上海交通大學

SHANGHAI JIAO TONG UNIVERSITY

学士学位论文

BACHELOR'S THESIS

论文题目：Study on the mechanism of GABAergic interneuron abnormality caused by haploinsufficiency of ASD risk gene *Senp1*

学生姓名: Ruoqing Wang

学生学号: 517111910194

专　　业: Bioscience (Zhiyuan Honors Program)

指导教师: Zilong Qiu, Weidong Li

学院(系): School of Life Sciences and Biotechnology, Zhiyuan College

上海交通大学

本科生毕业设计（论文）任务书

课题名称：Study on the mechanism of GABAergic interneuron abnormality caused by haploinsufficiency of ASD risk gene *Senp1*

执行时间：2020 年 9 月 至 2021 年 5 月

教师姓名：李卫东　　职称：教授

学生姓名：王若晴　　学号：517111910194

专业名称：生物科学

学院(系)：致远学院

毕业设计（论文）基本内容和要求：

基本内容:

1：利用 *Senp1* 杂合小鼠开展动物行为学实验，初步验证自闭症风险基因 *Senp1* 的自闭症核心行为学表型。

2：利用免疫荧光染色技术，探索 *Senp1* 杂合突变对大脑特定脑区 GABA 能中间神经元形态、数目和类群比例的潜在影响。

3：针对 GABA 能中间神经元发生改变的潜在特定脑区，探究 SENP1 蛋白产生作用的分子机制。

4：针对 GABA 能中间神经元发生改变的潜在特定脑区，利用立体定位注射 AAV 病毒原位过表达 SENP1 蛋白，回补该脑区 SENP1 蛋白剂量。

5：验证 AAV 病毒注射后的 *Senp1* 杂合小鼠 GABA 能中间神经元、电生理、SENP1 蛋白表达量以及自闭症核心行为等表型是否改善。

要求：

1：提出 *Senp1* 半倍剂量缺失引起 GABA 能中间神经元功能异常的神经生物学机制模型。

2：参与发表一篇 SCI 论文。

毕业设计（论文）进度安排：

序号	毕业设计（论文）各阶段内容	时间安排	备 注
1	*Senp1* 杂合小鼠行为学检测	2020.09-2020.11	
2	观察GABA能神经元形态数量	2020.11-2021.01	
3	SENP1 下游蛋白探究	2021.01-2021.02	
4	病毒立体定位注射回补SENP1蛋白	2021.02-2021.03	
5	验证各层面表型是否改善	2021.03-2021.04	
6	总结数据，撰写论文	2021.04-2021.05	

课题信息：

课题性质 ：设计□　　论文√□

课题来源*：国家级□　　省部级□　　校级□　　横向□　　预研√□

指导教师签名：_____

年　　月　　日

学院（系）意见：

院长（系主任）签名：_____

年　　月　　日

学生签名：_____

年　　月　　日

上海交通大学
学位论文原创性声明

本人郑重声明：所呈交的学位论文《Study on the mechanism of GABAergic interneuron abnormality caused by haploinsufficiency of ASD risk gene *Senp1*》，是本人在导师的指导下，独立进行研究工作所取得的成果。除文中已经注明引用的内容外，本论文不包含任何其他个人或集体已经发表或撰写过的作品成果。对本文的研究做出重要贡献的个人和集体，均已在文中以明确方式标明。本人完全意识到本声明的法律结果由本人承担。

学位论文作者签名：

日期：　　年　　月　　日

上海交通大学
学位论文版权使用授权书

本学位论文作者完全了解学校有关保留、使用学位论文的规定，同意学校保留并向国家有关部门或机构送交论文的复印件和电子版，允许论文被查阅和借阅。本人授权上海交通大学可以将本学位论文的全部或部分内容编入有关数据库进行检索，可以采用影印、缩印或扫描等复制手段保存和汇编本学位论文。

保密□，在___年解密后适用本授权书。

本学位论文属于

不保密☑。

（请在以上方框内打“√”）

学位论文作者签名：　　指导教师签名：

日期：　　年　　月　　日　　日期：　　年　　月　　日

自闭症风险基因 *Senp1* 半倍剂量缺失引起 GABA 能中间神经元功能异常的机制研究

摘要

自闭症谱系障碍（Autism spectrum disorder, ASD），也称为自闭症，是一种常见的，高度遗传性和异质性的神经发育障碍。遗传学研究证明自闭症的遗传力约为 40%-90%。通过与医院进行合作，对比父母和患病子女的全基因组序列，我们筛选出了杂合新发突变的自闭症相关基因 *SENP1*（Sentrin-specific peptidase1），该基因编码去一种泛素化酶。现实中自闭症患者通常具有复杂的基因组特征，研究单个自闭症风险基因的主要目的是阐明自闭症的致病机理，寻找不同自闭症风险基因之间的共同点，进而寻找致病的核心要素。有研究表明，由自闭症风险基因表达的蛋白通常都参与突触形成和基因表达调控。本课题拟在小鼠中杂合敲除 *Senp1* 基因，通过行为学实验验证小鼠的自闭症相关表型，构建典型的自闭症小鼠模型，通过分子和免疫学实验探索该基因作用同路和调控的特定脑区，提出 *Senp1* 基因半倍剂量缺失引发 GABA 能中间神经元功能异常的机制模型，为自闭症的临床诊断和干预提供理论支持。

关键词：自闭症，新发突变，GABA 能神经元，神经元发育

STUDY ON THE MECHANISM OF GABAergic INTERNEURON ABNORMALITY CAUSED BY HAPLOINSUFFICIENT OF ASD RISK GENE *Senp1*

ABSTRACT

Autism spectrum disorder (ASD) is a common, highly hereditary and heterogeneous neurodevelopmental disorder. Genetic studies have shown that the heritability of autism is about 40%-90%. By cooperating with the hospital and comparing the whole genome sequence of parents and children with autism, we identified a *de novo* heterozygous mutation of the Sentrin-specific peptidase1 (*SENP1*) gene, coding the small ubiquitin-like modifiers (SUMO) deconjugating enzyme, as a potentially new candidate gene for ASD. Studies have shown that proteins expressed by autism risk genes are usually involved in synapse formation and gene expression regulation. In this work, we knock out *Senp1* gene in mice heterozygously, verify the autistic-like behaviors in in *Senp1* haploinsufficient mice (*Senp1*+/-), and propose a possible mechanism model of GABAergic interneuron function abnormality caused by *Senp1* haploinsufficiency. This work is expected to provide theoretical support for the clinical diagnosis and intervention of autism.

Key words: ASD, *de novo* mutation, GABAergic interneuron, neuron development

Contents

1. Introduction

Autism spectrum disorder (ASD), also known as autism, is a highly heritable neurodevelopmental disorder. The diagnosis rate of autism is increasing year by year. How to find the pathogenesis of autism and implement effective intervention methods has gradually become a major problem. Researchers can perform high-throughput sequencing on a large number of autistic family samples. By comparing the whole genome sequence of parents and children with the disease, more than 100 associated genes and genomic regions can be screened out. To facilitate research, researchers focus on single genes based on heterozygous *de novo* mutations.

Molecular experiments have shown that proteins expressed by ASD risk genes are usually involved in synapse formation and gene expression regulation. The distribution positions and functions of these proteins are diverse. It suggests that the underlying cause of autism may be related to the connection and development of the nervous system. If the pathogenic mechanism of a single autism risk gene can be clarified through experiments, it can provide a reference paradigm for the research of multiple autism risk genes that have been screened. It provides theoretical support for clinical drug interventions and greatly advances the research on autism. My project chose the typical autism risk gene *SENP1* to explore and find out how this gene affects GABAergic interneurons.

1.1 The genetic basis of ASD

The core symptoms of ASD are social deficits and repetitive stereotypes ([1], Lord, Brugha et al., 2020, 5). Many studies have been carried out from molecular genetics to immunity, neuroanatomy, and neurochemistry, from which people are trying to find the

cause of autism. Here, by focusing on ASD patients from Shanghai Xinhua hospital, we identified a *de novo* heterozygous mutation of the Sentrin-specific peptidase1 (*SENP1*) gene as an ASD-risk gene.

1.1.1 Concept of autism spectrum disorder

Although autism spectrum disorder is one of the highest heritable mental disorders ([2], Sandin, Lichtenstein et al., 2017, 1182-4), the genetic landscape of autism spectrum disorder was not revealed until the application of genome-wide sequencing technologies such as whole-exome sequencing and whole-genome sequencing ([3], De Rubeis, He et al., 2014, 209-15).

The major contributory genetic components of autism spectrum disorder include *de novo* variations and rare inherited variations, as well as structural variations ([4], Iakoucheva, Muotri et al., 2019, 1287-98). During the last decade a substantial proportion of the genetic architecture of autism spectrum disorder has come to light. These studies have delivered a trove of susceptibility genes. Some evidence show that synaptic dysfunction is a reason for autism ([5], Levy, Mandell et al., 2009, 1627-38). In the past ten years, autism spectrum disorder has evolved from one of the most mysterious and misunderstood common diseases to one of the successful cases in the post-genomics era ([4], Iakoucheva, Muotri et al., 2019, 1287-98).

1.1.2 *De novo* mutations in autism spectrum disorder

A *de novo* mutation is a new germline mutation not inherited from either parent. Most germline mutations occur during the mitotic cell division of spermatogonia and occur at a constant rate in the gonads ([6], Crow, 2000, 40-7). Therefore, most *de novo*

mutations are generated by fathers, and the total incidence of new mutations in offspring increases by 1-2 mutations per year with age.

The major contributory genetic components of ASD include *de novo* variations and rare inherited variations, as well as structural variations ([4], Iakoucheva, Muotri et al., 2019, 1287-98, 7], Searles Quick, Wang et al., 2021, 55-69).Among various genetic variants, the *de novo* variants of large effect, for example, protein-truncating variants, would account for 10-15% of total contributing genetic causes of ASD, which provide the first line of the clue for illustrating the neural circuits responsible for social behaviors disrupted in autism patients ([7], Searles Quick, Wang et al., 2021, 55-69). Considering the general population, *de novo* mutation has little effect on the risk of autism. Indeed, most individuals who are genetically at risk for common diseases, especially those with a small effect size, will never show symptoms ([8], Gaugler, Klei et al., 2014, 881-5). However, regardless of the exact proportion of the risks posed by these mutations, their most important contribution to autism may lie in elucidating the mechanism of the disease ([1], Lord, Brugha et al., 2020, 5).

1.2 SUMOylation in the regulation of synaptic development

The post-translational modification of proteins using smaller ubiquitin-related modifiers (SUMO) is one of the important physiological processes in eukaryotic cells. Hundreds of proteins have undergone the sumoylation reaction. They are involved in DNA repair, chromatin organization, signal transduction, macromolecule assembly and so on ([9], Flotho and Melchior, 2013, 357-85). SUMO can be removed from its substrate, which is called deSUMOylation, and can be catalyzed by several SUMO-specific proteases, such as SENP1, in mammals. SUMOylation and deSUMOylation maintain dynamic balance which is quite important for normal physiological function

of cells. Recent studies have shown that SUMOylation plays a vital role in regulating synaptic communication and brain development ([10], Rocca, Wilkinson et al., 2017, 877).

1.2.1 The function of SENP1

Till now there are six SUMO proteases have been found which are classified as SENP1-3 and SENP5-7 (sentrin/SUMO-specific protease) ([11], Xu, Chau et al., 2006, 345-52). SENP1 (Sentrin-specific peptidase1) plays a decisive role in post-translational SUMOylation modifications by releasing SUMO groups from proteins ([9], Flotho and Melchior, 2013, 357-85).

The SUMO family of vertebrates has three main members: SUMO-1, -2 and -3. They can all be catalyzed by SENP1. The binding of SUMO to certain proteins is very similar to ubiquitination, but depending on the type of protein modified, these modifications may lead to different results ([11], Xu, Chau et al., 2006, 345-52). Mammalian SENP1 is mainly located in the nucleus ([12], Kim, Sung et al., 2005, 6272-8).

Since SENP1 is one of major SUMOylation deconjugating enzymes, we reasoned that SENP1 may regulate synapse development by conjugating small ubiquitin-like modifier to candidate proteins, which performed critical functions in synapse development. In a previous study using mass spectrometry, researchers identified that SENP1 proteins existed in the post-synaptic complex and implicated in brain disorders ([13], Li, Zhang et al., 2017, 1150-61). Homozygous mutation of SENP1 has been implicated in severe neurometabolic diseases ([14], Tarailo-Graovac, Shyr et al., 2016, 2246-55).

1.2.2 SUMOylation affects the development of synapses

The most thorough and most researched functions of the SUMO modification is to regulate transcription, maintain protein stability, and regulate the transport process ([15], Hay, 2005, 1-12). Some studies have shown that there are SUMO-modified proteins in the nucleus, and SUMOylation plays an important role in regulating brain development and synaptic communication. Therefore, the abnormality of SUMOylation under certain pathological conditions can cause abnormal interference of information transmission between synapses ([16], Girach, Craig et al., 2013, 1294-301).

If the SUMO of protein in brain neurons is abnormal, certain neurological diseases will occur. The SUMOylation of MeCP2 can restore the abnormal phenotype of Rett syndrome, indicating that the abnormal function of SUMOylation of MeCP2 may be one of the reasons for Rett syndrome ([17], Tai, Liu et al., 2016, 10552). Alzheimer's disease is a neurodegenerative disease that produces amyloid precursor protein (APP) deposits in the patient's brain, where the aggregated APP can be used as a substrate for SUMOylation ([18], Tang, Lu et al., 2018, 1100-2). Studies have found that the deSUMOylation of FOXP1 can affect the growth of dendrites to form mature dendrites ([10], Rocca, Wilkinson et al., 2017, 877). These studies indicate that if the function of SUMOylation is blocked, it may lead to abnormal brain lesions.

Studies have found that the SUMOylation of FMRP is essential for maintaining the maturity and density of dendritic spines. In Fragile X syndrome (FXS) patients, FMRP protein content decrease severely, and it can lead to immature neuronal morphology, accompanied by abnormally long and slender filopodia ([19], Khayachi, Gwizdek et al., 2018, 757). In this project, FMRP is likely to be a downstream protein of SENP1 to participate in the regulation of neurons, and then produce autism phenotypes. If SENP1

indeed plays a critical role in removing the SUMO groups from FMRP protein, the SUMOylation level of FMRP in the brain of *Senp1* haploinsufficient mice may elevate.

1.3 The influence of GABAergic synaptic transmission on autism

There are approximately 20–30% interneurons in the neocortex ([20], Markram, Toledo-Rodriguez et al., 2004, 793-807). The cortical network is composed of local GABAergic inhibitory interneurons and glutamatergic excitatory projection neurons ([21], Tremblay, Lee et al., 2016, 260-92). In recent years, the research on the labeling and monitoring of interneurons has gradually increased, and the research on GABAergic interneurons has gradually become thorough. Studies have shown that the abnormal development of interneurons can lead to abnormal functions of the cerebral cortex and abnormal inhibitory circuits, which are related to schizophrenia, autism spectrum disorder and other mental diseases ([22], Yang, Yang et al., 2021).

1.3.1 Synapse concept and classification

Synapse refers to the structure in which the electrical or chemical signal is transmitted from one neuron to another neuron or to another cell. Presynaptic cells use chemical signals, that is, neurotransmitters, to transfer information to postsynaptic cells, which are called chemical synapses ([23], Missler, Südhof et al., 2012, a005694). There are two kinds of synapse including inhibitory synapse and excitatory synapse. Inhibitory synapses are synapses that transmit pre-synaptic excitement and have an inhibitory effect on post-synaptic excitement. Excitatory synapse refers to the connection between nerve cells that can excite post-synaptic neurons. Inhibitory postsynaptic potentials are

synaptic potentials that make post-synaptic neurons less likely to produce action potentials. GABA and glycine are common neurotransmitters involved in IPSPs. There are two types of inhibitory receptors including ionotropic receptors and metabotropic receptors. Inhibition of postsynaptic potentials has many applications in scientific research and medical fields. Drugs can treat neurological and psychological diseases by affecting neurotransmitters, which in turn affect the receptors of postsynaptic neurons, ion channel, G protein and so on.

1.3.2 GABAergic interneuron and autism

The balance of excitement and inhibition in the brain is closely related to the two basic types of neurons, glutamatergic projection neurons and GABAergic neurons. GABAergic interneurons can be divided into non-overlapping categories by several markers, such as parvalbumin (PV), somatostatin (SST), vasoactive intestinal peptide (VIP) ([22], Yang, Yang et al., 2021). In this project we focus on parvalbumin and somatostatin. GABAergic interneurons can regulate the dynamic circuits of the cerebral cortex, regulate the firing rate of pyramidal cells, the generation of cortical rhythm, and maintain the balance of excitability and inhibitory which is necessary for information transmission ([24], Goldberg and Coulter, 2013, 337-49).

Mutations of a few ASD risk genes including contactin-associated protein-like 2 (CNTNAP2) ([25], Alarcón, Abrahams et al., 2008, 150-9), multiple ankyrin repeat domains protein 3 (SHANK3) ([26], Moessner, Marshall et al., 2007, 1289-97), and Methyl-CpG binding protein 2 (MeCP2) ([27], Liu, Li et al., 2016, 98-102) are related to the dysfunction of interneurons. Many gene mutations related to the development and activity of interneurons are highly correlated with abnormal neurodevelopment,

indicating that abnormal interneuron development leads to abnormal function of inhibitory circuit and dysfunction of interneurons, and then leads to abnormal neurodevelopment.

1.4 Research goals and innovations

Finding the pathogenesis of autism is an important thing. Only by finding out the cause of the disease can we implement effective interventions on patients. We need to provide life and medical protection for autistic patients as soon as possible. This project uses a variety of technical methods to explore the pathogenic mechanism of the autism risk gene *Senp1*, and hopes to contribute to the exploration of autism through the exploration of the pathogenic mechanism of single-gene mutations.

1.4.1 Research goals

In recent years, many studies abroad have used large-scale sequencing methods, that is, a large number of samples are used to screen out single gene mutation sites that cause autism, and some potential pathogenic genes have been obtained ([28], Fu, O'Connor et al., 2013, 216-20). Although the samples are sufficient, the Research on the pathogenic mechanism and function of these genes is not thorough. Compared with foreign countries, there is a lack of autism families in China, so it is difficult to confirm whether there are autism genes unique to East Asian races compared to North America. With partial data support, in-depth research on these genetic samples is necessary and urgently needed.

For a single typical autism gene, we can focus on its function, the neural circuits involved in regulation and the affected brain regions, and explore the mechanism of

action that produces the autism phenotype when the gene is heterozygous knocked out. Next, we can get a set of mature and process-oriented autism gene research paradigm，which will promote and integrate different single disease-causing genes to obtain a complete autism research system, provide follow-up measures for the screening of single gene mutations, and also provide clinical diagnosis in the future.

The diagnosis of autism is based on the patient's behavior. Clinically, it generally relies on identifying a series of core diagnostic features of autism to give scoring standards. Interventions for autism include human intervention and drug intervention. Usually, medical staff will coordinate and guide the communication methods of autistic patients, and encourage patients to participate in games and daily activities to improve their social and communication skills ([29], Sanchack and Thomas, 2016, 972-9). In terms of drug treatment, almost all drugs that have been proven to be beneficial for autism are involved in the treatment of the complications of autism, rather than directly treating the typical features of autism ([1], Lord, Brugha et al., 2020, 5). Studying the root causes of autism due to genetic mutations will help us find more effective drugs for treating autism.

1.4.2 Innovations

Mental diseases have always been a major problem in the medical profession, such as Alzheimer's disease and Parkinson's syndrome. Mental diseases caused by gene mutations are a special kind of disease. We can directly knock out genes to construct animal models for research, which greatly simplifies the research methods. The heritability of other mental diseases is not as high as autism spectrum disorder, so they will be more difficult to study. We start from a simple point of view to study autism

caused by single gene mutations, and explore whether there are specific brain regions and proteins that cause autism. After finding out the abnormal reactions caused by single gene mutations, we can more conveniently study the abnormalities of neural circuits. This can not only provide a reference for a wider range of autism, but also provide research ideas for other mental diseases. The technologies used in this subject are all basic biotechnology, but the subject of inquiry is of great significance. In this project, we showed that *Senp1* haploinsufficient mice exhibited defects of social behaviors and increased stereotypic behaviors, without deficiency in learning and memory tasks.

1.5 Summary

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder. While the core symptoms of ASD are social deficits and repetitive stereotypes. Through the study of non-ill parents and sick children, we have found a gene that may cause autism, called *SENP1*, which has the function of deSUMOylation. Study shows that SUMOylation and deSUMOylation balance is vital for neurodevelopment and FMRP is closely related to the transmission of information between synapses which can be SUMOylated by SENP1. GABAergic interneurons have chiefly inhibitory action at receptors in the adult vertebrate. Studies have shown that presynaptic excitement of GABAergic interneuron regulates fear memory expression ([30], Zhang, Tan et al., 2016, 716-28). Considering the connection between GABAergic interneurons and some phenotypes of autism, we chose to start with SENP1 to study whether the decrease in protein content can affect the normal physiological functions of GABAergic interneurons. From genes to proteins, to neurons, and finally combined with the phenotype of autism, we will jointly explore the pathogenic mechanism of gene mutations leading to autism.

2. Materials and methods

Many materials and test methods are used in this project. Here I list the viruses, animals, antibodies and chemicals used in the experiment, and the main test methods, including perfusion brain extraction and slice, immunofluorescence assays, behavioral test, stereotactic injection of AAV virus, western blot, immunoprecipitation assays, and statistical analysis.

2.1 Materials

The reagents and materials used in the project are provided by Dr. Zilong Qiu, Center for Excellence in Brain Science and Intelligence Technology, Institute of Neuroscience.

2.1.1 Viruses

AAV9-hSyn-EGFP (Serotype 2/9, titer 8.5×10^{14} vg/mL), AAV9-hSyn-SENP1-EGFP (Serotype 2/9, titer 4.1×10^{13} vg/mL) and AAV9-hSyn-FMRP-EGFP (Serotype 2/9, titer 6.2×10^{13} vg/mL) were purchased from Institute of Neuroscience Gene Editing Core.

2.1.2 Animals

Male C57BL/6N and *Senp1* +/- mice at the age of 2-4 months old were used for experiments. C57BL/6N mice were purchased from SLAC laboratory (Shanghai). *Senp1* +/- mice were acquired from Jinke Cheng's lab at Shanghai Jiao Tong University.

C57BL/6N mice are also called wild type (WT) mice; *Senp1* +/- mice are also called *Senp1* haploinsufficient mice. All animals were housed under a 12:12 h light-dark cycle with water and food *ad libitum* in the animal facility at the Institute of Neuroscience.

2.1.3 Antibodies

Antibodies include mouse anti-FMRP (Sigma, Cat#SAB4200597), rabbit anti-FMRP (Abcam, Cat#ab17722), rabbit anti-SENP1 (Abcam, Cat#ab108981), mouse anti-GAPDH (Cell Signaling Technology, Cat#97166), rabbit anti-Parvalbumin (Abcam, Cat#ab181086), mouse anti-Somatostatin (Santa Cruz Biotechnology, Cat#sc-55565), donkey anti-rabbit, Alexa Fluor 555 (Thermo Fisher Scientific, Cat#A-31572), donkey anti-rabbit, Alexa Fluor 488 (Thermo Fisher Scientific, Cat#A-21206), donkey anti-mouse, Alexa Fluor 555 (Thermo Fisher Scientific, Cat#A-31570), and donkey anti-mouse, Alexa Fluor 488 (Thermo Fisher Scientific, Cat#A-21202).

2.1.4 Chemicals

Chemicals include DAPI (Thermo Fisher Scientific, Cat#D1306), optimum cutting temperature formulation (SAKURA, Cat#4583), tween-20 (Sangon, Cat#A100777), triton-100 X (Sangon, Cat#A110694), loading buffer (Applygen, Cat#B1012), and sodium pentobarbital (Sigma, Cat#P3761).

2.2 Methods

Methods include perfusion brain extraction and slice, immunofluorescence assays, behavioral test, stereotactic injection of AAV virus, western blot, immunoprecipitation

assays, and statistical analysis.

2.2.1 Perfusion brain extraction and slice

Senp1 +/+ and *Senp1* +/- mice were anaesthetized with sodium pentobarbital (Sigma, Cat#P3761, 50 mg/kg). The limbs of the mouse were fixed on the foam board with a needle. Use tweezers to pick up the skin of the abdomen, and cut the fur of the mouse upwards along the abdominal cavity until the thoracic cavity is exposed. Use scissors to cut off the bones of the thoracic cavity, pull the lung lobe to one side to expose the heart, and cut the right atrial appendage of the mouse. When the blood flowed out, insert the needle into the left ventricle of the mouse heart and slowly inject 0.1 M PBS (phosphate buffer saline). With the needle position unchanged, the prepared 15ml 4% PFA solution was slowly injected into the mice. During the process, the mice could be seen stiff, which was due to the protein denaturation caused by the PFA. When the mouse is completely stiff, it indicates that the perfusion fixation has been completed. Cut the mouse head with scissors, cut open the mouse skull with tweezers, exposing the brain, and carefully remove the brain with a steel spoon. Put it into 4% PFA solution for post-fixation. After perfusion, brains were post-fixed overnight in 4% PFA at 4°C and sequentially dehydrated in 15% and 30% sucrose/PBS solution respectively. After the fixation, the brain was placed in a 15% sucrose solution for dehydration, shaken on a shaker overnight at 4°C, and then replaced with a 30% sucrose solution the next day to repeat the dehydration step until the brain completely sinks to the bottom of the solution. Took out the metal mold, extruded Optimum Cutting Temperature formulation (OCT) (SAKURA, Cat#4583) to cover the bottom of the mold, took out the dehydrated brain tissue from the sucrose solution, wiped the solution on the surface, and put it into the mold flatly, and then added enough OCT until the entire brain was embedded. Put the mold in the refrigerator at -80°C for several hours and then took it out. At this time,

the brain had been completely embedded in the frozen OCT. The frozen brain was sectioned at 40 μm from parietal lobe to occipital lobe with a Microtome Cryostat (Leica, CM1950) at -25°C. Added 4ml of PBS solution to each well in the six-well plate, put the sliced brain slices in human PBS solution with a toothpick. The brain slices were stored at 4°C.

2.2.2 Immunofluorescence assays

Floating brain sections (40 μm) were rinsed in PBS then blocked overnight at 4°C in PBS containing 5% Bovine albumin (BSA) and 0.2% Triton X-100, followed by incubating with mouse anti-FMRP primary antibodies (Sigma-Aldrich, Cat#SAB4200597, 1:1000) at 4°C for overnight and donkey anti-mouse Alexa Fluor 555 secondary antibodies (Thermo Fisher Scientific, Cat#A-31570, 1:1000) at 4°C for 1 h. All primary and secondary antibodies were diluted with PBS containing 5% BSA and 0.4% Triton X-100. All brain sections were finally counter-stained with DAPI (Sigma, Cat#d9542, 5mg/mL, 1:1000). Sections were washed 3 × 10 min in PBS before incubating with secondary antibodies. For other antibody combinations, sections were rinsed with PBS, blocked and treated with primary and secondary antibodies as described above. Images were captured by objective fluorescent microscope (Olympus, VS120, 10 ×) or confocal microscope (Olympus, FV3000 IX83, 10 ×, 20 × and 60 ×).

2.2.3 Behavioral test

Adult male *Senp1* +/+ and *Senp1* +/- mice were tested in the following sequence: open field locomotion, three-chamber sociability test, novel object cognition, restrictive repetitive behavior test and Barnes maze test. Behavioral assays were recorded using cameras. The experimenter handled the mice for constitutive 4 days, 4 min each time

to familiarize the mice with the smell of the experimenter before the test. In the fifth day, mice were put into the open field (40 × 40 cm^2) for 10 min and the movement of mice was recorded. In the next week, mice were test for three-chamber sociability and novel object cognition. The test apparatus (40 × 60 cm^2) contained three adjacent chambers side by side. In the first day mice were tested for social approach. Before the three-chamber experiment started, mice were put into the middle chamber for 10 min to habituate. Then a novel male mouse (stranger A) was put into one of the side chambers for 10 min. The time the subject mouse spent in each chamber was recorded and analyzed. In the next day mice were tested for social novelty. Stranger A and a novel male mouse (stranger B) were put into two side chambers for 10 min respectively, and the time the subject mouse spent in each chamber was recorded and analyzed (Noldus, EthoVisionXT).

In novel object cognition test, strange mice were replaced with novel objects. A week later, mice were test for restrictive repetitive behavior. 36 black glass marbles were arranged in a symmetrical 6 × 6 grid on top of 7-cm deep bedding in a clean standard mouse cage (40 × 40 cm^2). Mice were put into the cage for 3 min to habituate, then placed in the cage for a 10-min exploration period. The camera took a picture at the beginning and end of the exploration to count the number of buried marbles. 'Buried' was defined as greater than 50% covered by bedding. The next day after marble burying test, mice were placed into a clean standard mouse cage (40 × 40 cm^2) for recording cumulative time spent in grooming in the next 30 min.

The Barnes maze test was carried out next week. The maze was consisted of a circular platform, 2 m in diameter with 20 evenly spaced holes at the edges. Mice were trained to find a specific small dark chamber under the platform which was the only habitable one called the "escape box." In day 0, mice were placed on the platform for 3 min to

habituate followed by pre-training for 3 min to find the escape box and then rest for 1 min. In day 1, mice were placed on the center of the platform for free exploration. The number of errors made to find the escape box was recorded within 3 min followed by 1 min rest. Mice were trained in 2 sessions daily with an inter-trial interval of 1 min for 12 days. The number of errors and latency time made before finding the escape hole were noted. AAV-injection mice were allowed for 1 month to recovery after surgery prior to behavioral test.

2.2.4 Stereotactic injection of AAV virus

After the anesthetized with sodium pentobarbital (Sigma, Cat#P3761, 50 mg/kg), viruses were injected into the retrosplenial agranular cortex (RSA) bilaterally according to standard mouse brain atlas (Paxinos and Franklin Mouse Brain Atlas, 2nd edition) at the following coordinates: anteroposterior (AP), -1.46 mm; mediolateral (ML), 0.5 mm; dorsoventral (DV), -0.45 mm; anteroposterior (AP), -2.5 mm; mediolateral (ML), 0.75 mm; dorsoventral (DV), -0.5 mm angled 90° toward the midline in the coronal plane. Wipe and disinfect the mouse head with a cotton swab moistened with alcohol, and gently scrape the hair on the top of the head with a blade to expose the scalp. Put the mouse back up, head forward, and hang the teeth on the bulge of the iron frame. Use ear rods to insert the mouse's ear holes on both sides. Fix the head and place the body on the metal platform. Cover with a paper towel to prevent the body temperature from dropping too quickly. Prepare the required oil pump, remove all the gas in the needle tube, connect the needle tube to the glass electrode and fix it on the instrument. The tweezers and scissors were sterilized with alcohol, the mouse scalp was clamped with the tweezers, and the mouse scalp was cut along the midline of the body with the scissors to expose the skull. Use a glass tube to determine the position to be injected on

the surface of the skull and make a mark. Use an electric drill to drill slowly at the mark until the surface of the brain could be seen. Use a PCR tube with small amount of virus to be injected, fix the plasticine under the needle tube, use an oil pump to suck in the virus to be injected, then remove the PCR tube, and place the glass electrode above the drilled hole in the skull, according to the required depth pierce into the brain, and then start the oil pump to slowly inject the virus. About 200 nl virus was injected with a speed of 30 nl/min by a micro-injector and micro-infusion pump (PHD 2000, Harvard Apparatus). After the injection, the glass electrode was stopped in the original position for 10 minutes to prevent the virus from leaking, and then slowly pulled out. Wipe the bone debris on the surface of the skull with iodine, remove the mouse from the instrument, lift the scalp with tweezers and suture it with a medical suture needle, sew 3-4 stitches along the cut scalp thread. The mice were kept on a warm blanket to maintain the body temperature until fully awake.

2.2.5 Western blot and Immunoprecipitation assays

To assess protein intensity *in vivo*, the embryonic cortex and RSA of 3-month mice were dissected. After being homogenized at 1000 rpm for 1 min, tissues were lysed by ultrasound for 30 min in ice-cold RIPA lysis buffer. For Western blot, proteins in supernatant were combined with loading buffer (Applygen, Cat#B1012) and was heated to 100 °C for 30 min. The protein lysate was electrophoresed using 8%-12% SDS-PAGE gels and ran for 100 min at 120V. The proteins were transferred to a transfer membrane (Millipore Immobilon-P, Cat#IPVH00010) for 100 min at 200 mA. The membrane was blocked with 5% TBST containing Bovine albumin (BSA), washed 3×10 min with 1×TBST with 0.1% Tween-20 (Sangon, Cat#A100777), and then was incubated for 12 h with the primary antibody at 4 °C. The membrane was washed 3×10

min with 1×TBST with 0.1% Tween-20, incubated with the secondary antibody for 1 h. Signals were detected using an Ai600 system following manufacturer's protocol. For immunoprecipitation, 650 µl supernatants were added with 20 µl untreated and pre-blocked protein G-agarose beads for 1 h upside down at 4 °C. Proteins with beads were centrifuged at 12000 rpm for 10 min at 4 °C, and supernatants were incubated with primary antibodies or their corresponding IgGs as IP control for 4 h upside down at 4 °C and then for 1h at 4 °C with 30 µl of pre-blocked protein G-agarose beads. Following 12000 rpm for 10 min at 4 °C, precipitates attached by remaining 20 µl beads were added with 40 µl loading buffer, and proteins were eventually eluted by boiling the beads to 100°C for 30 min before SDS-PAGE.

2.2.6 Statistical Analysis

Data were plotted as mean ± standard error of mean (SEM) or Standard Deviation (SD). Statistical tests were analyzed with GraphPad Prism (GraphPad Software). For comparisons between two groups, data sets were analyzed with Student's t-test (two-tailed, paired or unpaired). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$.

2.3 Summary

There are four main research methods used in the project, and they are immunofluorescence staining technology, western blotting, stereotactic injection technology and behavioral test. Through immunofluorescence staining technology, we can clarify the approximate distribution and number of a certain protein or neuron, as well as the morphological characteristics of specific neurons, so as to visually show the

molecular and cellular differences of different genotypes of mice. Through western blot, we can quantitatively determine the content of a certain type of protein to be studied, so as to explore the mechanism of action between protein molecules, or the difference in protein expression between mice of different genotypes. Through stereotactic injection technology, we can not only sparsely label certain types of neurons to observe their microscopic morphology and the interconnection between neurons, but also specifically replenished the required proteins to clarify the changes and expressions of protein content. In the end, all phenotypes are tested using behavioral experiments.

3.Results

The male patient carrying the *SENP1* mutation exhibited typical deficits in social behaviors including communication and interaction, measured by ADOS and CARS scores. To investigate whether the *SENP1* gene contributes to social behavior, we use laboratory mouse as an animal model to examine whether genetic deletion of the *Senp1* gene may affect social behaviors in mouse. Since homozygous deletion of the *Senp1* gene causes lethality of mouse, we examine a battery of behavioral tests for the *Senp1* haploinsufficient mice which carry heterozygous deletion of the *Senp1* gene.

In this study, we showed that *Senp1* haploinsufficient mice (*Senp1*+/-) exhibited defects of social behaviors and increased stereotypic behaviors, without deficiency in learning and memory tasks. Interestingly, we found that the inhibitory neurons seem to be specifically affected in the retrosplenial agranular (RSA) cortex of *Senp1* haploinsufficient mice. Since SENP1 has the function of deSUMOylation, we chose FMRP as its downstream protein to explore whether the content of FMRP in *Senp1* haploinsufficient mice will change and how the proteins affect GABAergic interneuron. Remarkably, we could rescue the defects of social behaviors of *Senp1* haploinsufficient mice by introducing SENP1 or FMRP only into the RSA region. These results indicate that *Senp1* is an ASD risk gene, and its heterozygous mutation can produce an autism phenotype. Decrease in the content of SENP1 protein will reduce the deSUMOylation function of the cell, which in turn reduces the content of its downstream protein FMRP. Because FMRP is very important for the transmission of information between neurons, it will affect the normal physiological functions of GABAergic interneurons, and then produce autism phenotypes.

3.1 *Senp1* haploinsufficient mice show autistic-like behaviors

As the basis of this project, we need to detect whether *Senp1* heterozygous mutations can produce autism phenotypes. Since ASD has two core phenotypes, namely, social disorder and repetitive stereotyped behavior, we tested these two separately. In addition, we also tested whether *Senp1* haploinsufficient mice will produce other abnormal phenotypes, such as memory impairment. The premise of the experiment is that all living things have the instinct to explore unknown things. When a normal mouse faces strange and familiar things, it will choose to explore strange things.

3.1.1 *Senp1* haploinsufficient mice have social communication defects

In order to detect whether *Senp1* haploinsufficient mice have social disorders, we designed three-chamber test. First, let the test mice in a 40 cm x 60 cm box for 10 minutes for habituation. Put stranger1 in the cage on the left, and use 10 minutes to make the test mice and stranger 1 (S1) familiar with each other. After these two tasks are completed, we put stranger 2 (S2) in the cage on the right, and record the movement trajectory in the next 10 minutes. Under normal circumstances, normal mice will show a tendency to explore stranger mice stranger 2, while mice with social disorders have no obvious tendency to stranger 1 and stranger 2. Specifically, in the ten minutes recorded, the sniffing time of normal mice next to the right cage was significantly longer than that of the left cage, while the sniffing time of mice with social disorders was no significant difference.

The left figure is a schematic diagram of the action trajectories of wild-type mice and *Senp1* haploinsufficient mice (Figure 3.1 A), and the right figure is the statistics of

sniffing time of 8 mice of two genotypes (Figure 3.1 B). It can be seen that wild-type mice have a clear tendency to stranger 2, and the sniffing time of the right cage is significantly longer than that on the left. However, there was no significant difference in the sniffing time of heterozygous mice beside the left and right cages. Experimental results prove that *Senp1* haploinsufficient mice do have social disorders.

This experiment is a behavioral experiment. 16 mice need to be tested separately, including 8 heterozygous mice and 8 wild-type mice. The experimental data uses t test for statistics. Another possibility for the results of this experiment is that *Senp1* haploinsufficient mice can't distinguish between stranger 1 and stranger 2 because of memory impairment, so there is no significant difference in the sniffing time between the two cages. In order to rule out this situation, we designed the novel object cognition experiment.

3.1.2 *Senp1* haploinsufficient mice novel object cognition is normal

In order to detect whether the memory function of *Senp1* haploinsufficient mice is normal, we designed a novel object cognition experiment. The experiment is similar to the three-chamber experiment. First, let the test mice in the box for 10 minutes for habituation. Replace the two stranger mice in the previous experiment with two objects with different shapes, sizes and colors, namely object 1 (O1) and novel object (NO). In the ten minutes after placing the novel object, record the movement trajectory of the tested mice. Mice with normal memory function will show a tendency towards novel objects. Within ten minutes, the mouse will have more sniffing time on the right cage.

The experimental results showed that there was no significant difference between the performance of wild-type mice and *Senp1* haploinsufficient mice (Figure 3.1 C, D). The

sniffing time of mice on the right side was longer than that on the left side. The results indicate that the memory function of *Senp1* haploinsufficient mice is normal. In other words, the abnormal reaction of heterozygous mice in the three-chamber experiment was caused by social disorders.

3.1.3 *Senp1* haploinsufficient mice show repetitive behavior

Because the two core phenotypes of autism are social disorders and repetitive stereotypes, and we have confirmed that *Senp1* haploinsufficient mice have social disorders, then we need to detect whether there are repetitive stereotypes. In order to detect whether heterozygous mice have repetitive stereotyped behaviors, we designed the buried marble experiment and grooming time detection. In the buried marble experiment, the mouse was placed in a box with 36 glass marbles, and the number of marbles buried by the mouse in the next 10 minutes will be recorded. Because of the absence of repetitive stereotyped behavior in wild-type mice, the number of buried marbles was significantly less than that of autistic mice. In the grooming time detection, we observed and recorded the total time of the mouse's grooming time in 30 minutes. In general, mice with repetitive stereotyped behavior will have longer grooming time, while wild-type mice will have shorter grooming time.

We will take pictures of the box before and after the experiment (Figure 3.1 E). When performing data statistics, if the proportion of beads that are covered is greater than 50%, they are regarded as "buried marbles" and included in the final result. The statistical results of 8 pairs of mice show that the number of buried marbles in *Senp1* haploinsufficient mice is significantly higher than that of wild-type mice (Figure 3.1 G). This is a preliminary verification of the abnormal phenotype of *Senp1* haploinsufficient

mice. The grooming behavior of the mice was tested within 30 minutes, and the results showed that the grooming time of *Senp1* haploinsufficient mice was significantly improved compared with that of wild-type mice (Figure 3.1 H). The results of these two experiments both proved that *Senp1* haploinsufficient mice have repeated stereotyped behaviors.

Based on the above results (Figure 3.1 A-E, G-H), we can preliminarily determine that *Senp1* haploinsufficient mice have social disorders and repetitive stereotypes, that is, *Senp1* haploinsufficient mice have autism phenotypes. Next, we will test whether the phenotype of *Senp1* haploinsufficient mice has changed in other aspects.

3.1.4 *Senp1* haploinsufficient mice are normal in another tests

We next performed the Barnes maze experiment. *Senp1* haploinsufficient mice are known to have social disorders and repetitive stereotyped behaviors. We further tested whether their learning and memory functions are normal. Place the mice on a disk with holes around and let them explore freely. There is high-intensity light above the disk. Due to the light-shielding properties of mice, they will choose feasible holes to hide themselves, called "target holes". There is only one target hole, which exists at a fixed angle, and there are different signs on the surrounding walls to indicate the direction (Figure 3.1 F). There is a dark and safe box under the target hole. The experiment needs to be carried out for six consecutive days, and the test will be carried out on day 12. On day 0, the mice can explore freely on the disk and are guided into the target hole 90 s later. From day1 to day5, the test was conducted twice a day to record the time required for the mice to find the target hole. The time will be limited to 3 minutes. If the mouse still does not find the correct target hole within 3 minutes, we will guide them into it. After entering the target hole, the mice were allowed to acclimate in the dark box for

one minute, which would strengthen their memory of the target hole.

Experimental results showed that after 5 days of training, the time required for *Senp1* haploinsufficient mice and wild-type mice to find the target hole gradually decreased. On day 12, the memory of the mice is weakened, so it takes longer to find the target hole (Figure 3.1 I). This experiment shows that there is no abnormality in the learning and memory function of *Senp1* haploinsufficient mice. Like wild-type mice, they can obtain the memory of the target hole during training.

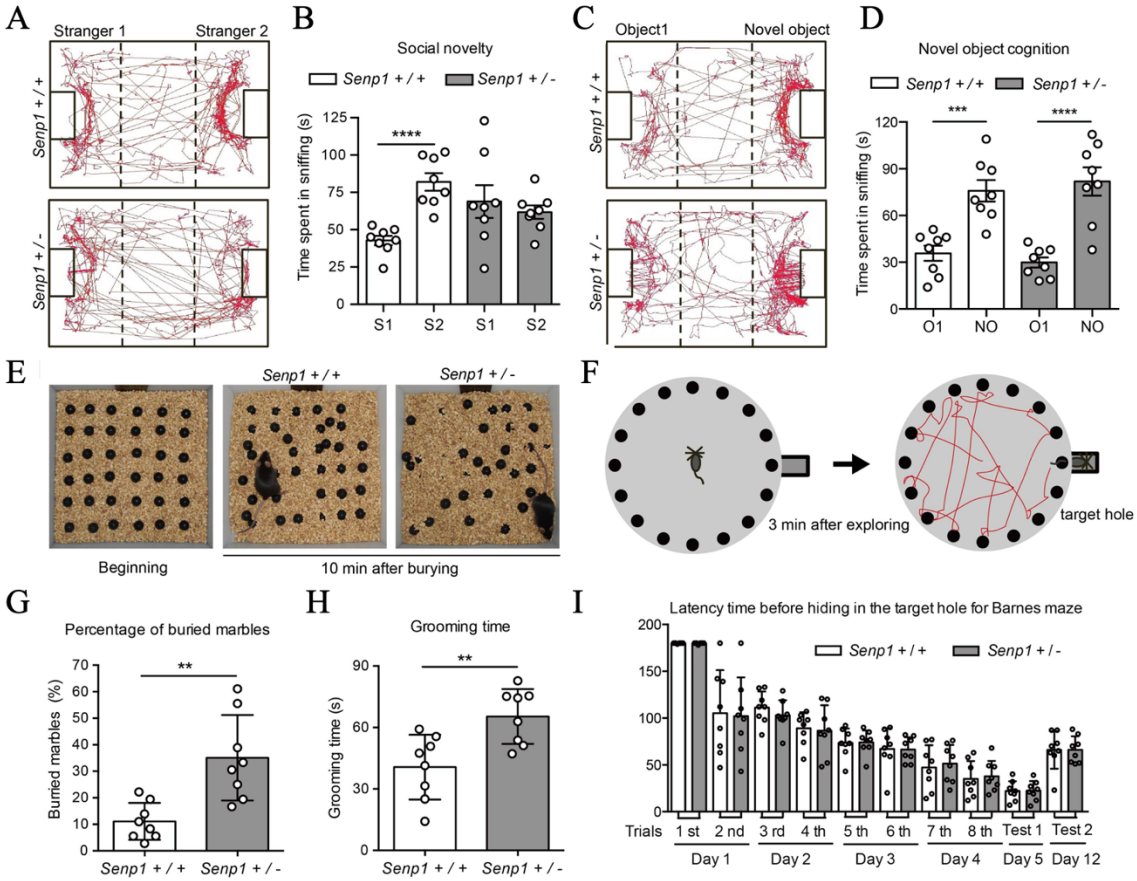

Figure 3.1 *Senp1* haploinsufficient mice show autistic-like behaviors

A. Representative locomotor traces of mice in the three-chamber test for social novelty. The red curve is the trajectory of the mouse in 10 minutes, and the rectangles on the left

and right sides are the cages where two stranger mice are placed. B. In the social novelty, the sniffing time of mice to stranger 1 and stranger 2. S1 means stranger 1, and S2 means stranger 2. Use t test to analyze the data (n = 8, for each genotype). C. Representative locomotor traces of mice in the three-chamber novel object cognition. The red curve is the trajectory of the mouse in 10 minutes, and the rectangles on the left and right sides are the cages where two different objects are placed. D. In the novel object cognition, the sniffing time of mice for different objects. O1 is object1, and NO is novel object. Use t test to analyze the data (n = 8, for each genotype). E. Representative images showing the beginning and the end of the marble burying test. F. Quantification of percentage of buried marbles within 10 min (n = 8, for each genotype). G. Quantification of grooming time within 30 min (n = 8, for each genotype). H. Schematic illustration for a mouse to perform a trial within 3 min of Barnes maze test. I. Quantification of latency time before hiding in the target hole (n = 8, for each genotype).

** $p < 0.01$. n is the number of mice used in each experiment. Bars represent means ± SD.

3.2 *Senp1* haploinsufficient mice show increase of GABAergic neuron

In order to continue to explore the effects of heterozygous mutations in *Senp1*, we carried out immunofluorescence staining experiments. It is known that GABAergic interneurons are closely related to the phenotype of autism, so we decided to study on GABAergic interneurons. GABAergic interneurons can be divided into non-overlapping categories by different markers, including parvalbumin (PV), and somatostatin (SST) and so on. This topic focuses on the research of these two types of markers.

The brains of wild-type and *Senp1* haploinsufficient mice were perfused respectively. After fixation and dehydration, the mouse brain was sliced into 40-micron thick slices. Floating brain sections were rinsed in PBS then blocked overnight at 4° C in PBS containing 5% Bovine albumin (BSA) and 0.2% Triton X-100。

3.2.1 *Senp1* haploinsufficient mice show increase in RSA

We use PV and SST antibodies to stain the brain slices, observe and record them with a microscope. After the blocking is completed, add the primary antibody solution at a dilution ratio of 1:1000. After incubating overnight, add the secondary antibody solution at a dilution ratio of 1:1000. Finally, add DAPI according to the dilution ratio of 1:300.

The experimental results showed that the number of GABAergic interneurons in the RSA (retrosplenial agranular cortex) increased significantly (Figure 3.2 A). The area marked by the dashed frame is the RSA brain area. The green signal marked PV and SST neurons. Manually count the single neurons marked in the figure, and use t test for data analysis. Since the proportion of neurons labeled by the antibody is roughly fixed, the number of neurons can be qualitatively displayed. The statistical results show that compared with wild-type mice, the numbers of PV neurons and SST neurons in the RSA brain regions of *Senp1* haploinsufficient mice are significantly increased (Figure 3.2 B). The 14 groups of data were counted, including 7 wild-type mice and 7 *Senp1* haploinsufficient mice. Statistics show significant differences, indicating that heterozygous mutations in *Senp1* gene will increase the number of GABAergic interneurons in the RSA brain area of mice.

3.2.2 *Senp1* haploinsufficient mice show no change in other areas

In order to explore whether the RSA brain area is a specific brain area for autism spectrum disorder, we also performed statistics on other brain areas in the cerebral cortex. For convenience, no schematic diagrams of other areas are shown. The statistics of six brain regions were carried out, including ACC (accumbens nucleus), CA1 (field CA1 of hippocampus), CA2 (field CA2 of hippocampus) and CA3 (field CA3 of hippocampus), DG (dentate gyrus), RSG (retrosplenial granular cortex) and S1BF (primary somatosensory cortex). Compared with wild-type mice, *Senp1* haploinsufficient mice showed no changes in the number of PV and SST neurons in these six brain regions (Figure 3.2 C-H). Since the distribution of GABAergic interneurons is roughly concentrated in these brain regions, it can be explained that when *Senp1* is heterozygously mutant, GABAergic interneurons only increase specifically in the RSA brain region, suggesting that the inhibitory synaptic connections in RSA may be altered in *Senp1* haploinsufficient mice.

The retrosplenial cortex (RSC), composing of two parts — RSA (retrosplenial agranular) and RSG (retrosplenial granular), is implicated in the top-down modulation of sensorimotor information from primary sensory cortices ([31], Bicanski and Burgess, 2020, 453-70). In the past decade, research on the retrosplenial cortex has gradually increased. This core cortex supports a range of cognitive functions, including episodic memory, navigation, imagination, and planning for the future. Studies have shown that the dysfunction in this area can lead to a series of mental illnesses ([32], Vann, Aggleton et al., 2009, 792-802).

This experiment shows that RSA may play a vital role in autism spectrum disorder. Our next research will focus on this brain area, and explore how the *Senp1* heterozygous

mutation affects the normal physiological function of the RSA brain area through the regulatory circuit. According to the deSUMOylation function of SENP1 protein, find the downstream protein replaced by SENP1 protein.

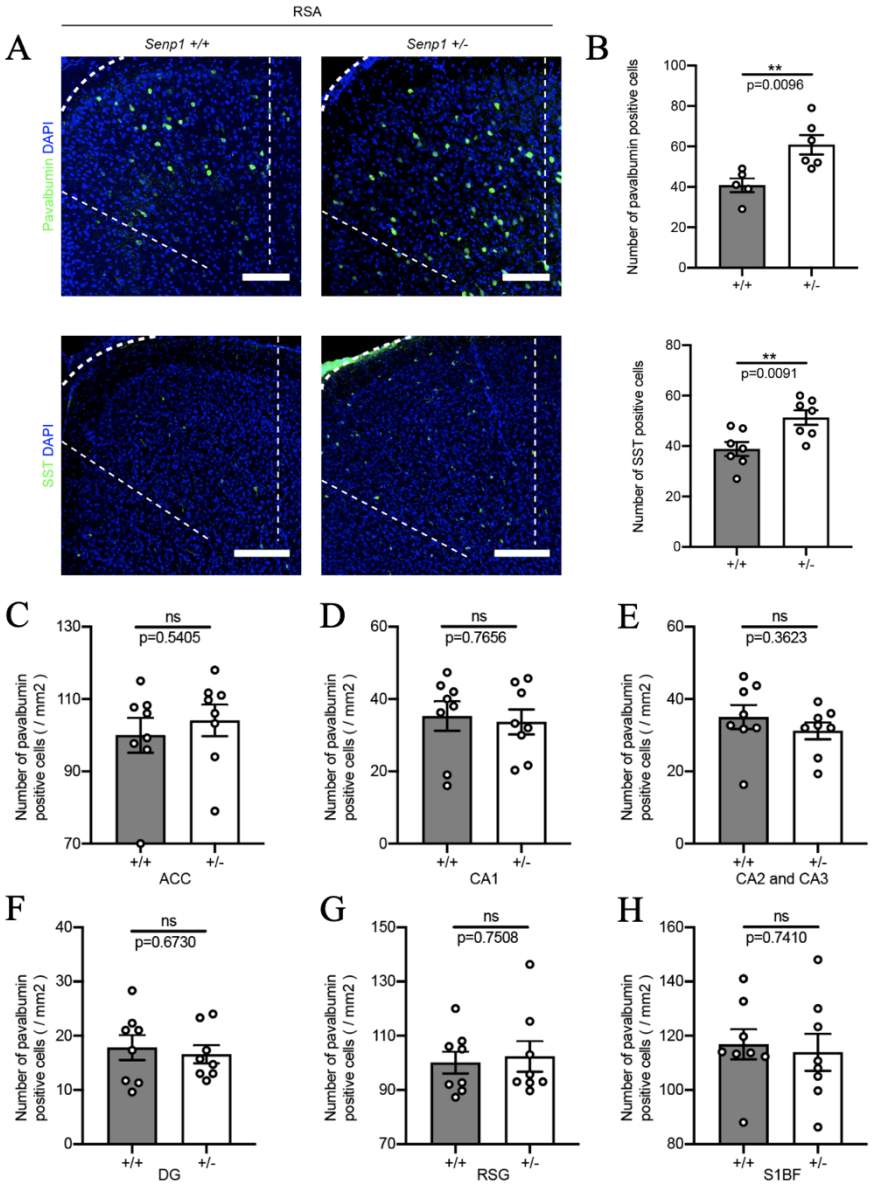

Figure 3.2 *Senp1* haploinsufficient mice show RSA-specific increase of GABAergic interneuron

A. Representative coronal immunofluorescence staining sections of cortex stained for

anti-parvalbumin (Abcam, Cat#ab181086) (green), anti-somatostatin (Santa Cruz Biotechnology, Cat#sc-55565) (green) and DAPI (Thermo Fisher Scientific, Cat#D1306) (blue) at 3 months of age. RSA, retrosplenial agranular cortex; Scale bar, 200μm. B. Quantification of average number of neurons within RSA brain region (n = 7, for each genotype). C. Quantification of average number of neurons within ACC (accumbens nucleus) (n = 8, for each genotype). D. Quantification of average number of neurons within CA1 (field CA1 of hippocampus) (n = 8, for each genotype). E. Quantification of average number of neurons within CA2 (field CA2 of hippocampus) and CA3 (field CA3 of hippocampus) (n = 8, for each genotype). F. Quantification of average number of neurons within DG (dentate gyrus) (n = 8, for each genotype). G. Quantification of average number of neurons within RSG (retrosplenial granular cortex) (n = 8, for each genotype). H. Quantification of average number of neurons within S1BF (primary somatosensory cortex) (n = 8, for each genotype).

** $p < 0.01$; ns means no significant. n is the number of mice used in each experiment. Bars represent means ± SD.

3.3 SENP1 protein modifies FMRP expression through SUMOylation

We have already understood that *Senp1* heterozygous mutations can cause abnormalities in GABAergic interneurons in the RSA brain area. Next, we need to explore the molecular mechanism that produces this result. Since SENP1 is one of major SUMOylation deconjugating enzymes, we reasoned that SENP1 may regulate synapse development by conjugating small uniqitin-like modifier (SUMO) to candidate proteins, which performed critical functions in syanpse development. Among many synaptic proteins which are implicated in SUMOylation modification, we found that fragile X syndrome mental retardation protein (FMRP) was an intriguing candidate of SENP1-regulated deSUMOylation, as mutation of FMR1, the coding gene of FMRP,

causing Fragile X syndrome (FXS), which shares autistic symptoms with ASD ([19], Khayachi, Gwizdek et al., 2018, 757, 18], Tang, Lu et al., 2018, 1100-2). The SUMOylation and deSUMOylation is a dynamic process, dysregulation either of which will sure lead to compromise of proper functions of FMRP. Thus we set out to investigate whether FMRP is dysregulated in the brain of *Senp1* haploinsufficient mice. We designed immunofluorescence staining experiments and western blotting experiments to verify whether FMRP is regulated by SENP1.

3.3.1 The decrease of SENP1 leads to the decrease of FMRP

We first examine whether FMRP is expressed in the RSA of mouse brain. We took out the mouse brain by perfusion, and then took out the tissue of the RSA brain area. After the homogenate, the protein sample was obtained, and then the western blotting experiment was carried out (Figure 3.3 A). After heating the protein samples of wild-type mice and *Senp1* haploinsufficient mice, we can get RSA lysate, and the protein samples were added to 10% precast gel. We used antibodies for detection including rabbit anti-FMRP (Abcam, Cat#ab17722), rabbit anti-SENP1 (Abcam, Cat#ab108981), and mouse anti-GAPDH (Cell Signaling Technology, Cat#97166). The results showed that compared with wild-type mice, the SENP1 protein content of *Senp1* haploinsufficient mice in the RSA brain area decreased significantly. It is worth noting that we can see that the FMRP protein content of *Senp1* haploinsufficient mice in the RSA brain area has also decreased (Figure 3.3 B).

In order to see the expression of SENP1 and FMRP in RSA more intuitively, we conducted an immunofluorescence staining experiment. We found that FMRP closely colocalized with SENP1 in the RSA neurons of the mouse brain (Figure 3.3 C, D).

These results show that the protein level of FMRP in the RSA region clearly downregulated in *Senp1* haploinsufficient mice comparing to its of wild-type mice, strongly suggest that FMRP was regulated by SENP1 in the RSA of the brain. We also quantitatively measured the content of these two proteins in the RSA brain area. The results showed that compared with wild-type mice, the contents of SENP1 and FMRP in *Senp1* haploinsufficient mice were significantly decreased (Figure 3.3 E).

These two experiments can quantitatively show that when *Senp1* is heterozygously mutated, the protein content of SENP1 and FMRP in the RSA brain area will be reduced. Due to the deSUMOylation function of SENP1, we assume that FMRP is regulated by SENP1. That is to say, in heterozygous mice, when the content of SENP1 decreases, the deSUMOylation regulation of FMRP decreases, and the SUMOylated FMRP will increase.

3.3.2 Increased FMRP-SOMO bands in RSA

If SENP1 indeed plays a critical role in removing the SUMO groups from FMRP protein, the SUMOylation level of FMRP in the brain of *Senp1* haploinsufficient mice may elevate. To determine the level of FMRP SUMOylation in the RSA region, we collected the RSA lysate from *Senp1* haploinsufficient mice and WT mice, then performed immunoprecipitation assay using antibody against FMRP. We found that in the RSA lysate from *Senp1* haploinsufficient mice, there is clearly an up-shifting band with FMRP positive signals, but not in WT mice (Figure 3.3 F), suggesting that FMRP-SUMO signals are enriched in the RSA region of *Senp1* haploinsufficient mice. Together, these evidences suggest that SENP1 regulates the SUMOylation of FMRP in the brain, hereby over SUMOylation of FMRP protein in the lack of SENP1 would lead to its degradation ([19], Khayachi, Gwizdek et al., 2018, 757).

This experiment shows that we have found a downstream protein regulated by SENP1, namely FMRP. FMRP plays an important role in the transmission of information in the nervous system. When the content of SENP1 protein decreases, excessive ubiquitination will increase the degraded FMRP, resulting in a decrease in the overall content of FMRP protein. This may be one of the reasons for the abnormality of GABAergic interneurons. SENP1 protein indirectly regulates the physiological functions of GABAergic interneurons, thereby regulating the phenotype of autism.

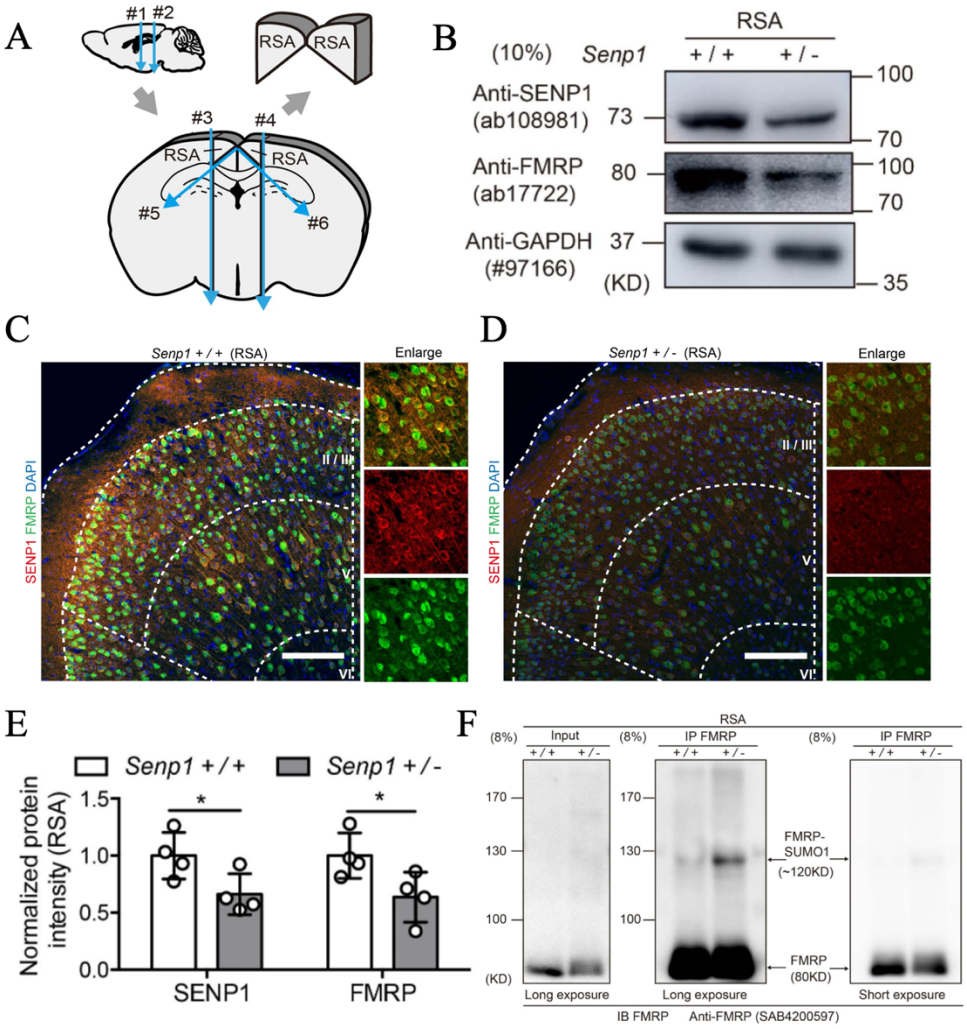

Figure 3.3 SENP1 protein modifies FMRP expression through SUMOylation

A. Schematic illustration for dissecting RSA region from the mouse brain. B. Representative western blot pictures illustrating protein intensity of SENP1, FMRP and GAPDH in the RSA region from *Senp1* haploinsufficient mice and wild-type mice. Antibodies include rabbit anti-FMRP (Abcam, Cat#ab17722), rabbit anti-SENP1 (Abcam, Cat#ab108981), and mouse anti-GAPDH (Cell Signaling Technology, Cat#97166). C. Representative immunofluorescence staining sections of RSA stained for SENP1 (red), FMRP (green) and DAPI (blue) at 3 months of age of wild-type mice. Scale bar, 200μm. D. Representative immunofluorescence staining sections of RSA stained for SENP1 (red), FMRP (green) and DAPI (blue) at 3 months of age of *Senp1* haploinsufficient mice. Scale bar, 200μm. E. Quantification of normalized SENP1 and FMRP protein intensity in RSA (n = 4, for each genotype). F. Representative immunoblot with anti-FMRP antibody using RSA extracts of 3 months old mouse immunoprecipitated with anti-FMRP antibody or control IgG.

* $p < 0.05$. Bars represent means ± SD.

3.4 Protein supplementation rescues the abnormal phenotype

We next wonder whether re-introducing SENP1 or FMRP may be able to rescue the autistic-like behaviors of *Senp1* haploinsufficient mice. Using AAV-based strategy, we injected AAV harboring vector control, SENP1 or FMRP cDNA bi-laterally in RSA region of *Senp1* haploinsufficient mice at 2 months of age and examined behaviors one month later. When the mice were two months old, they were injected with the virus. The virus will be expressed in the next month. When the mice were three months old, we carried out behavioral experiments to explore whether the autistic phenotype of the mice was rescued. The behavioral experiment takes about two weeks, after which the mice will be sacrificed for perfusion brain extraction and immunofluorescence staining experiments (Figure 3.4 A).

We injected three viruses, including AAV9-Vector, AAV9-SENP1 and AAV9-FMRP. Among these virus, AAV9-Vector is the control group, and the other two viruses express SENP1 and FMRP proteins respectively. The protein content of the RSA brain region of *Senp1* haploinsufficient mice will be supplemented after virus injection (Figure 3.4 B). We used Western blotting to detect whether the protein content of RSA brain regions in *Senp1* haploinsufficient mice was rescued. Western blot experiments were performed on the RSA lysates of wild mice and *Senp1* haploinsufficient mice injected with the virus. The results showed that the both of the protein in *Senp1* haploinsufficient mice was lower than that of wild-type mice, while the protein content of *Senp1* haploinsufficient mice was not much different from that of *Senp1* haploinsufficient mice with AAV9-Vector injection, and the protein content of *Senp1* haploinsufficient mice injected with AAV9-SENP1 was higher than that of the control group. The experimental results show that the virus we injected can indeed increase the protein content of the RSA brain region of *Senp1* haploinsufficient mice, and the increased content is close to the normal value (Figure 3.4 E).

We need to explore whether the number of GABAergic interneurons and the autism-like phenotype of *Senp1* haploinsufficient mice can be improved after the protein content of the RSA brain region is rescued to a normal value.

3.4.1 The number of GABAergic neurons in RSA is rescued

In order to detect the changes in the number of GABAergic interneurons in the RSA brain regions of *Senp1* haploinsufficient mice after virus injection, we carried out another immunofluorescence staining experiment. One month after the virus injection, the *Senp1* haploinsufficient mice were perfused to take their brains, and the brains were sliced into 40-micron slices. After staining the brain slices, count the GABAergic

interneurons in the RSA brain area. We injected three different viruses, including AAV-Vector, AAV-SENP1 and AAV-FMRP. The statistical results of the number of GABAergic interneurons in the RSA brain area showed that the number of the control group was high, but the number of parvalbumin-positive interneurons in the two groups injected with SENP1 and FMRP proteins decreased significantly (Figure 3.4 C, D). The statistical results of the number of GABAergic interneurons in the RSG brain area showed that there was no significant difference in the number of parvalbumin-positive neurons between the control group and the other two groups.

The experimental results show that protein replenishment in the RSA brain area can improve the abnormal increase in the number of GABAergic interneurons in autistic mice, and that both SENP1 protein and FMRP protein replenishment can rescue abnormal phenotypes. It is noteworthy that this also shows that the RSA brain area may be an important brain area related to autism. In addition, no difference in the number of parvalbumin-positive neurons between the control group and the experimental group was observed in the RSG brain area, which also shows the accuracy of the virus injection experiment.

3.4.2 The autistic-like behaviors are rescued

We first performed behavioral tests on *Senp1* haploinsufficient mice injected with the virus, including three-chamber experiments and repeated stereotyped behavior detection tests. When the mice were three months old, they were tested for differences in their propensity for stranger 1 and stranger 2. The results showed that the mice in the control group showed the same phenotype as the *Senp1* haploinsufficient mice. They had no significant difference in the sniffing time between stranger 1 and stranger 2, so they had social deficits. The *Senp1* haploinsufficient mice injected with AAV-SENP1

and AAV-FMRP showed a tendency toward stranger 2, which proved that protein injection can rescue the autism phenotype of *Senp1* haploinsufficient mice (Figure 3.4 H, J). In the repeated stereotyped behavior test, we recorded the number of buried marbles and the time of grooming in three different groups of mice. The results showed that the control group still had repetitive stereotyped behavior, while the *Senp1* haploinsufficient mice injected with the two proteins were rescued (Figure 3.4 F, G, I).

We found that injection of AAV harboring vector control clearly did not change the deficits of social novelty test of *Senp1* haploinsufficient mice, whereas injection of AAV-SENP1 into the RSA of *Senp1* haploinsufficient mice significantly increased the time *Senp1* haploinsufficient mice spent in sniffing novel partners, suggesting that the SENP1 in the RSA is crucial for social behaviors in mouse. Moreover, injection of AAV-FMRP in the RSA region of *Senp1* haploinsufficient mice also alleviated the social deficits.

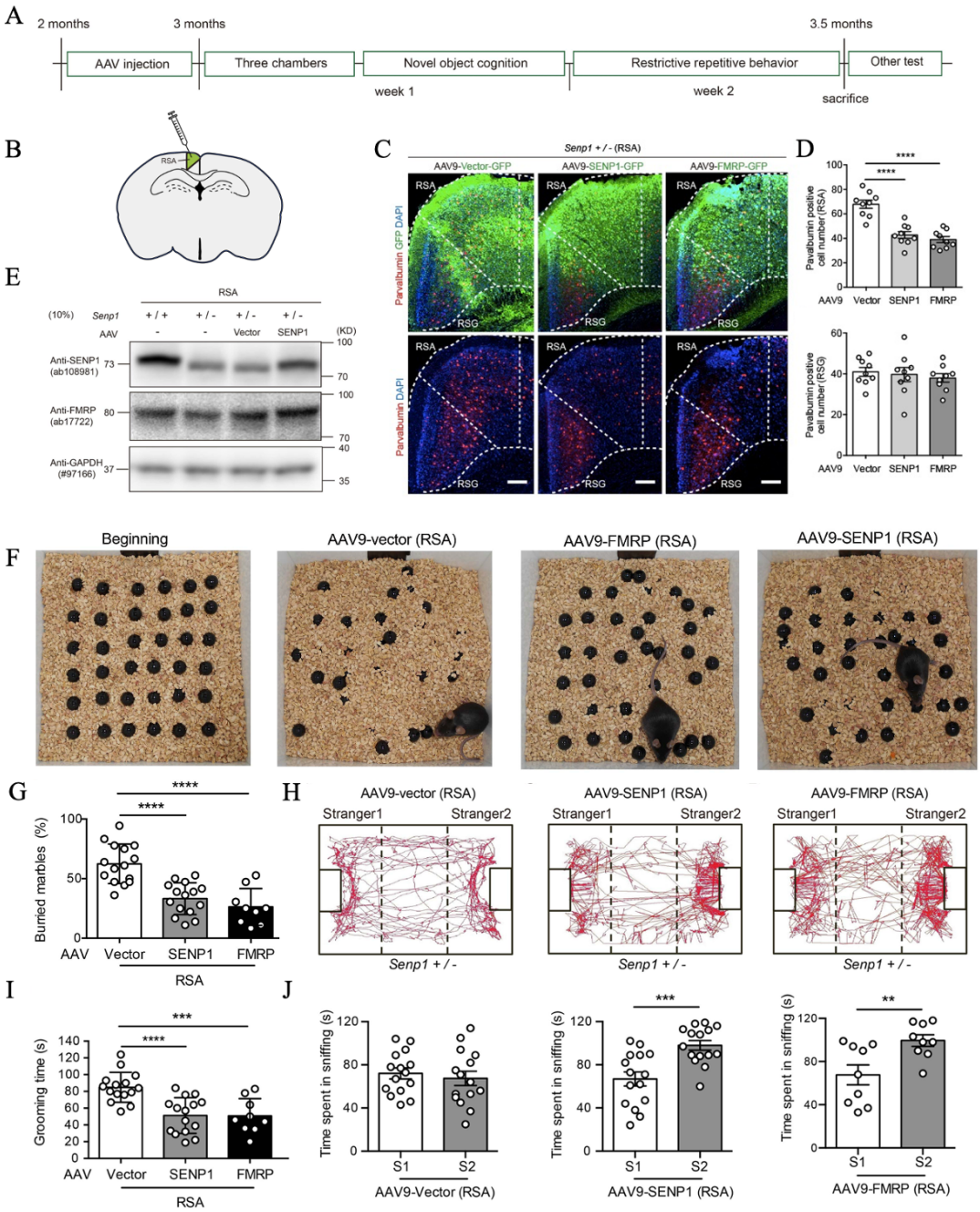

Figure 3.4 AAV delivery of SENP1 and FMRP in RSA region rescues autistic behaviors of *Senp1*-haploinsufficient mice

A. Strategy of behavioral tests after AAV injection for *Senp1* +/- mice. The mice were

injected with the virus when they were two months old, were subjected to behavioral experiments when they were three months old, and were sacrificed two weeks later for immunofluorescence staining experiments. B. Schematic illustration for injecting virus to RSA region. C. Representative coronal immunofluorescence staining sections of RSA and RSG stained for parvalbumin (red), GFP (green) and DAPI (blue) at 3.5 months of age. Scale bar, 200μm. D. Quantification of average parvalbumin-positive neuron number within each brain region (n = 9, for each experiment). E. Representative western blot pictures illustrating protein intensity of SENP1 and FMRP for RSA tissues of mice with or without injection of AAV-vector or AAV-SENP1. F. Representative figures showing from beginning to the end of the marble burying test for *Senp1* haploinsufficient mice after AAV injection. G. Quantification of percentage of buried marbles within 10 min. *Senp1* haploinsufficient mice injected with various AAVs (AAV9-vector in RSA n=15; AAV9-SENP1 in RSA n=15; AAV9-FMRP in RSA n=9). H. Representative locomotor traces of mice in the three-chamber test for social novelty. *Senp1* haploinsufficient mice were injected with various AAVs (AAV9-vector in RSA n=15; AAV9-SENP1 in RSA n=15; AAV9-FMRP in RSA n=9). I. Quantification of grooming time within 30 min. *Senp1* haploinsufficient mice injected with various AAVs (AAV9-vector in RSA n = 15; AAV9-SENP1 in RSA n= 15; AAV9-FMRP in RSA n=9). J. Quantification of time mice spent in sniffing familiar and novel partners. *Senp1* haploinsufficient mice injected with various AAVs (AAV9-vector in RSA n = 15; AAV9-SENP1 in RSA n= 15; AAV9-FMRP in RSA n=9).

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Bars represent means ± SD.

3.5 Summary

It is known that the etiology of autism is closely related to gene mutations. We started with constructing genetic mutation strains of mice to study the effects of single gene

mutations on neurons. In this project, we first cooperated with the hospital to obtain samples of patients with heterozygous mutations in *SENP1*. Based on such a *de novo* single gene heterozygous mutation, we constructed a corresponding mouse model. Knock out the *Senp1* gene heterozygously in mice to explore whether the mice will develop autism-like phenotype. Through behavioral experiments, we have clarified the autistic phenotype of *Senp1* haploinsufficient mice, which means that *Senp1* haploinsufficient mice show obvious social disorders and repetitive stereotyped behaviors, which are consistent with the main phenotype of autism. After successfully obtaining mice with autism, we explored the cell-level effects of *Senp1* mutations. Considering that GABAergic interneurons are a type of neurons that are vital to the normal function of the brain, and their dysfunction is closely related to autism, we focused on exploring the function of GABAergic neurons. In the immunofluorescence staining experiment, we labeled parvalbumin-positive and somatostatin-positive neurons, and found that their numbers in the cerebral cortex of *Senp1* haploinsufficient mice increased significantly. What's more noteworthy is that this increase in number is specific to the brain area, that is, GABAergic neurons will increase significantly in the RSA brain area, but will not change in other brain areas.

In order to further explore the effect of the heterozygous mutation of *Senp1* gene on the brain area of RSA, we started with the function of the SENP1 protein to find its downstream proteins. It is known that if the FMR1 gene expressing FMRP protein is mutated, it will produce symptoms similar to autism, and the FMRP protein can be regulated by SUMOylation. We explored whether SENP1 protein can regulate FMRP protein. Through immunofluorescence staining experiments and western blotting experiments, we determined that in *Senp1* haploinsufficient mice, when the content of SENP1 protein decreases, the content of FMRP protein also decreases. This result implies that FMRP is likely to be regulated by SENP1. When the SENP1 protein content

decreases, the function of removing SUMO from the FMRP protein will also decrease, and the FMRP protein content of SUMOylation will increase. When the protein is SUMOylation, it is likely to be degraded and decreased, so the content of FMRP protein will be reduced. Western blot experiments also proved that FMRP-SUMO bands appear in *Senp1* haploinsufficient mice. This result further shows that FMRP is indeed regulated by SENP1. When the protein content of SENP1 decreases, the content of FMRP-SUMO will increase.

It is known that FMRP is essential for the normal physiological function of neurons. If the content of FMRP protein is reduced, it is likely to cause abnormalities in GABAergic interneurons, and then produce autism phenotypes. In order to verify this idea, we designed a protein-replenishing experiment. By specifically injecting AAV virus into the RSA brain area, the protein content of SENP1 and FMRP in the RSA brain area of *Senp1* haploinsufficient mice was filled back and forth. One month after the virus was expressed, we used behavioral experiments to test whether their autism phenotype improved. The results showed that the social deficits and repetitive stereotyped behavior of *Senp1* haploinsufficient mice were improved after replenishing protein specifically in the RSA brain area. This experimental result proves that SENP1 plays a vital role in the RSA brain area, and its downstream protein FMRP is also one of the indispensable proteins. After the behavioral experiment, we sacrificed the mice, and then performed immunofluorescence staining to detect whether the number of GABAergic interneurons in the RSA brain area of the mice was improved. The results showed that when the protein content of SENP1 and FMRP in the RSA brain region of *Senp1* haploinsufficient mice returned to normal values, the number of GABAergic interneurons was also rescued. The abnormal increase in the number of GABAergic interneurons in autistic mice will decrease with the expression of the virus. This experiment illustrates the necessity of SENP1 protein and its downstream protein FMRP.

More importantly, it shows that the RSA brain area is an important brain area closely related to autism.

In general, in *Senp1* haploinsufficient mice, the content of SENP1 protein is reduced, resulting in a decrease in FMRP protein that is subject to its deSUMOylation. FMRP is essential to the normal physiological function of GABAergic interneurons, so this type of neuron is also affected, resulting in social deficits and repetitive stereotyped behaviors (Figure 3.5 A, B). We used the method of constructing an animal model to initially explain how the heterozygous mutation of *Senp1* causes autism in mice.

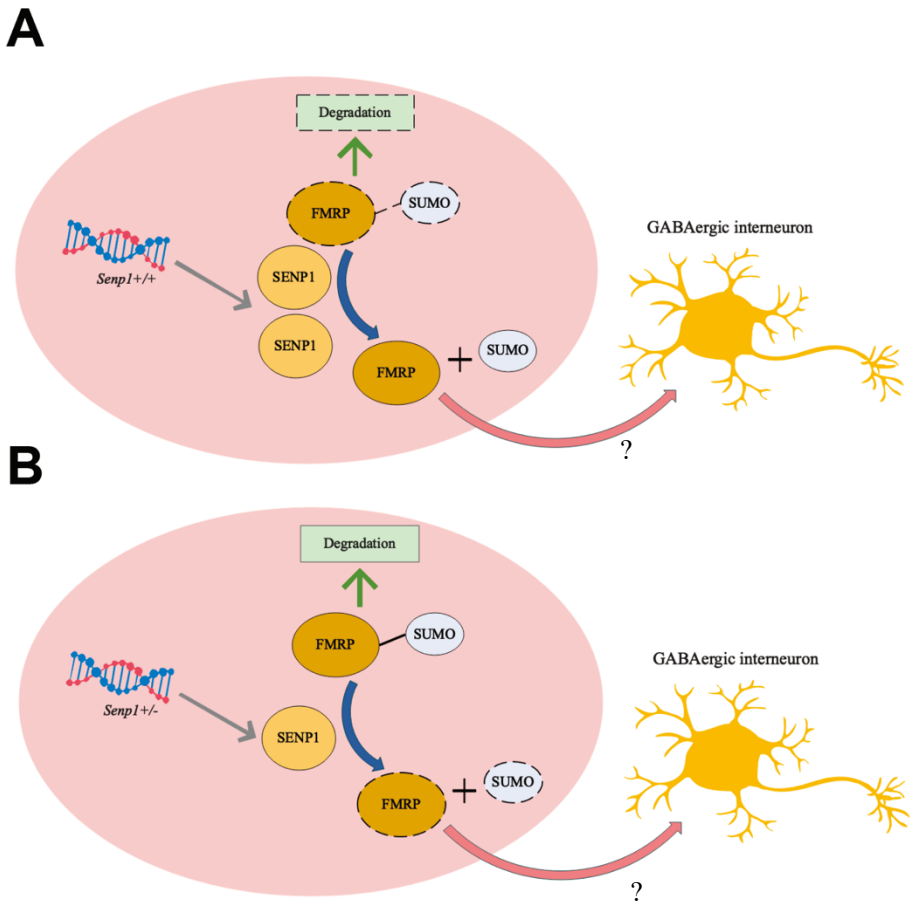

Figure 3.5 How *Senp1* heterozygous mutations cause abnormalities

A. Schematic illustration for how SENP1 protein regulates the SUMOylation of FMRP protein, which in turn regulates GABAergic interneurons in wild-type mice. There is sufficient SENP1 protein to allow FMRP to perform deSUMOylation. B. Schematic illustration for how the decrease of SENP1 protein reduces the content of FMRP, which in turn makes GABAergic interneurons dysfunction in *Senp1* haploinsufficient mice.

4 Discussion

4.1 Conclusion

In this project, we found that in *Senp1* haploinsufficient mice, mimicking the human ASD patient, the number of GABAergic interneurons in the RSA region of retrosplenial cortex was specifically increased, which causally leading to deficits in social novelty tests and could be rescued by re-introduction of SENP1 or its downstream molecule FMRP during adulthood. This data suggests that the RSA region plays a critical role in regulating social behaviors in the mouse brain.

4.1.1 The main conclusions of this project

Based on autism, my project mainly explores whether a heterozygous mutation of *Senp1*, a risk gene for autism, can produce autism in mice, and how to produce autism. The experiment uses a variety of technical methods to explore the principles of the autism phenotype. Through immunofluorescence staining experiments, we can visually see the distribution and number of neurons marked by different markers. Through Western blotting, we can qualitatively analyze the amount of protein in the sample and the change in protein content. Through stereotactic injection of the virus, we can specifically replenish the required protein in the required brain area, and then explore the relationship between the protein and the phenotype. Through behavioral experiments, we can link mice with autism, which allows us to use simple methods to measure the pathological characteristics of animals.

The techniques used in the experiment are relatively basic, but the experimental results are very reliable. Taking human diseases as an entry point, and verifying on mouse

models before exploring the specific effects of gene mutations, this can provide some suggestions for the study of autism.

The main conclusion we got is mainly how the autism risk gene *Senp1* affects the FMRP protein, which in turn affects GABAergic interneurons, and finally produces the autism phenotype. At the very beginning of the experiment, we used behavioral experiments to verify the autism phenotype of heterozygous mice, which is also the basic conclusion of the entire experiment. Since both humans and mice are mammals, there is a certain degree of credibility in using mice to simulate human autism. However, because the species gap between humans and mice is larger than that between humans and monkeys, it is ideal to use monkeys as animal models. Limited to the experimental time and experimental conditions, we used mice as an animal model for preliminary exploration. After obtaining mice with autism phenotypes, we explored the GABAergic interneurons in the cerebral cortex of *Senp1* haploinsufficient mice based on existing research results. The results show that when mice develop autism due to heterozygous mutations in the *Senp1* gene, GABAergic interneurons in the RSA brain area of the cerebral cortex will increase. Subsequent western blot experiments showed that SENP1 protein regulates the function of downstream protein FMRP through deSUMOylation. Therefore, we can basically clarify the mechanism of SENP1 protein and how its insufficient dose affects GABAergic interneurons. In order to make the experimental results more reliable, we have designed a protein replenishment experiment. When the SENP1 and FMRP proteins were supplemented in the RSA brain area of *Senp1* haploinsufficient mice, their autism phenotype and the number of GABAergic interneurons in the RSA brain area were rescued. These conclusions are mutually corroborating, explaining the importance of the SENP1 protein, and also point out an important brain area that may be related to autism, that is, the RSA brain area.

4.1.2 Weaknesses of experimental design

Due to time and experimental technology limitations, this experiment still has many shortcomings. At the beginning of the experiment, the hope was to explore the overall cause of autism by searching for autism risk genes. The study of the abnormalities caused by single gene mutations is a very basic but not very practical thing, but if the pathway of action of a single gene can be fully described, it will also be helpful for molecular research.

The first is that the research on the changes in the number of GABAergic interneurons is not perfect. GABAergic interneurons can be divided into non-overlapping categories by different markers, but the PV-positive and SST-positive pointed out in the experiment are only two of them. The subject did not explore whether similar changes occurred in other types of neurons, nor did it explain that the changes in these two types of neurons are inevitable phenomena in GABAergic interneurons. In addition, it is not clear whether changes in the number of neurons will affect certain functions and trigger the autism phenotype. For the changes in the number of these two types of GABAergic interneurons, we need to further consider the functional changes behind this change. The specific mechanism of FMRP protein affecting GABAergic neurons also needs further study.

Secondly, the research on the specific mechanism of FMRP protein content reduction is not perfect. It is known that the decrease of SENP1 protein content will reduce the protein content of FMRP, so is the connection between these proteins achieved through the single path of deSUMOylation? What physiological significance does the SUMOylation FMRP have? We cannot rule out that FMRP-SUMO molecules may act on neurons in other ways, leading to the appearance of autism. It is possible that SENP1

indirectly acts on FMRP by regulating other proteins, thereby reducing the protein content of FMRP.

Research on the RSA brain area indicates that this brain area is likely to closely related to autism. If there is a dysfunction in RSA, it is likely to cause autism. In the protein replenishment experiment, we injected three AAV viruses into the RSA brain area. More rigorously, we should also inject the virus into other unrelated brain regions to explore whether supplementing proteins in other brain regions can also improve the autism phenotype. We have obtained the conclusion that the protein content of FMRP in the RSA brain region of heterozygous mice is reduced, but at the same time we should test other brain regions to explore whether SENP1 specifically regulates the content of FMRP in the RSA brain region.

In the protein replenishment experiment, we confirmed that the number of PV-positive neurons in the RSA brain area of *Senp1* haploinsufficient mice will decrease after being injected with the virus, which is close to the normal value. Due to the limitation of the experimental sample and time, we could not get the statistical results of the number of different somatostatin-positive neurons. If the number of somatostatin-positive neurons is rescued, it can more clearly explain the impact of GABAergic interneurons. At the same time, we should pay attention to whether the function of neurons interacting with GABAergic interneurons is affected. In addition, we need to pay attention to whether the precursor neurons of GABAergic interneurons are affected.

4.2 Unresolved issues

This project describes that heterozygous mutation of *Senp1* gene can cause social disorders and repetitive stereotyped behaviors in mice, pointed out the downstream

protein FMRP regulated by SENP1, and discovered a brain area that may be related to autism, that is, the RSA brain area. Due to design ideas and insufficient technical methods, there are many problems that need to be solved next.

First, we need to explore the relationship between social disorders and repetitive stereotyped behaviors in mice with human autism. Since this project is based on human diseases and uses mice as an animal model for research, it is necessary to correlate the research results with human diseases. Since monkeys and humans are both primates, we can use monkeys as experimental models to further explore the effects of *Senp1* heterozygosity on animals, and then combine the experimental conclusions with humans to explore effective intervention methods suitable for humans. In addition, whether the two phenotypes of social disorders and repetitive stereotypes in mice can correspond to human autism is also a problem that needs to be resolved. If a sufficiently reliable and systematic standard for detecting autism in mice is constructed, it will greatly simplify research thinking.

In addition, *Senp1* is an autism risk gene screened from human patient samples, and its expression product protein SENP1 is a relatively conservative enzyme with deSUMOylation function. SENP1 regulates many different proteins in cells, including FMRP. We also need to study other pathways regulated by SENP1 to further clarify the organic diseases that occur in the brain when *Senp1* is heterozygously mutated. We need to combine the amount of protein and the function of neurons to explain why the final autism phenotype is produced. Continue to search for downstream proteins regulated by SENP1 through western blot experiments, and then look for other pathways of action.

Mental diseases are usually caused by neuronal dysfunction. The GABAergic interneurons pointed out in this project also have abnormal numbers. We need to

explore whether there are other types of neuronal dysfunction in *Senp1* haploinsufficient mice, and their interconnections. Since neurons transmit information relying on current and synaptic structure, we can study synapse-related proteins and record the firing characteristics of neurons. Using electrophysiological methods to record the current changes of GABAergic interneurons, explore their connections with peripheral neurons, and further clarify the influence of inhibitory synapses on autism.

In this project, we propose a brain area that may be closely related to autism, namely RSA brain area. In order to explain the connection between this brain area and autism, we need to explore other types of autism risk genes. We can construct mouse models of autism with heterozygous mutations in a single gene, and detect whether the RSA brain regions of these autism mouse models produce changes in the number of GABAergic interneurons, and then explain the contribution of RSA brain regions in autism. At the same time, we can specifically knock down the expression of *Senp1* gene in wild-type mice and detect whether the mice have autistic phenotypes, to further illustrate the importance of RSA in *Senp1* haploinsufficient mice.

4.3 Summary

My project mainly proposes an autism risk gene *Senp1*, and has made a preliminary verification of its pathogenic mechanism, that is, affects the normal function of GABAergic interneurons by affecting the deSUMOylation of the downstream protein FMRP, and finds a possibility autism-related brain area RSA. Due to various reasons, there are still many deficiencies and unsolved problems in the experiment. I also look forward to giving correct answers to these questions in the future.

It has been plagued many families with autism, and there is no absolutely effective medicine to treat it fundamentally. Compared with the United States, China's care for

families with autism is also relatively backward. If we can find the mechanism of the pathogenesis of autism through the study of single genes, and develop highly effective drugs, this will bring great hope to both autistic patients and their families. Neuroscience has always been the focus of the world's biologists, and mental diseases is a major problem in neurobiology. Parkinson's and Alzheimer's disease are also as unbreakable as a heavy mountain. What kind of neural circuits control memory and social interaction? What genes affect the thinking and intelligence of the brain? These problems need to be solved by us. Nowadays, single-gene research on autism is only a trivial experiment, but it is such a small step that has become a big step in scientific development.

The most peculiar discovery of this project is to propose a brain area that may be associated with autism. Although the current evidence is very inadequate, it gives us confidence in the study of autism in the future. We need to further explore the mechanisms of protein interaction, information transmission and neural circuits in the RSA brain area.

It is worth mentioning that our work has been included in the pre-printed version of biorxiv, in which I participated in some of the work.([33], Yang, Shi et al., 2021, 2021.01.24.427868)

References

[1] Lord, C., T. S. Brugha, T. Charman, J. Cusack, G. Dumas, T. Frazier, E. J. H. Jones, R. M. Jones, A. Pickles, M. W. State, J. L. Taylor and J. Veenstra-VanderWeele (2020). "Autism spectrum disorder." Nat Rev Dis Primers 6(1): 5.

[2] Sandin, S., P. Lichtenstein, R. Kuja-Halkola, C. Hultman, H. Larsson and A. Reichenberg (2017). "The Heritability of Autism Spectrum Disorder." Jama 318(12): 1182-4.

[3] De Rubeis, S., X. He, A. P. Goldberg, C. S. Poultney, K. Samocha, A. E. Cicek, Y. Kou, L. Liu, M. Fromer, S. Walker, T. Singh, L. Klei, J. Kosmicki, F. Shih-Chen, B. Aleksic, M. Biscaldi, P. F. Bolton, J. M. Brownfeld, J. Cai, N. G. Campbell, A. Carracedo, M. H. Chahrour, A. G. Chiocchetti, H. Coon, E. L. Crawford, S. R. Curran, G. Dawson, E. Duketis, B. A. Fernandez, L. Gallagher, E. Geller, S. J. Guter, R. S. Hill, J. Ionita-Laza, P. Jimenz Gonzalez, H. Kilpinen, S. M. Klauck, A. Kolevzon, I. Lee, I. Lei, J. Lei, T. Lehtimäki, C. F. Lin, A. Ma'ayan, C. R. Marshall, A. L. McInnes, B. Neale, M. J. Owen, N. Ozaki, M. Parellada, J. R. Parr, S. Purcell, K. Puura, D. Rajagopalan, K. Rehnström, A. Reichenberg, A. Sabo, M. Sachse, S. J. Sanders, C. Schafer, M. Schulte-Rüther, D. Skuse, C. Stevens, P. Szatmari, K. Tammimies, O. Valladares, A. Voran, W. Li-San, L. A. Weiss, A. J. Willsey, T. W. Yu, R. K. Yuen, E. H. Cook, C. M. Freitag, M. Gill, C. M. Hultman, T. Lehner, A. Palotie, G. D. Schellenberg, P. Sklar, M. W. State, J. S. Sutcliffe, C. A. Walsh, S. W. Scherer, M. E. Zwick, J. C. Barett, D. J. Cutler, K. Roeder, B. Devlin, M. J. Daly and J. D. Buxbaum (2014). "Synaptic, transcriptional and chromatin genes disrupted in autism." Nature 515(7526): 209-15.

[4] Iakoucheva, L. M., A. R. Muotri and J. Sebat (2019). "Getting to the Cores of Autism." Cell 178(6): 1287-98.

[5] Levy, S. E., D. S. Mandell and R. T. Schultz (2009). "Autism." Lancet 374(9701): 1627-38.

[6] Crow, J. F. (2000). "The origins, patterns and implications of human spontaneous mutation." Nat Rev Genet 1(1): 40-7.

[7] Searles Quick, V. B., B. Wang and M. W. State (2021). "Leveraging large genomic datasets to illuminate the pathobiology of autism spectrum disorders." Neuropsychopharmacology 46(1): 55-69.

[8] Gaugler, T., L. Klei, S. J. Sanders, C. A. Bodea, A. P. Goldberg, A. B. Lee, M. Mahajan, D. Manaa, Y. Pawitan, J. Reichert, S. Ripke, S. Sandin, P. Sklar, O. Svantesson, A. Reichenberg, C. M. Hultman, B. Devlin, K. Roeder and J. D. Buxbaum (2014). "Most genetic risk for autism resides with common variation." Nat Genet 46(8): 881-5.

[9] Flotho, A. and F. Melchior (2013). "Sumoylation: a regulatory protein modification in health and disease." Annu Rev Biochem 82: 357-85.

[10] Rocca, D. L., K. A. Wilkinson and J. M. Henley (2017). "SUMOylation of FOXP1 regulates transcriptional repression via CtBP1 to drive dendritic morphogenesis." Sci Rep 7(1): 877.

[11] Xu, Z., S. F. Chau, K. H. Lam, H. Y. Chan, T. B. Ng and S. W. Au (2006). "Crystal structure of the SENP1 mutant C603S-SUMO complex reveals the hydrolytic mechanism of SUMO-specific protease." Biochem J 398(3): 345-52.

[12] Kim, Y. H., K. S. Sung, S. J. Lee, Y. O. Kim, C. Y. Choi and Y. Kim (2005). "Desumoylation of homeodomain-interacting protein kinase 2 (HIPK2) through the cytoplasmic-nuclear shuttling of the

SUMO-specific protease SENP1." FEBS Lett 579(27): 6272-8.
[13] Li, J., W. Zhang, H. Yang, D. P. Howrigan, B. Wilkinson, T. Souaiaia, O. V. Evgrafov, G. Genovese, V. A. Clementel, J. C. Tudor, T. Abel, J. A. Knowles, B. M. Neale, K. Wang, F. Sun and M. P. Coba (2017). "Spatiotemporal profile of postsynaptic interactomes integrates components of complex brain disorders." Nat Neurosci 20(8): 1150-61.
[14] Tarailo-Graovac, M., C. Shyr, C. J. Ross, G. A. Horvath, R. Salvarinova, X. C. Ye, L. H. Zhang, A. P. Bhavsar, J. J. Lee, B. I. Drögemöller, M. Abdelsayed, M. Alfadhel, L. Armstrong, M. R. Baumgartner, P. Burda, M. B. Connolly, J. Cameron, M. Demos, T. Dewan, J. Dionne, A. M. Evans, J. M. Friedman, I. Garber, S. Lewis, J. Ling, R. Mandal, A. Mattman, M. McKinnon, A. Michoulas, D. Metzger, O. A. Ogunbayo, B. Rakic, J. Rozmus, P. Ruben, B. Sayson, S. Santra, K. R. Schultz, K. Selby, P. Shekel, S. Sirrs, C. Skrypnyk, A. Superti-Furga, S. E. Turvey, M. I. Van Allen, D. Wishart, J. Wu, J. Wu, D. Zafeiriou, L. Kluijtmans, R. A. Wevers, P. Eydoux, A. M. Lehman, H. Vallance, S. Stockler-Ipsiroglu, G. Sinclair, W. W. Wasserman and C. D. van Karnebeek (2016). "Exome Sequencing and the Management of Neurometabolic Disorders." N Engl J Med 374(23): 2246-55.
[15] Hay, R. T. (2005). "SUMO: a history of modification." Mol Cell 18(1): 1-12.
[16] Girach, F., T. J. Craig, D. L. Rocca and J. M. Henley (2013). "RIM1α SUMOylation is required for fast synaptic vesicle exocytosis." Cell Rep 5(5): 1294-301.
[17] Tai, D. J., Y. C. Liu, W. L. Hsu, Y. L. Ma, S. J. Cheng, S. Y. Liu and E. H. Lee (2016). "MeCP2 SUMOylation rescues Mecp2-mutant-induced behavioural deficits in a mouse model of Rett syndrome." Nat Commun 7: 10552.
[18] Tang, M., L. Lu, F. Xie and L. Chen (2018). "SUMOylation of Fragile X Mental Retardation Protein: A Critical Mechanism of FMRP-Mediated Neuronal Function." Neurosci Bull 34(6): 1100-2.
[19] Khayachi, A., C. Gwizdek, G. Poupon, D. Alcor, M. Chafai, F. Cassé, T. Maurin, M. Prieto, A. Folci, F. De Graeve, S. Castagnola, R. Gautier, L. Schorova, C. Loriol, M. Pronot, F. Besse, F. Brau, E. Deval, B. Bardoni and S. Martin (2018). "Sumoylation regulates FMRP-mediated dendritic spine elimination and maturation." Nat Commun 9(1): 757.
[20] Markram, H., M. Toledo-Rodriguez, Y. Wang, A. Gupta, G. Silberberg and C. Wu (2004). "Interneurons of the neocortical inhibitory system." Nat Rev Neurosci 5(10): 793-807.
[21] Tremblay, R., S. Lee and B. Rudy (2016). "GABAergic Interneurons in the Neocortex: From Cellular Properties to Circuits." Neuron 91(2): 260-92.
[22] Yang, J., X. Yang and K. Tang (2021). "Interneuron development and dysfunction." Febs j.
[23] Missler, M., T. C. Südhof and T. Biederer (2012). "Synaptic cell adhesion." Cold Spring Harb Perspect Biol 4(4): a005694.
[24] Goldberg, E. M. and D. A. Coulter (2013). "Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction." Nat Rev Neurosci 14(5): 337-49.
[25] Alarcón, M., B. S. Abrahams, J. L. Stone, J. A. Duvall, J. V. Perederiy, J. M. Bomar, J. Sebat, M. Wigler, C. L. Martin, D. H. Ledbetter, S. F. Nelson, R. M. Cantor and D. H. Geschwind (2008). "Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene." Am J Hum Genet 82(1): 150-9.
[26] Moessner, R., C. R. Marshall, J. S. Sutcliffe, J. Skaug, D. Pinto, J. Vincent, L. Zwaigenbaum, B. Fernandez, W. Roberts, P. Szatmari and S. W. Scherer (2007). "Contribution of SHANK3 mutations to

autism spectrum disorder." Am J Hum Genet 81(6): 1289-97.

[27] Liu, Z., X. Li, J. T. Zhang, Y. J. Cai, T. L. Cheng, C. Cheng, Y. Wang, C. C. Zhang, Y. H. Nie, Z. F. Chen, W. J. Bian, L. Zhang, J. Xiao, B. Lu, Y. F. Zhang, X. D. Zhang, X. Sang, J. J. Wu, X. Xu, Z. Q. Xiong, F. Zhang, X. Yu, N. Gong, W. H. Zhou, Q. Sun and Z. Qiu (2016). "Autism-like behaviours and germline transmission in transgenic monkeys overexpressing MeCP2." Nature 530(7588): 98-102.

[28] Fu, W., T. D. O'Connor, G. Jun, H. M. Kang, G. Abecasis, S. M. Leal, S. Gabriel, M. J. Rieder, D. Altshuler, J. Shendure, D. A. Nickerson, M. J. Bamshad and J. M. Akey (2013). "Analysis of 6,515 exomes reveals the recent origin of most human protein-coding variants." Nature 493(7431): 216-20.

[29] Sanchack, K. E. and C. A. Thomas (2016). "Autism Spectrum Disorder: Primary Care Principles." Am Fam Physician 94(12): 972-9.

[30] Zhang, J., L. Tan, Y. Ren, J. Liang, R. Lin, Q. Feng, J. Zhou, F. Hu, J. Ren, C. Wei, T. Yu, Y. Zhuang, B. Bettler, F. Wang and M. Luo (2016). "Presynaptic Excitation via GABAB Receptors in Habenula Cholinergic Neurons Regulates Fear Memory Expression." Cell 166(3): 716-28.

[31] Bicanski, A. and N. Burgess (2020). "Neuronal vector coding in spatial cognition." Nat Rev Neurosci 21(9): 453-70.

[32] Vann, S. D., J. P. Aggleton and E. A. Maguire (2009). "What does the retrosplenial cortex do?" Nat Rev Neurosci 10(11): 792-802.

[33] Yang, K., Y. Shi, X. Du, J. Wang, Y. Zhang, S. Shan, Y. Yuan, R. Wang, C. Zhou, Y. Liu, Z. Cai, Y. Wang, L. Fan, H. Xu, J. Yu, J. Cheng, F. Li and Z. Qiu (2021). "SENP1 in the retrosplenial agranular cortex regulates core autistic-like symptoms in mice." bioRxiv: 2021.01.24.427868.

Acknowledgements

感谢我的学校上海交通大学提供给我毕业设计的机会，让我可以在大学四年里安心学习，在科研道路上一步一步地走下去。感谢中科院神经所提供给我做实验的平台，让我有机会实现自己的想法，锻炼自己的能力。

感谢我的校外指导老师仇子龙老师和校内指导老师李卫东老师，感谢他们给我提供的学术上的指导和帮助，让我的毕业设计可以顺利地进行下去。仇老师是一个和善对待学生、认真负责的老师，李老师是一个和蔼沉稳，一丝不苟的老师。感谢仇老师和李老师对我的关照，给我的毕业论文提供了不可或缺的助力。

感谢陈峰老师，他对待学生十分用心，给我们的毕业论文提出了宝贵的指导意见。感谢学院的潘林敏老师，她十分可靠细致，对我们的毕业论文工作有很大的帮助。感谢毕业设计助教陈步云学长，有了他对我们的毕业论文答辩和审查都进展顺利。

感谢我的师兄杨侃博士，他对我的实验和论文进行了细致的指导，手把手教我做实验和处理数据，带领我认识到了实验的魅力。他认真细致，对待科学有严谨和尊敬的态度，也用这种态度来要求我，使我受益匪浅，感受良多。他总是对我的想法给与适当的肯定和合理的建议，引导我在科研的道路上独立地走下去。

感谢我的爸爸妈妈和弟弟，他们无条件地支持我，鼓励我。他们虽然不懂科研，但会一直认真地问我关于我的课题，听我讲述实验室的事情，给我提供最温暖的港湾，坚信我会成为推动人类科学进步的人。

感谢我的师妹刘雨婷，她陪我一起在实验室努力地工作，从早上八点到晚上八点，我们合作完成了不少的实验，一直互相帮助。我们两个经常交流对实验的看法，提出自己的观点。她是我在实验室最好的朋友，在这一年的奔波中彼此陪伴。

感谢我的室友张嘉琪和甄馨远，在我满身疲惫从实验室回到宿舍，她们两个会我聊天说话，一起分享日常，一起逛街，一起吃饭，消除了学业和科研上的压力。

感谢五号线和一号线地铁，我每天都从闵行校区去往徐汇一个来回，在地铁上消耗三个多小时的时间，是一号线和五号线一直陪伴着我。感谢玉兰苑，我每天晚上八九点回到学校后只有玉兰苑还提供着晚餐。感谢巧克力、奶茶和可乐，在无数个难熬的日子里提供给我大量多巴胺，让我继续坚持下去。感谢 Glory of the king，是它在闲暇时光给我喜怒哀乐。

感谢我自己，我的努力让我变成更好的我。