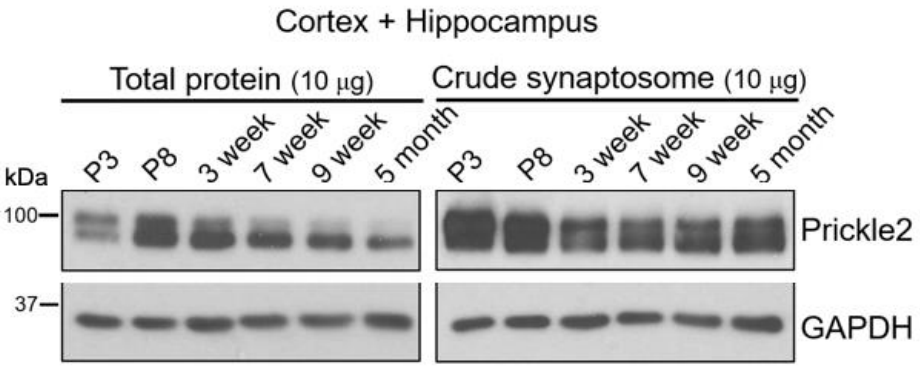


Figure 1. Prickle2 is constantly expressed in mice's cortex and hippocampus. Prickle2 expression level in homogenate fraction and crude synaptosome fraction from WT mice at different ages. 10 μg proteins were added. (n=3).

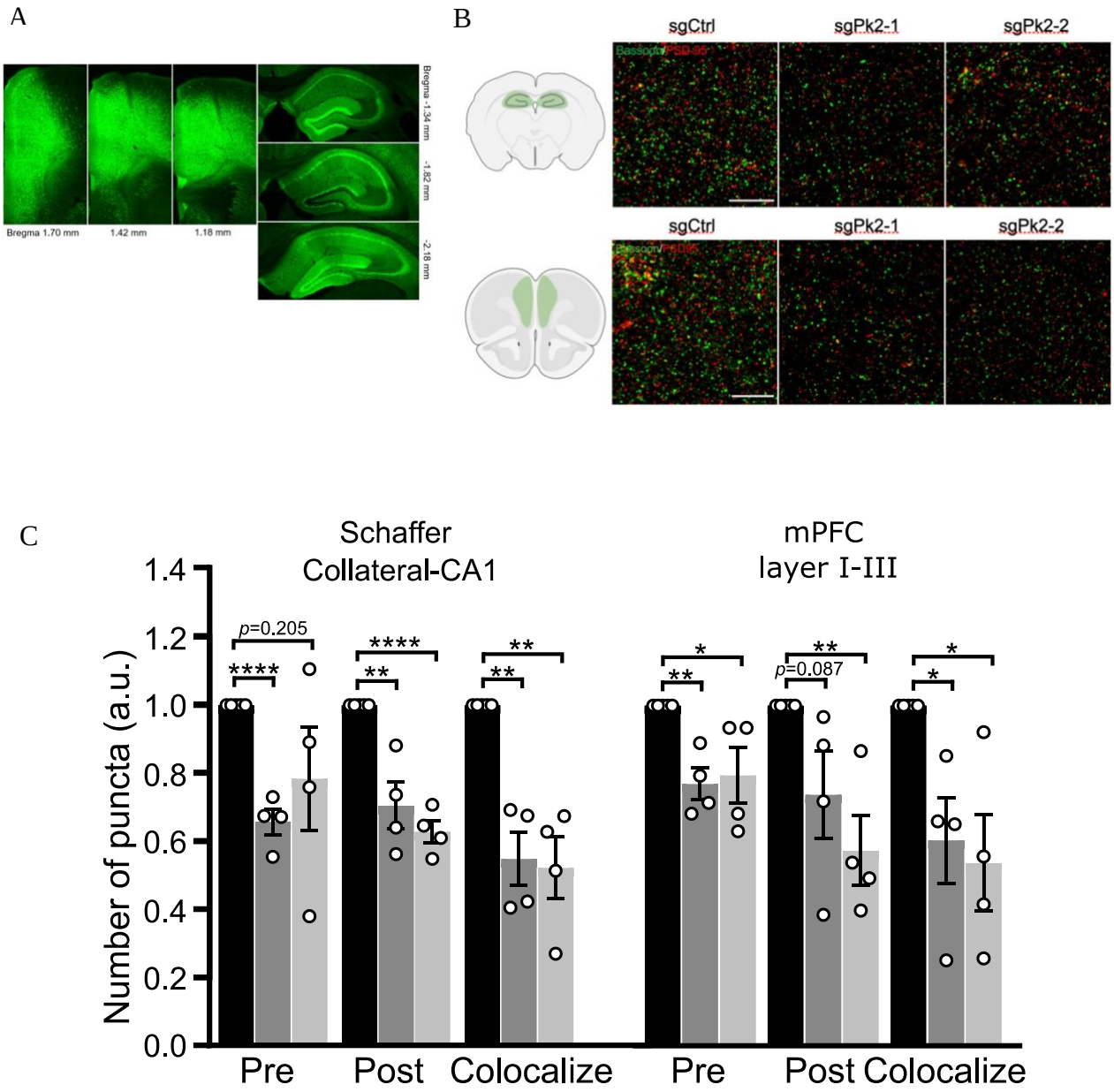


Figure 2. Prickle2 is required for excitatory synapse formation. (A) Expression of Cas9 (green) in medial prefrontal cortex and dorsal hippocampus of Cre-dependent Cas9 mice after AAV virus was injected. (B) Expression of presynaptic marker Bassoon (green) and postsynaptic marker PSD-95 (red) in dorsal hippocampus and medial prefrontal cortex of Prickle2 knocked-out mice and the control mice. (C) Quantification of presynaptic puncta, postsynaptic puncta, and co-localized puncta shown in (B) in dorsal hippocampus and medial prefrontal cortex. (n = 4). * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.001, **** indicates p-value < 0.0001.

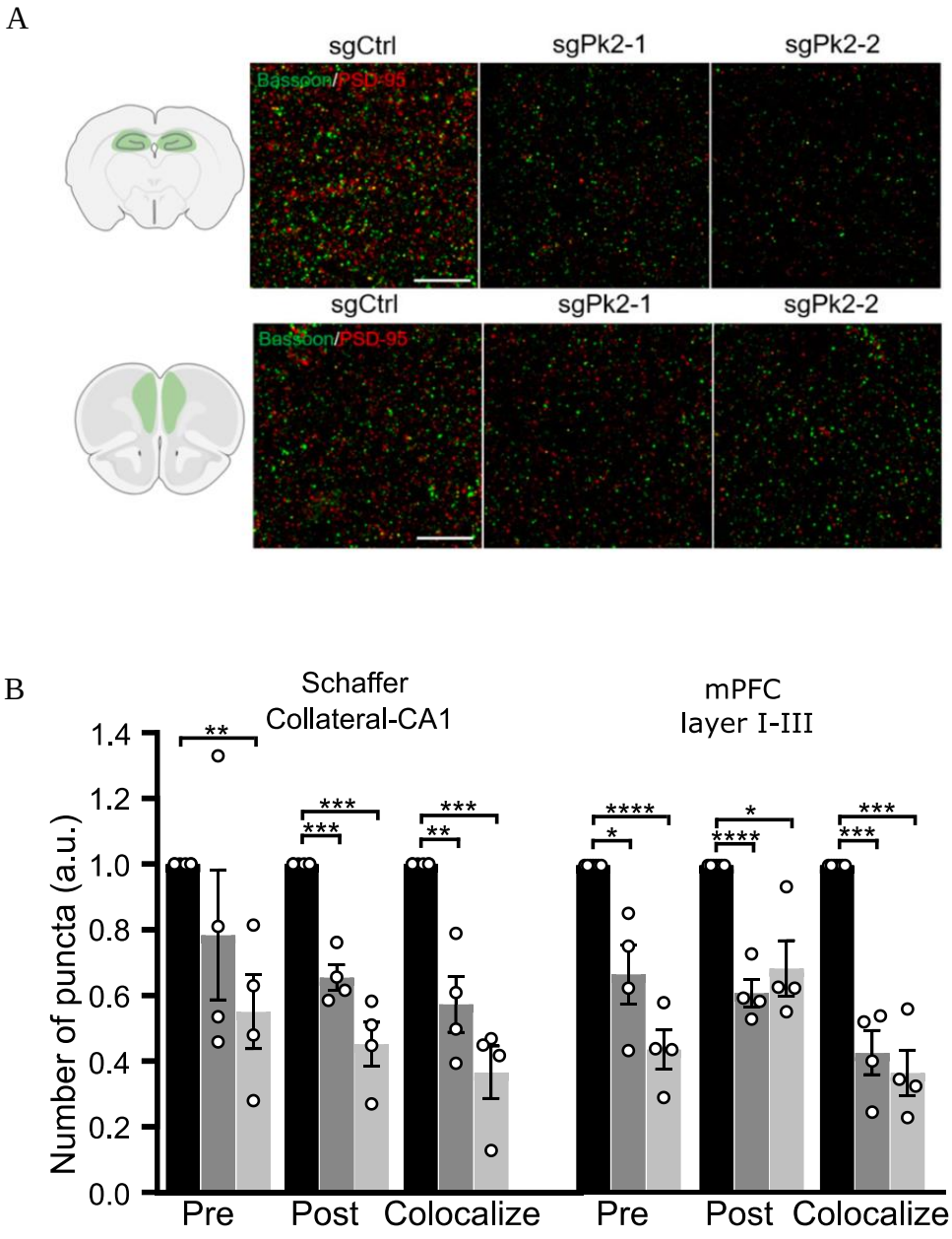


Figure 3. Prickle2 is required for excitatory synapse maintenance. (A) Expression of presynaptic marker Bassoon (green), postsynaptic marker PSD-95 (red), and co-localized puncta. (B) Quantification of presynaptic puncta, postsynaptic puncta, and co-localized puncta in the dorsal hippocampus and in the medial prefrontal cortex. (n = 4). * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.001, **** indicates p-value < 0.0001.

## 2.3 Discussion

Our results showed a constant expression level of Prickle2 in the hippocampus and cortex of mice aged from postnatal 3 to 5-month-old, suggesting Prickle2 may play a role in both developing and adult mice. However, our data does not rule out the possibility that the expression level of Prickle2 would decrease or even disappear in older mice. The average lifespan of mice is about 2 to 3 years, so future studies may apply to test whether Prickle2 is expressed in mouse genome throughout their whole lifespan.

Moreover, deletion of Prickle2 reduced the level of excitatory synapses in mice dorsal hippocampus and medial prefrontal cortex of developing and adult mice. This result is consistent to the reduced synaptic activity and deficits in hippocampal-related behavior of Prickle2-dysruped mice (Sowers et al., 2013). Together, these finding suggest Prickle2 is required for both excitatory synapse formation and maintenance. And next, we want to confirm the necessity of Prickle2 in synapse formation with Prickle2 mutant mice.

## Chapter 3. Prickle2 $^{E8Q/E8Q}$ Mice Shows Abnormal Synapse Formation and Function

Prickle2 is concentrated at the postsynaptic density fraction in mice hippocampal CA1 region (Hida et al., 2011). In addition, Prickle2 interacts with PSD-95 and forms protein complexes with PSD-95 and NMDA receptors (Hida et al., 2011). The NMDA receptors are glutamate receptors that play essential roles in neuronal development and plasticity (Caroll and Zukin, 2002). PSD-95 is one of the MAGUK family proteins that act as the major scaffold proteins in the postsynaptic density, and PSD-95 also stabilizes the expression of NMDA receptors (Won et al., 2016; Chen et al., 2011).

Several Prickle2 mutations has been found in patients with ASD or epilepsy, and some mutations were found in both ASD and epilepsy patients. Prickle2 p.E8Q mutation (Prickle2$^{E8Q}$) was found in patients with ASD, and Prickle2 p. R148H, p. V605D mutations were found in patients with epilepsy (Bassuk et al., 2008; Tao et al., 2011; Sowers et al., 2013). In addition, Prickle2 p. V153I mutation and Prickle2 deletion were found in both ASD and epilepsy patients (Bassuk et al., 2008; Tao et al., 2011; Sowers et al., 2013).

Here, we generated mice carrying Prickle2$^{E8Q}$ using CRISPR gene editing to check if the mutation affects the function of Prickle2. If it affects, we would further check whether it leads to density change in excitatory synapse and affects the density of synaptic proteins including AMPA receptors, NMDA receptors and MAGUK family proteins, and also Vangl2 and Celsr3, which regulate synapse formation.

## 3.1 Methods

The protocol of Perfusion and cryosectioning, Immunohistochemistry (IHC), Subcellular fractionation and western blots (WB) were the same as what described in Chapter 2. Antibodies used are listed in Table 1.

### *Animals*

All animal experiments performed here were approved by the University of California San Diego Institutional Animal Care and Use committee. Prickle2$^{E8Q}$ mice were generated by Dr. Ting Yu using CRISPR gene editing.

### *Transfection*

On the day before transfection, the concentration of HEK293T cells was measured. Cells were plated into each well in a 6-well plate with 2 ml Dulbecco's Modified Eagle's medium (DMEM. Cell density was 30% -50% confluent on the day of transfection. DNA was diluted in 100 µl of Reduced-Serum Medium (Opti-MEM) and mixed with polyethyleneimine (PEI) diluted in 100ul Opti-MEM. The cells were then incubated with the DNA solution at 37 °C for 48 hours, following by lysate extraction.

### *HEK293T cell Lysate extraction and Immunoprecipitation*

HEK293T cells were washed with 500 µl 1xPBS and collected with 1ml IP lysis buffer (20mM Tris (PH 7.5); 150mM NaCl; 2mM EGTA; 0.1% Triton x100; 1xProtease and Phosphatase inhibitor Cocktail (ThermoFisher Scientific, Cat#78445)) at 4 °C for 30 minutes. Cell lysate was collected via centrifugation at 13200 RPM for 15 minutes at 4 °C. 50 µl of the

lysate used as input western blot was created by mixing the cell lysate with 4xLDS sample buffer (3:1) and 2M DTT (40:1). The rest of the cell lysate was incubated with antibody (see Table1 for the detail of antibody used) for 2 hours at 4 °C. Beads were washed with PBS once and washed with IP lysis buffer twice 30 minutes before adding into the cell lysate. The cell lysate was incubated with beads for 2 hours at 4 °C. After incubation, cell lysate with beads was collected by centrifugation at 3000 RPM for 3 minutes, and the supernatant was aspirated. The beads were then washed three times with IP wash buffer (0.5% Triton x 100) at 3000 RPM for 3 minutes each. The supernatant was aspirated and 1xLDS buffer with 2M DTT (1:40) were added into the tube containing beads and denatured at 95 °C for 5 minutes for western blot.

Table1. Antibodies used in WB and IHC

| **Primary antibodies** | | | |
|---|---|---|---|
| Antibody | Company | Cat # | Application |
| Mouse anti-Prickle2 | MilliporeSigma | MABN1529 | WB (1:2000) |
| Goat anti-Vangl2 | Santa Cruz biotechnology | sc-46561 | WB (1:500) |
| Rabbit anti-Celsr3 | Zou lab customized | / | WB (1:1000) |
| Mouse anti-GluR1 | MilliporeSigma | MAB2263 | WB (1:10000) |
| Mouse anti-GluR2 | ThermoFisher Scientific | 32-0300 | WB (1:500) |
| Mouse anti-N1 | Antibodies Incorporated | 75-272 | WB (1:1000) |
| Mouse anti-NR2B | NeuroMab | 75-097 | WB (1:2000) |
| Mouse anti-PSD-93 | MilliporeSigma | MABN497 | WB (1:1000) |
| Mouse anti-SAP-97 | Antibodies Incorporated | 75-030 | WB (1:1000) |
| Mouse anti-SAP-102 | Antibodies Incorporated | 75-058 | WB (1:1000) |
| Goat anti-PSD-95 | Abcam | ab12093 | WB (1:1000) |
| Guinea pig anti-Bassoon | Synaptic Systems | 141 004 | IHC (1:500) |
| Rabbit anti-Flag | MilliporeSigma | F7425 | WB (1:1000) |
| **Secondary antibodies** | | | |
| Antibody | Company | Cat # | Application |
| Donkey anti-Goat IgG | Jackson ImmunoResearch | 705-035-147 | WB (1:10000) |
| Donkey anti-Rabbit IgG | Jackson ImmunoResearch | 711-035-152 | WB (1:10000) |
| Donkey anti-Mouse IgG | Jackson ImmunoResearch | 715-035-151 | WB (1:10000) |
| Donkey anti-Goat IgG Alexa Fluor 568 | ThermoFisher Scientific | A-11057 | IHC (1:300) |
| Donkey anti Guinea pig Alexa Fluor 488 | Jackson immunoresarch | 706-545-148 | IHC (1:300) |

## 3.2 Results

### *Prickle2$^{E8Q/E8Q}$ mice shows Hippocampal Synaptic Dysfunction at postnatal 14*

We checked the excitatory synapse density in the stratum radiatum of mice CA1 region at postnatal 14, which is the peak of synapse formation (Figure 4A). As the result, Prickle2$^{E8Q/E8Q}$ mice showed dramatic reductions in the density of excitatory synapses compare to their WT littermates, suggesting Prickle2$^{E8Q}$ mutation may disrupt the formation of excitatory synapses in vivo (Figure 4B)

### *Prickle2 $^{E8Q/E8Q}$ mice shows changed level of synaptic proteins*

To further understand the impact of Prickle2$^{E8Q}$ on synaptic formation, the level of Vangl2, Celsr3, and synaptic proteins in the crude synaptosome fraction was quantified. The brains of WT and Prickle2$^{E8Q/E8Q}$ mice at P14 were harvested and fractionated to get the crude synaptosome (Figure 5A). In Prickle2$^{E8Q/E8Q}$ mice, the level of Vangl2 was increased (Figure 5B). Since Vangl2 inhibits excitatory synapse formation, the increased level of Vangl2 may explain why Prickle2$^{E8Q}$ mutation reduced the number of excitatory synapses in Prickle2$^{E8Q/E8Q}$ mice (Figure 4).

Moreover, since Prickle2 interacts with PSD-95 and forms a complex with PSD-95 and NMDA receptors, we think Prickle2$^{E8Q}$ mutation may disrupt the interaction and alter the concentration of synaptic proteins (Hida et al., 2011). PSD-95 is one of the most abundant proteins in the MAGUK family, which is a group of scaffold proteins that are highly enriched in synapses and are responsible for organizing the numerous protein complexes required for synaptic development and plasticity (Chen et al., 2011). We tested the expression level of the members of the MAGUK family, PSD-95, PSD-93, SAP97, and SAP102 in WT and

Prickle2$^{E8Q/E8Q}$ mice. The level of all the proteins in the MAGUK family were more or less reduced, though for SAP102 there was little change (Figure 5B).

The expression levels of subunits of NMDA receptors, NR2B and NR1, and AMPA receptors, GLUR1 and GLUR2, were also tested. The expression levels of all of the NMDA and AMPA receptors tested were decreased in Prickle2$^{E8Q/E8Q}$ mice (Figure 5B). These level changes may alter synaptic protein organization and lead to defects of synaptic development and signaling.

*Prickle2$^{E8Q}$ increases the interaction between Prickle2 and Vangl2*

We also saw a decrease in Prickle2 level in the crude synaptosome of P14 mice (Figure 5B). This reminds us of a previous study that showed the interaction between Vangl2 and Prickle2 leads to proteasomal degradation of Prickle2 (Nagaoka et al., 2019). Indeed, Prickle2 degradation was increased when Vangl2 was present in HEK293T cells (Figure 6A). Since Prickle2 $^{E8Q}$ protein was less stable than WT Prickle2 in mice crude synaptosome fraction, we also tested the expression level of Prickle2 $^{E8Q}$ with Vangl2 in HEK293T cells, and we saw less expression level of Prickle2 $^{E8Q}$ compare to WT Prickle2 (Figure 6B). This reduced Prickle2 level indicates Prickle2$^{E8Q}$ mutation may increase the interaction between Prickle2 and Vangl2 and lead to further degradation of Prickle2. To confirm that, immunoprecipitations of Prickle2 and Prickle2$^{E8Q}$ with Vangl2 were performed. Compare to the level of WT Prickle2, more Prickle2$^{E8Q}$ protein was precipitated with Vangl2, suggesting the interaction between Prickle2 and Vangl2 did increase with the Prickle2$^{E8Q}$ mutation and therefore increased the proteasomal degradation of Prickle2 (Figure 6C).

Moreover, since both Vangl2 and Celsr3 play regulatory roles in synapse formation, we also tested whether Prickle2$^{E8Q}$ changes the interaction between Celsr3 and Prickle2 by immunoprecipitating WT Prickle2 and Prickle2 $^{E8Q}$ with Celsr3 (Figure 6C). The interaction between Celsr3 and Prickle2 seemed slightly weaker in Prickle2$^{E8Q}$ samples compared to WT Prickle2 samples, but the difference was not as significant as the interaction between Vangl2 and Celsr3 (Figure 6C).

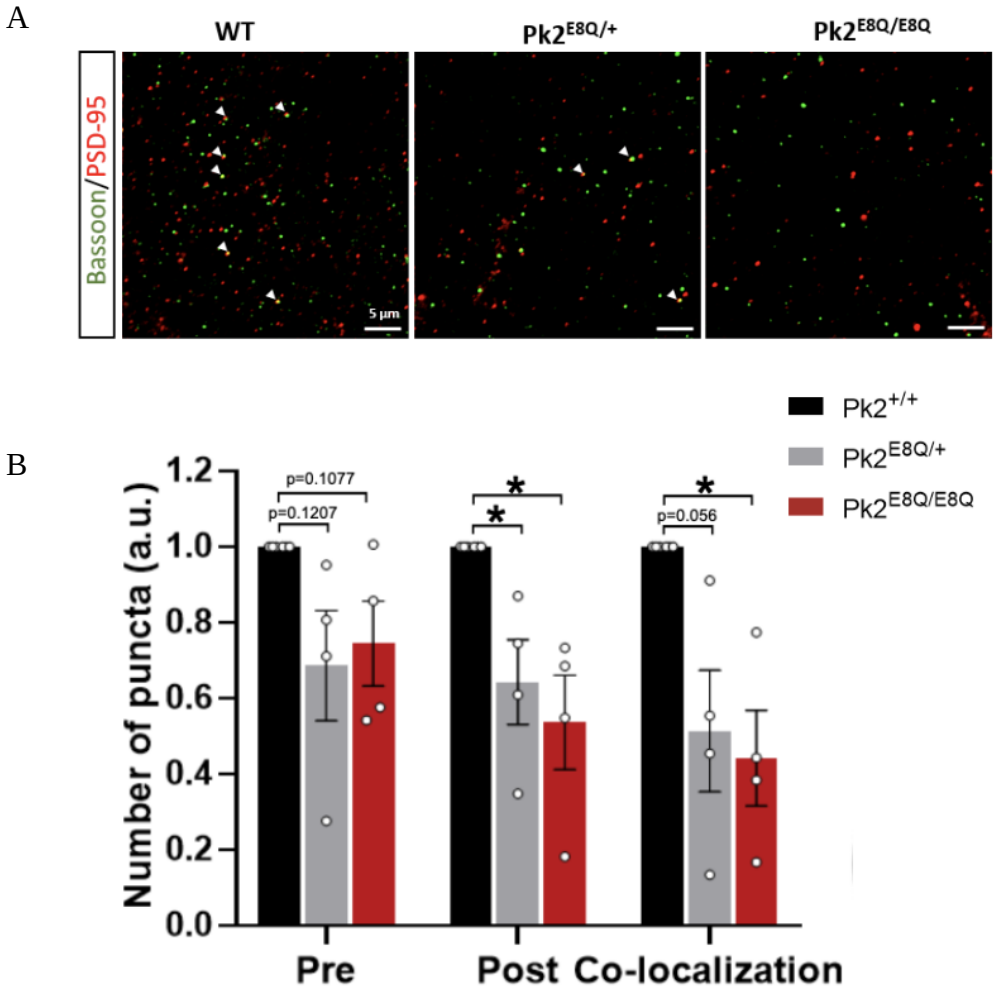


Figure 4. Prickle2$^{E8Q/E8Q}$ mice shows reduced number of excitatory synapse. (A) Expression of presynaptic puncta Bassoon (green), postsynaptic puncta PSD-95 (red), and colocalized puncta in the stratum radiatum of WT, Prickle2$^{E8Q/+}$, and Prickle2$^{E8Q/E8Q}$ mice. (B) Quantification of presynaptic puncta, postsynaptic puncta, and colocalized puncta. (n=4). * indicates p-value < 0.05.

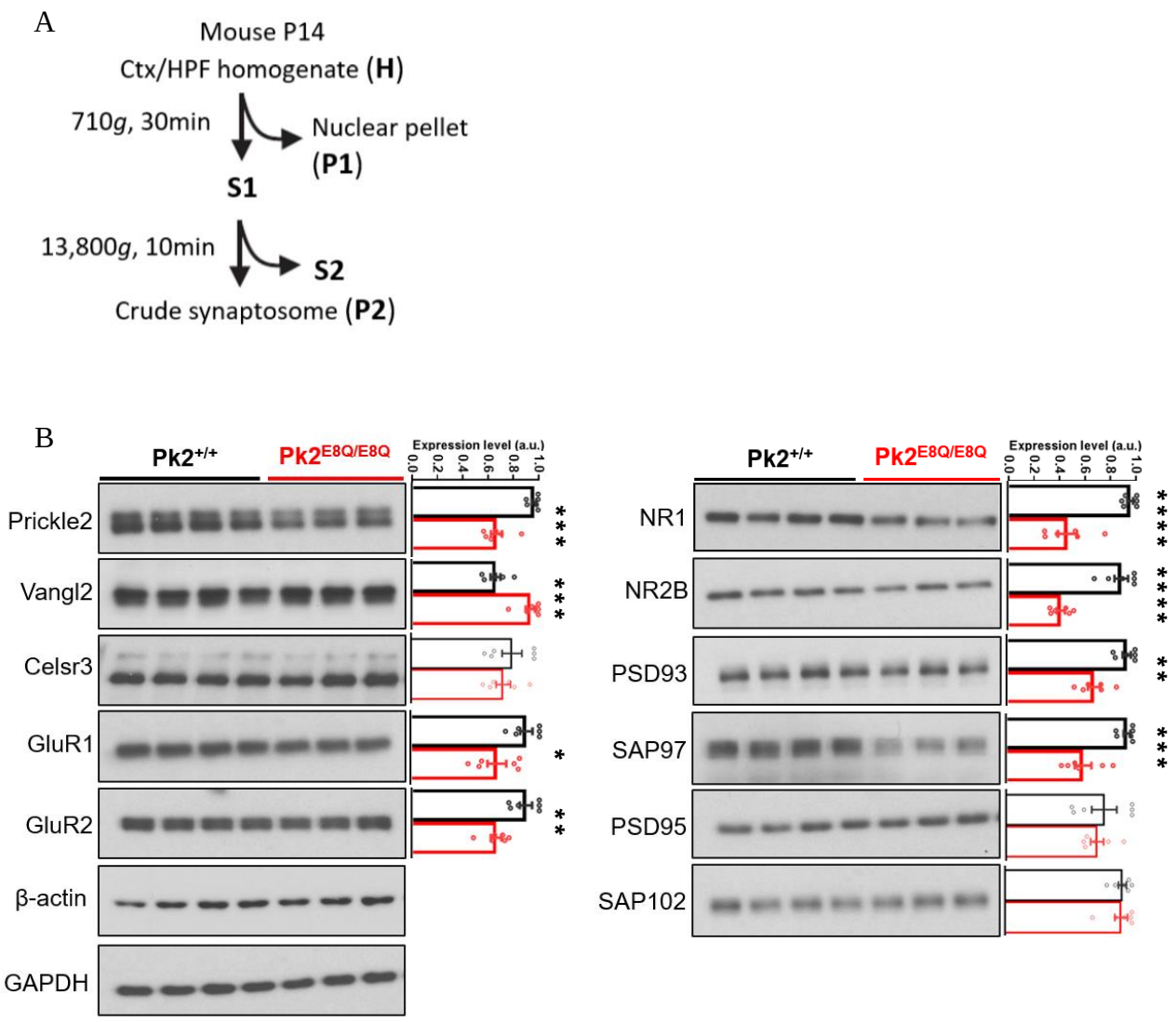


Figure 5. Prickle2$^{E8Q/E8Q}$ mice shows level change of synaptic proteins. (A) Asaay of crude synaptosome extraction. (B) Expression level of synaptic proteins in crude synaptosome extracted from Prickle2 WT and Prickle2 $^{E8Q/E8Q}$ mice. (n= 6). * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.001, **** indicates p-value < 0.0001.

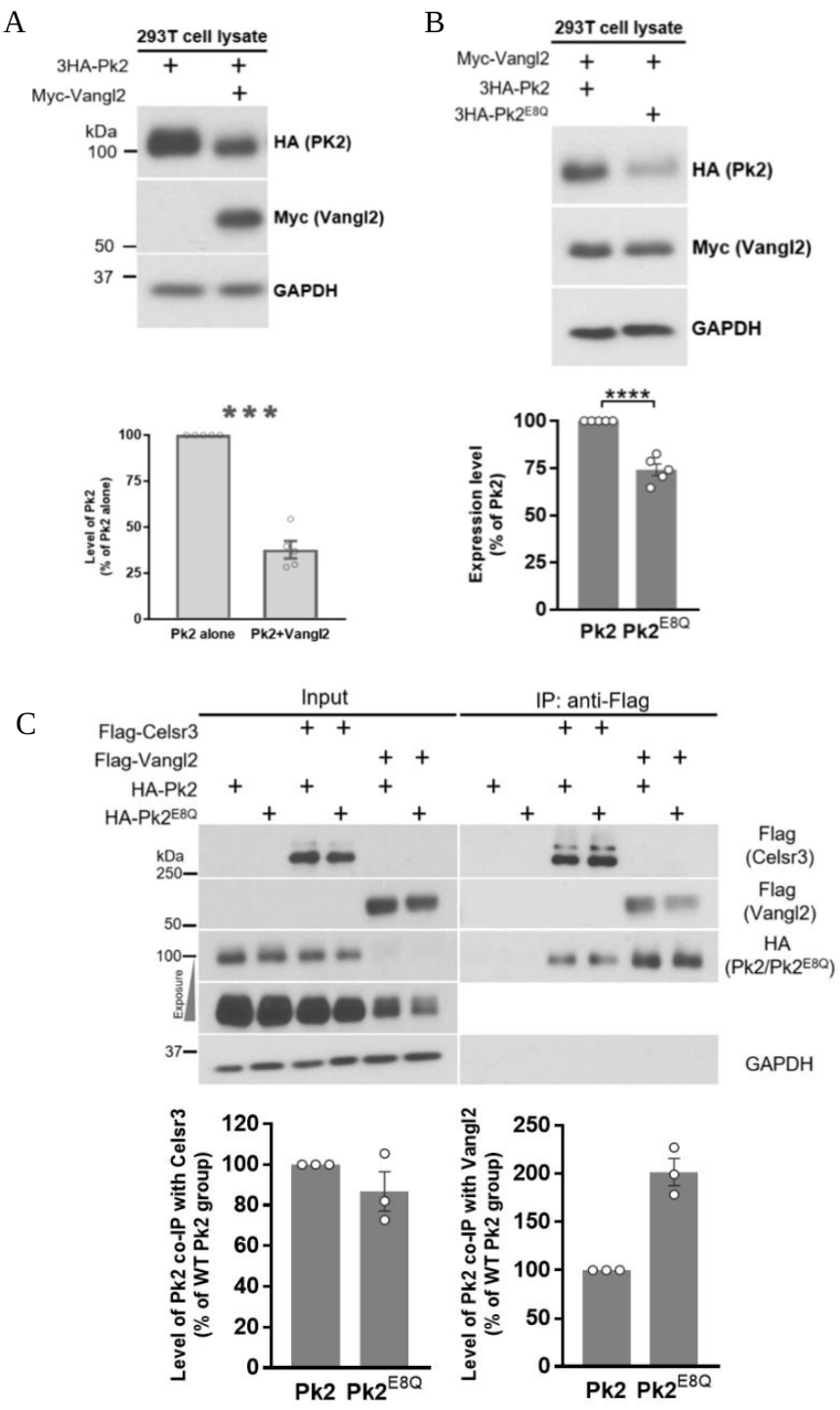


Figure 6. Prickle2 $^{E8Q}$ mutation increases the interaction between Prickle2 and Vangl2. (A) Expression levels of Prickle2 alone and of Prickle2 with Vangl2 in HEK293T cells. (n=5). (B) Expression levels of Prickle2 with Vangl2 and of Prickle2$^{E8Q}$ with Vangl2 in HEK293T cells. (n=5). (C) Immunoprecipitation using anti-Flag antibody and the quantification. Prickle2 and Prickle2$^{E8Q}$ were precipitated with Flag-Celsr3 or Flag-Vangl2. (n=3). *** indicates p-value < 0.001, **** indicates p-value < 0.0001.

## 3.3 Discussion

The result showed that Prickle2$^{E8Q}$ mutation reduced the number of excitatory synapses, probably due to the reduced level of subunits of NMDA receptors, AMPA receptors, and MAGUK family proteins that play essential roles in excitatory neurotransmission and synapse formation (Carroll and Zukin, 2002; Chater and Goda, 2014). However, the relationship between Prickle2 and these proteins and how Prickle2 changes their expression levels remain elusive. Since NMDA receptors and AMPA receptors are glutamate receptors that regulate excitatory neurotransmissions and synaptic plasticity, while MAGUK family proteins play essential roles in synapse formation, it would be of great interest for future research to determine whether Prickle2 can directly interact with these proteins and whether Prickle2 is involved in the formation of these proteins (Carrol and Zukin, 2002; Chater and Goda, 2014). The results may lead to the discovery that Prickle2 contributes to the regulation of higher brain functions such as neurotransmissions and synaptic plasticity mediated by NMDA receptors, AMPA receptors, and MAGUK family proteins. Another possible explanation for the reduced excitatory synapse density in Prickle2$^{E8Q/E8Q}$ mice might be the increased level of Vangl2, which inhibits synapse formation.

Moreover, Prickle2$^{E8Q}$ mutation also makes Prickle2$^{E8Q}$ less stable than WT Prickle2 both in vitro and in vivo. A previous study has found that the interaction between Vangl2 and Prickle2 leads to proteasomal degradation of Prickle2 (Nagaoka et al., 2019). Indeed, our result showed Prickle2$^{E8Q}$ mutation increased the interaction between Prickle2 and Vangl2, which may lead to further degradation of Prickle2 and therefore makes Prickle2 less stable. Therefore, mutation on Prickle2 may disrupt normal Prickle2 function and leads to abnormal synapse development.

## Chapter 4. A Possible Mechanism of How Prickle2 Functions at Synapses

Prickle1 and Prickle2 are abundantly expressed in the mice brain and they share similar structure (Katoh, M and Katoh, M., 2003). Both Prickle1 and Prickle2 contain one N-terminal Prickle Espinas Testin (PET) domain and three Lin1-1, IS1-1, and Mec-3 (LIM) domains that are found homologous in species including human, drosophila, and mouse (Katoh, M and Katoh, M., 2003). The PET domain is an approximate 100 amino acid motif and has been suggested to regulate protein-protein interactions and engage in the membrane insertion of Prickle (Sweede et al., 2008). Whereas LIM is a double zinc-finger domain that increases protein folding and tolerance to proteolysis and temperature change and may facilitate and stabilize the membrane insertion of the PET domain (Sweede et al. 2008; Katoh, M and Katoh, M., 2003).

Moreover, similar to Prickle2, mutation of Prickle1 has also been found to contribute to ASD and epilepsy. Endogenous Prickle1 interacts with a protein called SYNAPSIN1 that is involved in synaptogenesis and formation of synaptic vesicles, and Prickle1 mutation ($Prickle1^{+/-}$) can disrupt the interaction and cause ASD (Paemka et al., 2013). In addition, homozygous mutation in human Prickle1 ($Prickle1^{R104Q/R104Q}$) disrupts normal Prickle1 function and leads to progressive myoclonic seizure (Bassuk et al., 2008).

Our unpublished data shows Prickle1 and Prickle2 are both localized at postsynaptic density. To investigate the relationship between Prickle1 and Prickle2, we tested the interaction between Prickle1 and Prickle2 in HEK293T cells, and checked the colocalization between Prickle1, Prickle2, and PSD-95 in mice hippocampus and mPFC. Understanding the relationship between these proteins will shed light on a deeper mechanism of how Prickle2 regulates the function of synapses.

## 4.1 Methods

The protocol of Transfection, HEK293T cell Lysate extraction, Immunoprecipitation and western blot are the same as what described in Chapter 2 and Chapter3. Antibodies used for western blot are shown in Table 2:

Table 2. Antibodies used for testing the interaction between Prickle1 and Prickle2

| **Primary antibodies** | | | |
|---|---|---|---|
| Antibody | Company | Cat # | Application |
| Rabbit anti-Prickle1 | Proteintech | 22589-1-AP | WB (1:2000) |
| Mouse anti-Prickle2 | MilliporeSigma | MABN1529 | WB (1:2000) |
| Mouse anti-HA | BioLegend | 901513 | WB (1:1000) |
| **Secondary antibodies** | | | |
| Antibody | Company | Cat # | Application |
| Donkey anti-Rabbit IgG | Jackson ImmunoResearch | 711-035-152 | WB (1:10000) |
| Donkey anti-Mouse IgG | Jackson ImmunoResearch | 715-035-151 | WB (1:10000) |

*Analyzing Colocalization between Prickle1, Prickle2, and PSD-95*

To test whether Prickle1, Prickle2, and PSD-95 colocalize with each other, we used ImageJ and counted the number of synapses with Synapse counter downloaded from GitHub.

## 4.2 Results

*Prickle1 interacts with Prickle2 in HEK293T cells and colocalizes with Prickle2 in mice's stratum radiatum of CA1 region and I-III layers of mPFC*

Previous studies have shown that Prickle1 contributes to two neurodevelopmental disorders ASD and epilepsy, and our unpublished data shows both Prickle1 and Prickle2 are expressed in the postsynaptic density. However, the relationship between Prickle1 and Prickle2 remains elusive. Here, our immunoprecipitation assay showed that Prickle1 interacted with Prickle2 in HEK293T cells (Figure 7A). Moreover, Prickle and Prickle2 were colocalized with each other in the mouse genome (Figure 7B). We found that 73% Prickle1 was colocalized with 30% Prickle2 in the stratum radatum of CA1 region, and that 71% Prickle1 was colocalized with 30% Prickle2 in the layer I-III of mPFC (Figure 7C). The density of Prickle2 in these brain regions was much higher than the densities of Prickle1 and of PSD-95, leading to a different percentage of Prickle1 and Prickle2 that colocalized with each other (Figure 7C).

In addition, our result showed Prickle1 and Prickle2 were colocalized with PSD-95. In the stratum radiatum of CA1 region, 37% Prickle1 was colocalized with 33% PSD-95, and 30% Prickle2 was colocalized with 67% PSD-95 (Figure 7C). In the mPFC region, 42% Prickle1 was colocalized with 31% PSD 95, and 34% Prickle2 was colocalized with 60% PSD-95 (Figure 7C). However, only a small amount of Prickle1 was colocalized with PSD-95 without the presence of Prickle2, and the majority of Prickle1 colocalized with PSD-95 was also colocalized with Prickle2 (Figure 7C).

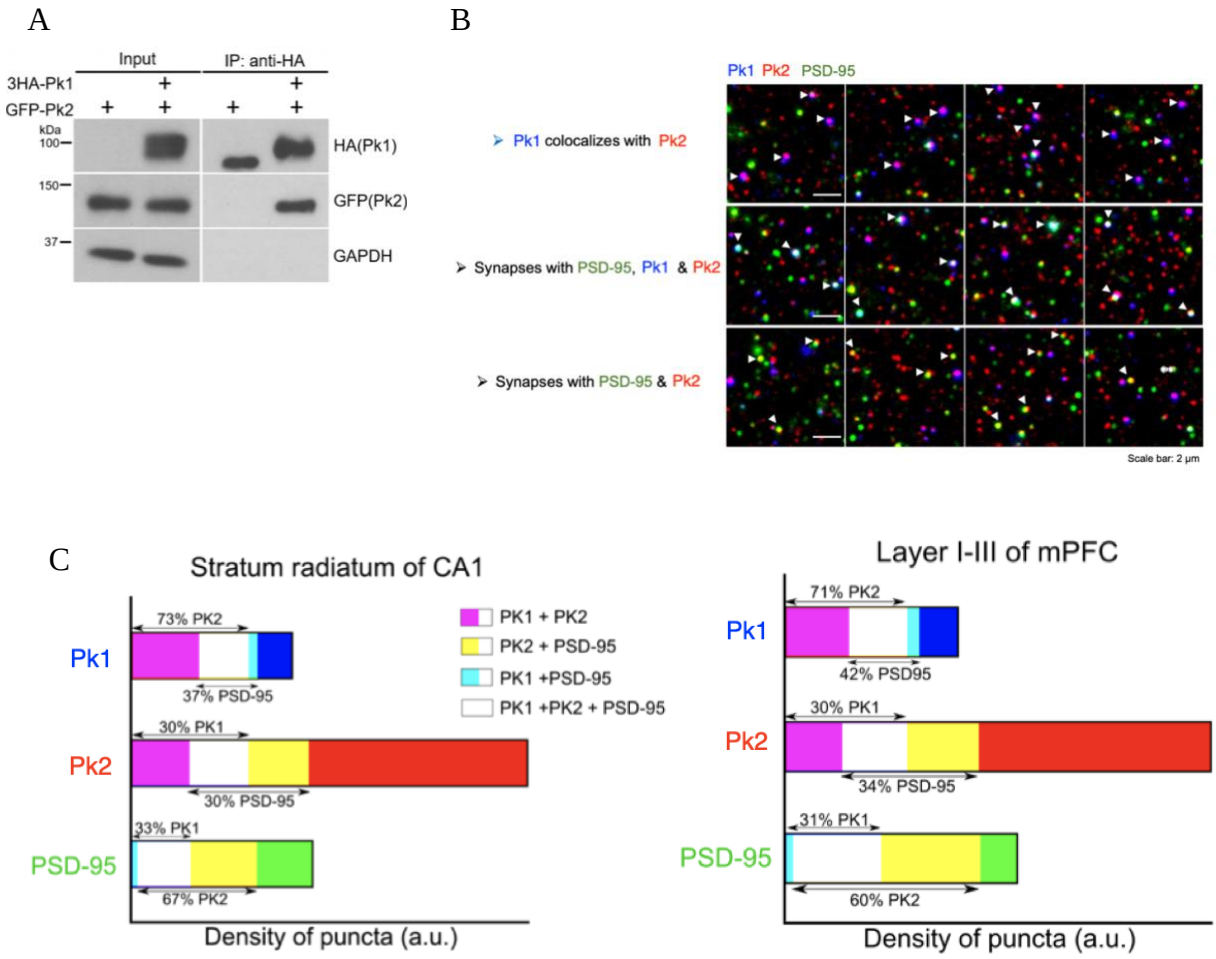


Figure 7. Prickle1 interacts with Prickle2 in HEK293T cells and colocalizes with Prickle2 in mice's stratum radiatum of CA1 region and layer I-III of mPFC. (A) Immunoprecipitation assay using transfected HEK293T cells. Anti-HA was used as the antibody. (B) Immunostaining of Prickle1, Prickle2, and PSD-95 in mice's stratum radiatum of CA1 region and Layer I to III of medial prefrontal cortex. (C) Density of Prickle1, Prickle2, and PSD-95 puncta in mice's stratum radiatum of CA1 region and Layer I to III of medial prefrontal cortex. Purple + white = colocalized Prickle1 and Prickle2; yellow + white = colocalized Prickle2 and PSD-95; cyan + white = colocalized Prickle1 and PSD-95; white only = colocalized Prickle1, Prickle2 and PSD-95; red = Prickle2 only; blue = Prickle1 only; green = PSD-95 only. Purple = colocalized Prickle1and Prickle2 without PSD-95; cyan = colocalized PSD-95 and Prickle1 without Prickle2; yellow = colocalized PSD-95 and Prickle2 without Prickle1. (n=25 for CA1 and n=45 for mPFC).

## 4.3 Discussion

Our result showed Prickle1 interacted with Prickle2 in HEK293T cells and colocalized with Prickle2 in mice's dorsal hippocampus and mPFC. Moreover, Prickle1 and Prickle2 were colocalized with PSD-95, and the majority of Prickle1 colocalized with PSD-95 were also colocalized with Prickle2 (Figure 7C). Prickle1 plays an essential role in axonal-dendritic development, and the mutation of Prickle1 contributes to neurodevelopmental disorders (Liu et al., 2013; Paemka et al., 2013). Since Prickle1 shares a similar structure with Prickle2 and they are both expressed in the postsynaptic density fraction in mice brain, it is possible that Prickle1 also plays a role in synapse formation and function. Understanding the role of Prickle1 at synapses may give a new insight into how the formation of excitatory synapses is regulated. Moreover, since we found that Prickle1 and Prickle2 interacted with each other in HEK293T cells and were colocalized in mice's dorsal hippocampus and mPFC, it is possible that Prickle1 works together with Prickle2 in regulating synapse formation, and the simultaneous disruption of these two genes may induce more reduction in synapse density compare to the disruption of a single one. In addition, we found that the amount of Prickle2 was about twice the amount of either Prickle1 or PSD-95, thus it is possible that the Prickle2 that did not colocalized with either Prickle1 or PSD-95 has different functions from the Prickle2 that colocalized with the other two proteins.

# Chapter 5. Conclusion

The synapse is an intracellular specialization that links presynaptic axonal terminals and postsynaptic dendritic spines. Once migration and aggregation are complete, axons and dendrites begin to grow, and they need to find appropriate synaptic targets. The directed recognition of axons to synaptic targets are facilitated by a specialization at the tip of axons called growth cone (Tamariz and Varela-Echavarria, 2015). And when axons reach their intended site, they start to establish an appropriate pattern of synapses. The characteristic and plasticity of the synapses may vary a lot, but the main function of the synapse is to transfer and process information between neurons.

Previous studies have found that Wnt proteins are involved in synapse formation (Shafer et al., 2011; Onishi et al., 2013; Thakar et al., 2017). Many of the Wnt proteins are localized at synapses, and Wnt canonical and noncanonical pathways showed opposing role on hippocampal synapse formation (Davis et al., 2008). Moreover, our lab found that two specific Wnt non-canonical proteins, Vangl2 and Celsr3, regulates excitatory synapses (Thakar et al., 2017). In this study, we investigated possible role of Prickle2, another Wnt non-canonical protein, in regulating synapse formation and function. We found that Prickle2 is required for synapse formation and maintenance, and Prickle2$^{E8Q}$ mutation leads to synaptic dysfunction and level change of synaptic proteins that are essential for synapse formation and neurotransmission. Moreover, we find the interaction between Prickle1 and Prikle2 in HEK293T cells and the colocalization between Prickle1, Prickle2 and PSD-95 in the hippocampus and mPFC of mice brain. This result suggests Prickle1 and Prickle2 may collaborate in regulating synapse formation in mice genome, but further investigation is needed to confirm that. Moreover, the specific role of Prickle1 on synapse formation and function is still not clear, and future experiment investigating the role of

Prickle1 on synapse formation may shed light on the mechanism of how Prickle proteins regulates synapse formation and function.

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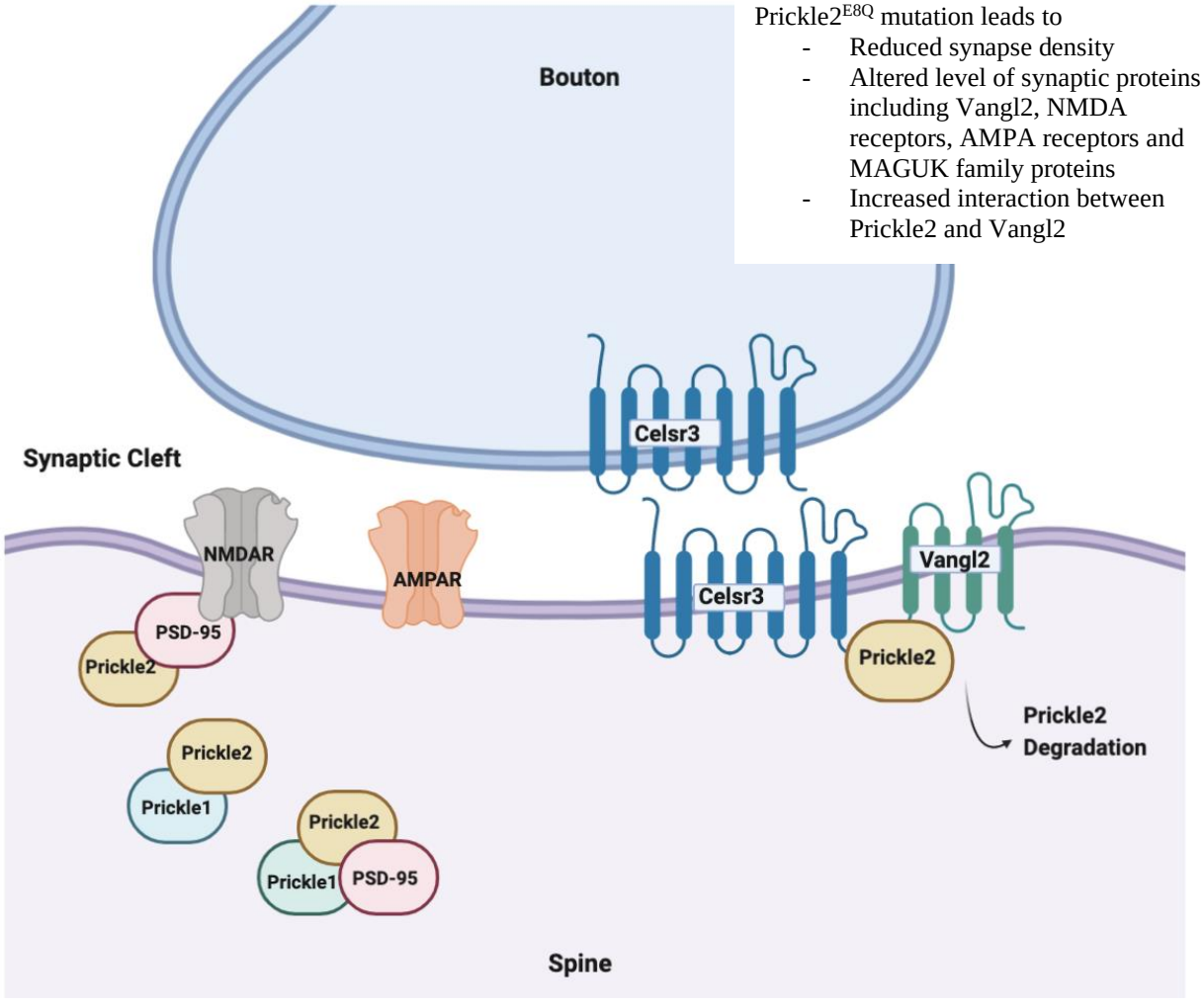


Figure 8. Summarized diagram of this thesis

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